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Short communication

Simplified procedures for the isolation of HF3, bothropasin, disintegrin-like/cysteine-rich protein and a novel P-I metalloproteinase from *Bothrops jararaca* venom

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ABSTRACT

HF3 and bothropasin are P-III hemorrhagic snake venom metalloproteinases (SVMPs) of *Bothrops jararaca*. The DC protein is composed of the disintegrin-like/cysteine-rich domains derived from the autolysis of P-III SVMPs. Here we describe simplified procedures for the isolation of HF3, bothropasin, the DC protein, and BJ-PI, a novel P-I SVMP. The isolated proteins were identified by mass spectrometry. BJ-PI is a potent caseinolytic enzyme devoid of hemorrhagic activity. HF3, bothropasin and BJ-PI show distinct fibrinogenolytic activities.

Snake venom metalloproteinases (SVMPs) are widely distributed in viperid venoms and play important roles in the main features of envenoming: severe local tissue damage and coagulopathy (Takahashi and Osaka, 1970; Shannon et al., 1989; Rucavado et al., 1995). SVMPs are zinc-dependent enzymes, which are members of the reprolysin subfamily of the M12 family of metalloproteinases (Bjarnason and Fox, 1995). They are secreted as preproenzymes and contain additional regulatory modules involved in their interactions with various protein targets (Fox and Serrano, 2008). Structurally they are classified into three classes, based on the presence or absence of various non-proteinase domains as observed via mRNA transcripts and proteins isolated from the venom: P-I, SVMPs composed of only a metalloproteinase domain; P-II, SVMPs composed of metalloproteinase domain and disintegrin domain; P-III, SVMPs composed of metalloproteinase domain, disintegrin-like domain and cysteine-rich domain, and in some instances lectin-like domains connected by disulfide bonds to the cysteine-rich domain (Fox and Serrano, 2008). Similar domain structures are found in the members of the A Disintegrin And Metalloproteinase (ADAM) and A Disintegrin And Metalloproteinase with Thrombospondin Motif (ADAMTS) protein families (Black and White, 1998; Tang and Hong, 1999).

HF3, a P-III class SVMP, is the most potent hemorrhagic toxin of *B. jararaca* venom showing a minimum hemorrhagic dose (MHD) of 15 ng on the rabbit skin (Assakura et al., 1986). Previously, we determined the amino acid sequence of HF3 by molecular cloning and showed that a recombinant protein composed of disintegrin-like/cysteine-rich domains inhibits collagen-induced platelet-aggregation (Silva et al., 2004). Moreover, we showed that HF3 recombinant proteins containing the cysteine-rich

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domain (disintegrin-like/cysteine-rich and cysteine-rich proteins) but not the disintegrin-like protein were able to significantly increase leukocyte rolling in the microcirculation (Menezes et al., 2008). Bothropasin is another P-III class SVMP of *B. jararaca* venom whose caseinolytic activity corresponds to 15% of the total activity of the crude venom (Mandelbaum et al., 1982). It causes hemorrhage on rabbit skin with a MHD of 1 μg. Bothropasin undergoes an autolysis process wherein the disintegrin-like and cysteine-rich domains (DC protein) remain intact while the metalloproteinase domain is cleaved at different sites (Assakura et al., 2003). Jararhagin, a homologous P-III class SVMP found in *B. jararaca* venom, is also proteolytically processed to generate the DC protein also known as jararhagin-C (Usami et al., 1994; Moura-da-Silva et al., 2003).

The isolation procedures used to obtain HF3 and bothropasin were rather troublesome and involved various chromatographic steps (Mandelbaum et al., 1982; Assakura et al., 1986). In this work we report a novel, less complex method to isolate these proteinases. The lyophilized *B. jararaca* venom (900 mg; Instituto Butantan, Brazil) was dissolved in 90 ml of 0.15 M NaCl and the turbid solution was clarified by centrifugation (4 °C, 10,000*g*, 20 min). Protein was precipitated at room temperature by adding ammonium sulfate to the supernatant. The fraction of 418 mg protein obtained between 30% and 60% of ammonium sulfate saturation was dissolved in 20 ml water, dialyzed against 0.02 M Tris–HCl buffer containing 1 mM $CaCl_2$ and 0.02% sodium azide (pH 7.5), at 4 °C, and chromatographed on a DEAE-cellulose DE-52 column (2.5 × 13.5 cm) previously equilibrated with the dialysis buffer, at 4 °C. After washing with 400 ml starting buffer at a flow rate of 60 ml/h, the column was developed with a linear gradient from 0 to 0.3 M NaCl in the initial buffer (total volume 1000 ml) at a flow rate of 60 ml/h, and fractions of 5 ml were collected. Fractions eluted with 0.15 M–0.20 M NaCl, containing HF3 and the DC protein, yielded 71 mg protein. They were concentrated by lyophilization, and part of the recovered protein (9 mg) was resuspended with 1.25 ml of 0.25 M ammonium bicarbonate containing 1 mM $CaCl_2$ and chromatographed in aliquots of 0.25 ml on a Superose 12 HR 10/30 column (GE Healthcare), at 0.1 ml min^{-1}, using a fast protein liquid chromatography system (FPLC) (Äkta Purifier, GE Healthcare). HF3 (0.9 mg) and the DC protein (3.8 mg) were obtained in different peaks of the Superose 12 chromatography (Fig. 1A). Fractions eluted with 0.20 M–0.25 M NaCl from the anion-exchange column yielded 83 mg protein and contained bothropasin. They were concentrated by lyophilization, and a sample containing 25 mg protein was resuspended with 1.25 ml of 0.25 M ammonium bicarbonate containing 1 mM $CaCl_2$ and chromatographed in aliquots of 0.5 ml on a Superose 12 HR 10/30 column (GE Healthcare), at 0.1 ml min^{-1}, using a fast protein liquid chromatography system (FPLC) (Äkta Purifier, GE Healthcare). Bothropasin (4 mg) was isolated in the first peak of the Superose 12 chromatography (Fig. 1A).

Moreover, a novel P-I class metalloproteinase, named BJ-PI, was isolated from *B. jararaca* venom by a one-step procedure using gel filtration chromatography on a Superformance (600 × 16 mm) column (Merck) equilibrated and eluted with 0.05 M Tris–HCl, 0.2 M NaCl, 0.5 mM $CaCl_2$, pH 7.5 (buffer A) and connected to an FPLC system (Äkta Purifier, GE Healthcare). The venom chromatography (30 mg venom dissolved in 1 ml buffer A), at a flow rate of 0.25 ml min^{-1}, yielded three main protein peaks and BJ-PI (4.2 mg) was isolated in peak II (Fig. 1A).

Isolated HF3, bothropasin, DC protein and BJ-PI showed a single protein band on SDS-PAGE under reduction conditions, according to Laemmli (1970) (Fig. 1B). Anti-HF3 antibodies (Assakura et al., 1986) recognized HF3, bothropasin and the DC protein by Western blot, carried out according to Burnette (1981) (Fig. 1C), but failed to recognize BJ-PI (not shown). Therefore, an antiserum to recognize BJ-PI was prepared as follows: BJ-PI (10 μg) was mixed with 1.6 mg $Al(OH)_3$ (Aldrox®, Wyeth) and PBS to give a final volume of 0.5 ml and the mixture was injected subcutaneously in the peritoneal region of Balb/c mice (20 g–22 g) weekly for 3 weeks. Three weeks later the animals were bled from the retro-orbital plexus and the antiserum was tested by ELISA (not shown) and Western blot assays for the recognition of BJ-PI (Fig. 1C). Besides BJ-PI, the antiserum anti-BJ-PI also recognized a protein band of ~50 kDa in the venom of *B. jararaca* which likely corresponds to bothropasin (not shown).

For protein identification, protein bands were excised, destained, and in-gel digested with trypsin (Hanna et al., 2000). The tryptic peptide mixture was analyzed by LC/MS/MS with a Finnigan LCQ Decca ion trap mass spectrometer system (Finnigan) or with a MALDI Q-TOF Premier mass spectrometer (Waters). Fig. 2A shows the sequences of the tryptic peptides that allowed the identification of HF3, bothropasin and the DC protein. According to the peptide sequences obtained from the DC protein isolated in this work, it may have originated from the autolysis of either bothropasin or jararhagin since their disintegrin-like/cysteine-rich domains are highly homologous (Paine et al., 1992; Assakura et al., 2003). Six peptides obtained from the digestion of BJ-PI (63 amino acid residues) showed identical sequence to the catalytic domain of bothrostatin, the precursor of a P-II class metalloproteinase from *B. jararaca* venom gland (Fernandez et al., 2005). The only exception was a glutamic acid residue of BJ-PI whose position is occupied by an aspartic acid residue in bothrostatin (Fig. 2B). These results suggest that BJ-PI may correspond to the processed form of the P-II precursor of bothrostatin. The analysis of the partial sequence of BJ-PI shows that it is distinct from the recently reported P-I proteinase bothrojaractivase, isolated from the same venom (Berger et al., 2008).

The hemorrhagic activity of the isolated proteinases was evaluated on the mouse skin (Paes Leme et al., 2009). HF3 showed a MHD 37.5 times higher than that of bothropasin (Table 1). BJ-PI was shown to be devoid of hemorrhagic activity using doses of up to 20 μg, however, it showed higher caseinolytic activity than bothropasin (1.6-fold) and HF3 (12-fold) as detected by a method described elsewhere (Menezes et al., 2006). Table 1 also shows a comparison of the proteolytic activity of the proteinases upon fibrinogen. A solution of human fibrinogen (Sigma) (50 μl) at 10 mg ml^{-1} in 0.1 M Tris–HCl buffer, 0.01 M $CaCl_2$, pH 8.8 (incubation buffer) was incubated with the enzymes (1 μg in 45 μl of incubation buffer) for 30 min, at 37 °C. The