Peptidomics of Three Bothrops Snake Venoms: Insights Into the Molecular Diversification of Proteomes and Peptidomes*§

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Snake venom proteomes/peptidomes are highly complex and maintenance of their integrity within the gland lumen is crucial for the expression of toxin activities. There has been considerable progress in the field of venom proteomics, however, peptidomics does not progress as fast, because of the lack of comprehensive venom sequence databases for analysis of MS data. Therefore, in many cases venom peptides have to be sequenced manually by MS/MS analysis or Edman degradation. This is critical for rare snake species, as is the case of Bothrops cotiara (BC) and B. fonsecai (BF), which are regarded as near threatened with extinction. In this study we conducted a comprehensive analysis of the venom proteomes of BC, BF, and B. jararaca (BJ) using a combination of solid-phase extraction and reversed-phase HPLC to fractionate the peptides, followed by nano-liquid chromatography-tandem MS (LC-MS/MS) or direct infusion electrospray ionization-(ESI)-MS/MS or MALDI-MS/MS analyses. We detected marked differences in the venom peptidomes and identified peptides ranging from 7 to 39 residues in length by de novo sequencing. Forty-four unique sequences were manually identified, out of which 30 are new peptides, including 17 bradykinin-potentiating peptides, three poly-histidine-poly-glycine peptides and interestingly, 10 L-amino acid oxidase fragments. Some of the new bradykinin-potentiating peptides display significant bradykinin potentiating activity. Automated database search revealed fragments from several toxins in the peptidomes, mainly from L-amino acid oxidase, and allowed the determination of the peptide bond specificity of proteinases and amino acid occurrences for the P4-P4’ sites. We also demonstrate that the venom lyophilization/resolubilization process greatly increases the complexity of the peptidome because of the imbalance caused to the venom proteome and the consequent activity of proteinases on venom components. The use of proteinase inhibitors clearly showed different outcomes in the peptidome characterization and suggested that degradomic-peptidomic analysis of snake venoms is highly sensitive to the conditions of sampling procedures.  

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snake venoms are the products of specialized secretory glands located above the upper jawbone in venomous snakes. Like most secretory proteins, venom toxins are synthesized in the cytoplasm of secretory cells in the gland and transferred to the rough endoplasmic reticulum, then to the Golgi apparatus, and finally transported via secretory granules to the lumen of the venom gland (1). The snake venomous secretion is an aqueous solution containing a high amount of proteins and peptides, however the mechanisms for controlling protein secretion into the gland lumen and for regulating its protein/peptide concentration, ionic strength, and pH are unknown. As in all eukaryotic cells the proteomes of the venom gland tissue are highly complex, comprising a much great number and diversity of multidomain proteins (2). Likewise, snake venom proteomes are highly complex and their protein integrity control and maintenance of homeostasis within the gland lumen, are crucial for the expression of biological activities, which favor the survival of the snake in its particular environment. In the gland lumen widespread, random proteolysis of venom components is minimized by the acidic pH which does not favor the activity of metalloproteinases (SVMPs)† and serine proteinases (SVSPs) (3). Moreover, the presence of pyro-

† The abbreviations used are: SVMP, snake venom metalloproteinase; BC, Bothrops cotiara; BF, Bothrops fonsecai; BJ, Bothrops jararaca; BK, bradykinin; BPP-CNP, bradykinin potentiating peptides and C-type natriuretic peptides; DMF, dimethylformamide; DVE, dried venom eluates; EDT, ethanedithiol; HOBt, 1-Hydroxybenzotriazole; LAAO, L-amino acid oxidase; NMM, N-Methylmorpholine; PEG, polyethylene glycol; pHpG, poly-His and poly-Gly peptides; sACE, somatic angiotensin-converting enzyme; SHR, spontaneously hypertensive rat; SVSP, snake venom serineproteinase; TBTU, O-([Benzotriazol-1-yl]-N,N,N’,N’-tetramethyluronium tetrafluoroborate.

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glutamate containing tripeptides in the venomous secretion contributes to the lack of proteolytic activity of SVMPs and prevents extensive degradation of venom components (4).

Numerous investigations into venom proteome and subproteomes have been reported with novel insights into the composition of venoms, their biological activities, and the evolutionary relationships of snakes as observed by venom proteomics (5–10). The lack of complete snake genome sequences is overcome, to a certain degree, by the assembly of venom gland transcriptome databases (2, 11–13) and by homology identification using sequencing information from MS/MS spectra (14, 15).

Although studies on snake venom proteomes are growing rapidly, the characterization of venom peptides do not progress as fast (8). The concentration of peptides in snake venoms as well as the size and complexity of their peptides are largely unknown because of the lack of systematic studies to characterize the amino acid sequence and abundance of bioactive peptides in venoms. In part this is because of the need of database searching of MS/MS spectra without enzyme specificity and of large scale de novo sequencing of novel peptides. The availability of a sequence database is essential for automated protein/peptide identification, and it can exponentially increase in size and search time if the search is carried out without enzyme restriction and with inclusion of variable modifications (16, 17). On the other hand, manual de novo interpretation may be difficult for noisy or low quality MS/MS spectra (17–19). Nevertheless, studies in peptidomics are receiving increasing attention owing to the significant roles that small polypeptides have in biological processes (20–27). In the case of venom peptides, such studies are important because of the potential to prospect bioactive molecules with therapeutic application. Therefore, in the last decade, various research groups made efforts to develop mass spectrometry based techniques in order to study venom peptides (20, 25, 28–33).

Snakes belonging to Bothrops genus are among the most studied neotropical vipers, both in terms of ecological features and toxin families. They are responsible for more fatalities in Central and South America than any other group of venomous snakes (34, 35). The venoms of Bothrops snakes contain multiple components that target blood hemostasis, the endothelial microcirculation, the extracellular matrix, and the cardiovascular system. Their protein concentration is high (ca. 100 mg/ml) and their proteomes show various degrees of complexity. They may be composed of some hundreds of different proteins which belong to 10–12 toxin families depending on the species (7, 14, 36). Two-dimensional electrophoresis coupled with liquid chromatography- tandem MS (LC-MS/MS) has been used for examining Bothrops venom complexity along with other approaches to selectively delineate subpopulations of venom toxins based on particular characteristics of the proteins such as antibody cross-reactivity, enzymatic activities, and post-translational modifications (5, 7, 8). In another work, the venom of B. jararaca (BJ) was analyzed by in-solution digestion with trypsin and ion-currents of tryptic peptides with FT-ICR LC/MS/MS were used to investigate the venom proteomic content showing 42 individual proteins representing 12 venom protein classes (37). Furthermore, the analysis of the venom peptidome of B. jararaca from sibling and nonsibling snakes showed for the first time sex-based differences among the bradykinin potentiating peptides (38).

Peptidomics is critical for the knowledge of the venom peptide arsenal of rare species because of the lack of studies and sequences in databases, as is the case of Bothrops cotiara (BC) and Bothrops fonsecai (BF), both regarded as near threatened with extinction snakes (39–41). B. cotiara and B. fonsecai inhabit similar, highly specialized habitats (Araucaria angustifolia pine forests), in different geographical regions of Brazil and are morphologically very difficult to distinguish. Interestingly, a previous proteomic study showed that the overall proteomes of BC and BJ venoms display significant compositional differences. Specifically, in the venom of BF phospholipase A2 is present in high abundance whereas in the BC venom is absent (6).

In most instances, proteomic/peptidomic analyses of snake venoms are conducted using pooled samples from many specimens to overcome the well documented individual variability that occurs both at the protein and peptide levels (38, 42, 43). Moreover, fresh venom samples are usually lyophilized to preserve the structural integrity of proteins. Nevertheless, the impact of lyophilization and resolubilization on the proteomes and peptidomes of snake venoms is largely unknown.

In this study we conducted a deep, comprehensive analysis of the venom peptidomes of BC and BF. The venom of BJ is one of the best characterized among venomous snakes and was also included in the study for comparison. The venoms were submitted to solid phase extraction, reverse phase (RP)-HPLC, matrix-assisted laser desorption ionization (MALDI)-MS/MS, and electrospray ionization (ESI)-MS/MS, automated database searches and de novo sequencing of peptides. New bradykinin potentiating peptides, poly-His and poly-Gly peptides (pHpGs) and, interestingly, a great number of fragments from l-amino acid oxidase as well as from other venom toxins were found in all venoms. We also demonstrate that the lyophilization/resolubilization process greatly increases the complexity of snake venom peptidomes because of the imbalance caused to the venom proteostasis and the consequent activity of proteinases upon other venom components. The in silico analysis of peptide sequences derived from venom proteins allowed the determination of the peptide bond specificity of venom proteinases and amino acid occurrences for the P4-P4’ sites. The results of these experiments will be discussed as well as their ramifications on our understanding of the structural stability of proteins in the venom solution and the molecular diversity of venom peptidomes.
**EXPERIMENTAL PROCEDURES**

**Venoms—** Lyophilized venom from BC, BF, and B. jararaca (BJ) was from Instituto Butantan (Sao Paulo, Brazil).

For the experiments with fresh venom of BC collected in the presence of proteinase inhibitors, venom from six adult specimens kept at the Laboratory of Herpetology (Instituto Butantan, Sao Paulo, Brazil) was milked into a beaker in ice bath, and immediately mixed with the inhibitor solution. Venom (250 μl) was mixed with 100 mM EDTA (14 μl) and MilliQ water (14 μl), or with 40 mM PMSF (14 μl) and MilliQ water (14 μl), or with 100 mM EDTA (14 μl) and 40 mM PMSF (14 μl), or with MilliQ water (28 μl). Venom solutions were centrifuged at 1,700 g at 4 °C for 30 min to remove debris, lyophilized and stored at −20 °C for further fractionation.

**Solid-phase Extraction—** For solid-phase extraction, 25 mg of lyophilized venoms of BC, BF, and BJ were dissolved and incubated for 30 min at room temperature in the following solutions: 1) 1 ml of 0.1% trifluoroacetic acid (TFA); 2) 1 ml of 2 mM phenylmethylsulfonyl fluoride, and 5 ml EDTA in order to inhibit SVSPs and SVMPs. BC venom (25 mg) that had been collected, mixed with inhibitors and lyophilized (see above) was also dissolved in solutions (1) or (2). Venom solutions were centrifuged at 10,000 × g for 10 min at room temperature and then fractionated in Sep-pak C18 cartridges previously conditioned with methanol and 0.1% TFA (Waters, Milford, MA) according to Menin et al. (31). After loading the venom solution the cartridge was washed with the venom dissolving solution (see above) and eluted with 0.1% TFA in H2O/acetoneitrile (ACN) (70:30). The eluates were dried in a SpeedVac concentrator and the dried venom eluates (DVEs) were stored at −20 °C for further analysis.

**RP-HPLC Separation—** RP-HPLC was performed using a binary pump system (10Avp, Shimadzu, Kyoto, Japan) coupled to a 10 mm × 250 mm Discovery C18 column (5 μm particle size; Supelco, Bellefonte, PA). The DVEs were dissolved in 500 μl of 0.05% TFA and 200 μl aliquots of each DVE were loaded into the column and eluted using a gradient of 5% B for 10 min, 5–40% B in 35 min, 40–80% in 5 min and 80% for 10 min at 1.5 ml/min (phase A: 0.05% TFA in water; phase B: 0.05% TFA in ACN). The eluted fractions were detected at 215 nm (SPD-10AV, Shimadzu, Kyoto, Japan), manually collected and dried in a SpeedVac concentrator.

**De novo Peptide Sequencing from MALDI Q-TOF MS/MS—** The RP-HPLC fractions were dissolved in 20 μl water/ACN/formic acid (49.8/49.8/0.5) and 1.5 μl were spotted onto the MALDI sample plate, dried and then mixed with 1.5 μl of a saturated solution of α-cyano-4-hydroxy-cinnamic acid (Sigma, St. Louis, MO) in 0.1% TFA in H2O/ACN (70:30). The samples were dried and submitted to MS/MS analysis in a MALDI Q-TOF Premier (Waters, Milford, MA). MS spectra were obtained until 15 counts had been accumulated from an m/z range of 800–4000. After MS acquisition, the 10 most intense ions were automatically selected for MS/MS analysis and spectra were obtained until 30 counts had been accumulated. Mass spectra were acquired and processed using the software Mass Lynx 4.1 (Waters, Milford, MA). For de novo sequencing, the spectra were smoothed and deisotoped with the module MaxEnt 3. Calibration was performed with PEG. Spectra were interpreted manually and with the help of the simple perl script denovo.pl (supplemental information). Briefly, from any mono charged fragment of a MS/MS spectrum, the script shows on screen the list of all possible amino acid residue differences forward or backward. Peptides were classified to protein classes by BLAST similarity search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Peptide Synthesis—** Synthetic BPP analogues were prepared in automated bench-top simultaneous multiple solid-phase synthesizer (PSSM 8 system; Shimadzu, Kyoto, Japan) using solid phase peptide synthesis by the Fmoc procedure (48). Briefly, sequential couplings of protected amino acids were performed with HOBt, TBTU, and NMM on L-proline-2-chlorotrityl resin (Novabiochem, Gibbstown, NJ). Fmoc cleavage was performed with 50% morpholine (v/v) in DMF. The resin-bound peptides were cleaved/deprotected with TFA/thioanisole/EDT/phenoI/water (82.5/5.0/2.5/5.0 v/v/v/v) at room temperature for 4 h. After filtration, the filtrate was concentrated under argon stream and precipitated with diethyl ether. All synthetic peptides were purified by RP chromatography using a semi-preparative HPLC system (Shim-pack Prep-ODS; Shimadzu, Kyoto, Japan).
and the purity and identity of the peptides were confirmed by MALDI-TOF MS in an Etan MALDI-TOF/Pro instrument (GE Healthcare, Uppsala, Sweden) and by analytical RP-HPLC using two different solvent systems.

**Animals**—Experiments were conducted with adult male Wistar rats (250–280 g body weight), bred at animal care facility of the Instituto Butantan (São Paulo, Brazil). The animals had free access to food and water and were submitted to a light/dark cycle (12 h each) before the preparation for the experiments. All animals were caged and handled under ethical conditions according to international rules of animal care as stated by the International Animal Welfare Recommendations, and in accordance with the guidelines established by our local institutional animal welfare committee (CEUAIB/Instituto Butantan, protocol #708/10).

**BK-potentiation on Blood Pressure in Anesthetized Rats**—The assay to evaluate the BK-potentiation on blood pressure were performed essentially as described elsewhere (49). Animals were anesthetized with a 12% sodium urethane solution (1.0 ml/100 g body weight; intraperitoneal injection), and received polyethylene catheters (PE-10 connected to a PE-50) into the femoral artery and vein for blood pressure measurements and intravenous (i.v.) bolus injections of peptides, respectively. The catheters were closed by a metallic pin and filled with isotonic saline solution. Pulsed arterial pressure (PAP), mean arterial pressure (MAP), and heart rate (HR) were continuously monitored by a solid-state strain gauge transducer connected to a computer using a data acquisition system (MP 100; BIOPAC Systems, Inc., Santa Barbara, CA). Rats were maintained anesthetized during the experiments. After blood pressure stabilization, standard hypotensive responses evoked by i.v. injections of BK (0.5 μg and 1.0 μg) were recorded. All peptides were dissolved in sterile isotonic saline immediately before use. Following, the injection of the synthetic peptide (60 nmol) was performed. Subsequently, injections of BK (0.5 μg) were repeated at 5, 10, 15, 20, 25, and 30 min after peptide administration. The peptides evaluated were BPP-10d (n = 5), BPP-10e (n = 4), BPP-10f (n = 4), BPP-11f (n = 5), and BPP-11h (n = 4). As positive control, BPP-10c (60 nmol, n = 4) was used (50). For statistical analysis, comparisons were made by one-way analysis of variance (ANOVA) with Newman-Keuls post-test using GraphPad statistical analysis software.

**RESULTS AND DISCUSSION**

**De Novo Sequencing of Peptides Present in the Venoms Fractionated in the Absence of Proteinase Inhibitors**—Venom samples were initially dissolved in 0.1% TFA and submitted to solid-phase extraction for concentration of their peptide fractions and depletion of proteins using Sep-pak C18 cartridges. The resulting DVEs containing the concentrated peptides were directly submitted to LC-MS/MS or fractionation by RP-HPLC and analysis by MALDI MS/MS (supplemental Table 1). BC, BF, and BJ venoms showed clearly distinct RP-HPLC profiles (Fig. 1) and no peptide was simultaneously found by de novo sequencing in the three venom species (Table I). Forty-four unique peptides were identified in the venoms by de novo sequencing of the MS/MS spectra containing complete or complementary b and y ion series (Table I; supplemental Spectra). Thirty of them are novel peptides including 10 LAAO fragments, 17 BPPs, and three pHpsGs. Several fragments of LAAO and novel BPPs, which are characterized by the presence of a pyroglutamatic acid (<E) residue at the N terminus and by the high proline content, were found in the three venoms. From the LC-MS/MS spectra, the LAAO peptide [I/L]TEPV[I/L]NFFAGEYTA[Q/K]AHGW[I/L]DST[I/L][Q/K]SRDAARDVNRAS, found at m/z 862.2 ([M+5H]+) (Fig. 2) was identified in the BC venom. It is not common to obtain high quality MS/MS spectra by CID fragmentation from highly charged peptides (> +3) (55, 56), however, for this 39-residue peptide with charge +5 the complete y-series and part of the b-series were obtained, allowing its complete sequencing. Moreover, other LAAO fragments have been found in the venoms of BC, BF and BJ (Table I), similarly as previously described in the venoms of B. moojeni (31, 57) and B. insularis (58).

All other peptides found by de novo sequencing were homologous or identical to sequences present in the BPP-CNP precursor which is composed of seven BPP molecules aligned in tandem after a hydrophobic signal peptide sequence, followed by a putative intervening sequence and a C-type natriuretic peptide at the C terminus (59). The BPP spectra are characterized by the typical m/z fragment at 213.1, corresponding to the y2 of the C-terminal PP (8, 20, 31). The recently described BPP-AP (60), containing the amino acids PPAP at the C-terminal, shows typical signals at m/z 187.1 (y2, AP), 284.2 (y3, PAP) and 381.2 (y4, PPAP). Twelve BPPs whose sequences had been reported before were found in this study (Table I) (20, 38, 60–63). The presence of some new BPPs and its fragments showing cleavage at the C-side position of the amino acid [Q/K], as BPP-10e and BPP-7b, BPP-11f and BPP-8a and BPP-10f and BPP-7c, indicate that these peptides may have derived from the proteolytic processing in the venoms and that [Q/K] is actually a Lys residue which would be susceptible to such cleavage by a serine proteinase.
Fig. 1. RP-HPLC chromatograms of DVEs from *Bothrops cotiara* (BC), *B. fonsecai* (BF), and *B. jararaca* (BJ) obtained by solid phase extraction.
Peptidomic Analyses of Bothrops Snake Venoms

Table I
Peptides identified in the DVEs of BC, BF, and BJ analyzed in the absence of proteinase inhibitors by ESI-Q-TOF or MALDI-Q-TOF and de novo sequencing from MS/MS spectra

*Peptides identified and sequenced from the LC-MS/MS analyses; **new LAAO fragments and new BPPs and pHpGs.

| Venom | HPLC fraction | m/z | z | Mass (Da) | Sequence | Protein or peptide | Peptide name |
|-------|----------------|-----|---|----------|----------|-------------------|-------------|
| BC    | 6              | 647.9| 2 | 1293.8   | (b ion: 217.1) VG/Q[K] | LAAO**    |
| BC    | 9              | 592.4| 2 | 1183.7   | ENWPPMPPP | BPP**     |
| BC    | 10–11          | 676.4| 2 | 1350.7   | ENWPPMPMPAP | BPP** |
| BC    | 11–13          | 839.4| 1 | 838.4    | ENWPS/Q[K]  | BPP**     |
| BC    | 12–14          | 864.1| 1 | 860.4    | ENAHAPS/Q[K]  | BPP** |
| BC    | 15             | 1296.7| 1 | 1295.5   | EARPPRG/Q[K[I/I]]PP | BPP** |
| BC    | 16–20          | 566.8| 2 | 1131.5   | ENWPS/Q[K]VPP | BPP** |
| BC    | 16–20          | 577.8| 2 | 1153.6   | ENAHAPS/Q[K]VPP | BPP** |
| BC    | 19–20          | 1370.7| 1 | 1369.7   | EGGWRPGP[1/I]PP | BPP     |
| BC    | **             | 468.7| 2 | 935.4    | TTD[1/L]PFSR | LAAO**  |
| BC    | **             | 624.8| 2 | 1247.6   | [I/L]VGMD/Q[K[1/L]PTSM | LAAO**  |
| BC    | **             | 503.3| 3 | 1506.9   | HHDHAAVGGGGGGA | pHpG** |
| BC    | **             | 617.4| 2 | 1232.7   | (b ion: 253.2) HAAVGGGGGGA | pHpG** |
| BC    | **             | 516.3| 3 | 1030.7   | [I/L]TEPV[1/L]NF | LAAO**  |
| BC    | **             | 862.1| 5 | 4305.4   | [I/L]TEPV[1/L]NFFAGEYTA/Q[K]AHGW | LAAO**  |
| BF    | 3              | 503.2| 3 | 1506.6   | HHDHAAVGGGGGGA | pHpG** |
| BF    | 3              | 754.3| 2 | 1506.7   | HHDHAAVGGGGGGA | pHpG** |
| BF    | 9–12, 14       | 592.3| 2 | 1182.7   | ENWPPMPMPPP | BPP** |
| BF    | 12, 14–15      | 676.4| 2 | 1350.7   | ENWPPMPMPAP | BPP** |
| BF    | 14             | 881.5| 1 | 880.5    | ERWPS/Q[K]  | BPP** |
| BF    | 19–23          | 587.8| 2 | 1173.6   | ERWPS/Q[K]VPP | BPP** |
| BF    | 19             | 1357.5| 1 | 1356.5   | ENWPPHP[1/I]PP | BPP |
| BF    | 24             | 682.4| 4 | 2725.4   | TA/Q[K]AHGW[1/L]DST[1/L][Q/K][S][Q/I/L] | LAAO** |
| BF    | 27             | 640.4| 2 | 1278.6   | EARPRH[P/Q][K][I/I]PP | BPP** |
| BF    | **             | 664.3| 2 | 1326.7   | VTVTY/Q[K][S][Q/K][I/E] | LAAO**  |
| BJ    | 17–23          | 1189.7| 1 | 1188.7   | EARPPHP[1/I]PP | BPP |
| BJ    | 17–23          | 595.3| 2 | 1188.5   | EARPPHP[1/I]PP | BPP |
| BJ    | 19–21          | 672.3| 2 | 1342.6   | EGRPPHP[1/I]/PP | BPP** |
| BJ    | 19–26          | 1095.6| 1 | 1094.6   | EGRPPHP[1/I]PP | BPP** |
| BJ    | 19–26          | 548.3| 2 | 1094.6   | EGRPPHP[1/I]PP | BPP** |
| BJ    | 20–23          | 679.3| 2 | 1356.7   | EGRPPHP[1/I]PP | BPP** |
| BJ    | 21–27          | 648.8| 2 | 1295.6   | EARPPRG/Q[K[I/L]PP | BPP** |
| BJ    | 21–27          | 649.3| 2 | 1296.7   | EGGLPP[1/R]PP | BPP** |
| BJ    | 23–27          | 640.4| 2 | 1278.7   | EARPRH[P/Q][K][I/I]PP | BPP** |
| BJ    | 27–29          | 1370.7| 1 | 1369.7   | EGWWPPGPGEIPPP | BPP** |
| BJ    | 27–29          | 685.8| 2 | 1369.6   | EGWWPPGPGEIPPP | BPP** |
| BJ    | 27–31          | 1215.6| 1 | 1214.6   | ENWPRPOIPP | BPP** |
| BJ    | 28–31          | 1299.7| 1 | 1298.7   | EWPRPTQIPPP | BPP** |
| BJ    | 28–31          | 650.3| 2 | 1298.6   | EWPRPTQIPPP | BPP** |
| BJ    | 29             | 1281.7| 1 | 1280.7   | EWGRGPGP[1/I]PP | BPP** |
| BJ    | 29–32          | 1101.6| 1 | 1100.6   | EWPRPPQIPP | BPP** |
| BJ    | 29–32          | 551.3| 2 | 1100.6   | EWPRPPQIPP | BPP** |
| BJ    | 32             | 1486.8| 1 | 1485.8   | EWAQWPQIPP | BPP** |
| BJ    | 32             | 1684.9| 1 | 1683.9   | EWAQWPRTQIPPP | BPP** |
| BJ    | **             | 894.0| 2 | 1785.9   | (b ion: 1099.6) PP[1/I][L]PP | BPP** |
| BJ    | **             | 900.9| 2 | 1799.9   | (b ion: 1113.4) PP[1/I][L]PP | BPP** |
| BJ    | **             | 598.2| 2 | 1195.6   | ENWHPGQIPP | BPP** |
| BJ    | **             | 575.3| 2 | 1148.7   | EGRHP[1/I]PP | BPP** |
| BJ    | **             | 564.8| 2 | 1127.7   | ENWRH[1/I][L]PP | BPP** |
| BJ    | **             | 603.8| 2 | 1205.6   | ENWRH[1/I]PP | BPP** |
| BJ    | **             | 659.4| 2 | 1316.7   | EGRAPHP[1/I]PP | BPP** |
| BJ    | **             | 380.5| 4 | 1517.8   | AN[1/I][L]GPMR[1/I]PE[Q/K][H]HR | LAAO**  |
| BJ    | **             | 560.7| 3 | 1679.0   | [I/L][L][AV][Q/K][Q][L][S][AT][SNP][Q/K][H] | LAAO**  |
| BJ    | **             | 522.2| 3 | 1563.7   | HHDHAAVGGGGGGA | pHpG** |

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Interestingly, we also found histidine and glycine rich peptides of the recently described pHpG class (19, 64), in the venoms of the three snakes. The pHpG peptides have been first described by Favreau et al. (19) in snake venoms of the genus Atheris by mass spectrometric analysis. The peptide found at m/z 522.2 ([M+3H]+) in the venom of BJ showed the sequence HHDHHAAVGGGGGGGGGGA (Bot.ja pHpG-1; Fig. 2) which is present in the snake brain BPP-CNP precursor (63). Favreau and colleagues (19) found by BLAST similarity search exactly the same sequence in the BPP-CNP precursors of BJ (63) and B. insularis (2). However, to the best of our knowledge, our result is the first experimental detection of this peptide in the venom. We found at m/z 754.3 ([M+2H]2+) and 503.2 ([M+3H]3+) the new sequence HHDHAAVGGGGGGGGA (Bot.cf pHpG-1) found in the venom of BJ at m/z 522.3 and charge +3. The spectrum was smoothed and deisotoped to singly charged states and after interpretation, ion series were labeled by the module BioLynx (MassLynx 4.1, Waters, Milford, MA) using a mass window of ± 1.0 Da because of a m/z shift of 0.2 from theoretical value; B, Spectrum of the peptide HHDHAAVGGGGGGGGA (Bot.ja pHpG-1) found in the venom of BJ at m/z 522.3 and charge +3. The spectrum was smoothed and deisotoped to singly charged states and after interpretation, ion series were labeled by the module BioLynx using a mass window of ± 0.3 Da.

Interestingly, we also found histidine and glycine rich peptides of the recently described pHpG class (19, 64), in the venoms of the three snakes. The pHpG peptides have been first described by Favreau et al. (19) in snake venoms of the genus Atheris by mass spectrometric analysis. The peptide found at m/z 522.2 ([M+3H]+) in the venom of BJ showed the sequence HHDHHAAVGGGGGGGGGGA (Bot.ja pHpG-1; Fig. 2) which is present in the snake brain BPP-CNP precursor (63). Favreau and colleagues (19) found by BLAST similarity search exactly the same sequence in the BPP-CNP precursors of BJ (63) and B. insularis (2). However, to the best of our knowledge, our result is the first experimental detection of this peptide in the venom. We found at m/z 754.3 ([M+2H]2+) and 503.2 ([M+3H]3+) the new sequence HHDHAAVGGGGGGGGA (Bot.cf pHpG-1) in the venoms of BC and BF, which differs from the Bot.ja pHpG-1 by only one Gly residue. In addition a fragment of the latter sequence (DHHAAVGGGGGGA) was detected at m/z 617.4 ([M+2H]2+) in the venom of BC.

It is difficult to interpret the biological meaning of the differences detected in the peptidomes, especially because of the lack of information available on peptidome characterization and envenoming by the snakes B. cotiara and B. fonscecai. The results of RP-HPLC and de novo sequencing of peptides indicate a higher complexity of BJ venom peptidome in number of sequences in comparison to the other two venoms. BC venom peptidome seems to be the less complex of the three snakes. The venoms of BC and BF were not as rich in BPPs as the venom of BJ, which is well known to be rich in active BPPs and the biological activity of several of these peptides have been extensively described (20, 38, 60–63). We found more common features between the peptidomes of BC and BF than BC and BJ or BF and BJ, and it is worth to mention that B. cotiara and B. fonscecai are morphologically very similar.
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(65). In fact, we found identical peptides in both venoms such as BPP-10d, BPP-12d and Bot.cf pHpG-1. We also observed homologous peptides with similar RP-HPLC retention times (Fig. 1) as BPP-7b and BPP-10e in the venom of BC and BPP-7c and BPP-10f in the venom of BF. On the other hand, the changes in the sequences of these homologous peptides also represent marked differences in the peptidomes and could be used to distinguish each species, in a similar manner as PLAs distinguished the proteome of BF from that of BC (6).

Protein Identifications in the Venoms Fractionated in the Absence of Proteinase Inhibitors—For the identification of peptides generated in the venom gland by the specific proteolytic processing of protein precursors or as a consequence of venom manipulation after milking and the possible proteolytic degradation of venom proteins, the spectra obtained by the LC-MS/MS analysis of the DVEs of venoms not treated with proteinase inhibitors were submitted to database search using MASCOT search engine without enzyme specificity. This analysis resulted in the identification of several peptides corresponding to 15, 11 and 12 protein hits in the venom of BC, BF, and BJ, respectively (supplemental Table 2). The abundance of peptides derived from venom proteins in these venoms indicates that the fragments were generated by proteolysis upon the process of lyophilization after milking and further resolubilization of the venoms for analysis. It is important to emphasize that BC has only one venom protein sequence (66) deposited in the currently available databases (NCBI: gi 262956; UniProtKB: P31988) whereas BF has none, thus the peptides identified in these venoms were likely derived from homologous proteins of the same toxin families described in other snake venoms. The identified proteins from which the peptides were cleaved off belong only to four protein classes: LAAO, BPP-CNP precursor, SVMP, and SVSP (supplemental Table 2).

Peptides from LAAO were detected in the three venoms showing significant identification scores. Moreover, the extensive degradation of LAAO in the venoms was illustrated by the amount of peptides identified by the LC-MS/MS analysis: 1) 49 peptides in the BC venom, including 28 unique peptides, which were assigned to the Bp-LAAO (67); 2) 41 peptides in the BF venom (20 unique peptides); 3) eight peptides in BJ venom (all unique). These results are in agreement with a previous proteomic analysis, which indicated that BC venom contains twice as much LAAO as BF venom (6). On the basis of the high content of peptide spectra detected in the LC-MS/MS analysis which resulted in no protein hit, we can infer that more LAAO fragments may be present in the lyophilized venoms analyzed in the absence of proteinase inhibitors, and the number of peptide identifications could possibly be higher if full amino acid sequences of LAAO present in these venoms were available in the protein databanks.

Although some LAAO peptides have been described in snake venoms by de novo sequencing in previous studies (31, 57, 58), we did not anticipate to find such a variety of LAAO fragments in the venoms because LAAOs are relatively large, glycosylated proteins that have been purified in intact form from several snake venoms (67–71) and there are no reports on the proteolytic processing of LAAO in the snake venom gland.

Precursor proteins containing various BPPs and a CNP were identified in various venom gland transcriptomic analyses (50, 59, 63, 72). They have not been isolated in intact form from the venoms and it is believed that they undergo proteolytic processing in the snake venom gland tissue to generate several bioactive peptides. The enzymes responsible for precursor processing as well as the detailed mechanism of peptide bond cleavage and trimming of spacer sequences have not been elucidated and recently we have identified new BPP sequences in B. jararaca venom which pointed out to novel, noncanonical cleavage sites at the precursor protein (8).

The inspection of the sequences of identified peptides (a total of 331 peptides of which 213 were unique peptides; supplemental Table 3), revealed that some of the peptides were likely cleaved off from proteins at cleavage sites that are compatible with SVMP and SVSP proteolytic specificities (supplemental Table 4) (14, 45, 73–87). Based on the results of 662 cleavage sites, there is observed preference across the P4-P4’ sites for Tyr, Ser, Pro, and Arg at P1 and for Ala, Gln, Gly, and Val at P1’, in decreasing order of frequencies (Fig. 3). Other high relative occurrences of cleavage were observed for Asp, Asn, and Gln at P4, Ser, Arg, Ile, and Lys at P3, Gly, Glu, and Ala at P2, Ala, and Asn at P2’, Leu and Val at P3’, and Ala and Gly at P4’. These cleavage positions are similar to those found by Paes Leme et al. (45) using a peptide library as substrates along with mass spectrometry to explore the peptide bond specificity of four SVMPs to determine their individual peptide cleavage consensus sequences. The occurrence of Pro at position P1 results mainly from BPP identifications in the venom of BJ.

Peptides derived from BPP-CNP precursors of BJ and B. insularis were identified in the BJ venom. Two unique peptides derived from the BPP-CNP precursor of BJ were identified in the venom of BF (Table II). These two peptides, SGSKAPAAPHR and SGSKAPAAPHRLS, were identical to sequences present in a BJ precursor identified by cDNA sequencing (59), and the former has also been reported in the venom of B. jararacussu (33). Interestingly, in the venom of BC no peptide showed hit to known BPP-CNP precursors indicating that if the venom gland of BC produces a BPP-CNP precursor protein then its sequence is significantly different from other known similar proteins. As previously reported by Zelantis et al. (8), we also found the peptides BPP-10c and BPP-13a plus <EQWA at N terminus in the venom of BJ (Table II). In addition, we found BPP-11c plus AP at C terminus (BPP-13d), Bot.ja pHpG-1 plus V at N terminus, and other known BPPs processed at alternative sites.

Other peptide sequences were derived from the proteolytic enzymes present in the venoms (Table II). The only identified
SVSP peptide, IHLGVHSKKVPNKDKQ, was found in the venom of BJ and matched a sequence of KN-BJ 2 (84), whereas SVMP peptides were identified in the three venoms. Interestingly, we found in BF venom at m/z 527.0 \(^3\)H110013 the peptide HLEKNKGLFSKDY, which is part of the prodomain of several SVMPs, including jararhagin and bothropasin (gi 82190823), two abundant hemorrhagic SVMPs found in the venom of BJ. In order to confirm the identification, we manually inspected the MS/MS spectrum, which presented the almost complete b-series and many y-signals (Fig. 4).

In comparison to the manual de novo sequencing, the database search approach allowed the identification of many more peptides. This was an expected result for the venom of BJ, whose venom gland transcriptome has been analyzed and published (11), but not so obvious for the other two venoms, from which few or no sequence is found in databases. Part of the sequences was found by both approaches, whereas several peptides were only found by database search, but the new peptides present in the venoms of the three snakes could only be described by de novo analysis. In fact, these techniques are complementary for the study of snake venom peptidomes. Nevertheless, manual de novo sequencing may be difficult and time consuming for noisy or low quality MS/MS spectra, but it is a valuable technique for the discovery of new molecules, as carried out in this work. It is a challenge for future studies to employ the software available for automatic de novo sequencing, in order to speed up the discovery of new venom peptides.

Protein Identifications in the Venoms Fractionated in the Presence of Proteinase Inhibitors—Viperid snake venoms possess high amounts of proteolytic enzymes (SVMPs and SVSPs), however there are protection mechanisms in the venom solution to avoid self-proteolysis (3, 88, 89). The proteolytic enzymes have optimal activity at alkaline pH (8–9), whereas the pH of the venom solution is slightly acidic. In our measurements of BC venom just after milking, the venom pH was around 5.4 (supplemental Fig. 1). Snake venoms also have a relatively high citrate concentration (30 to 150 mM), which should hamper the activity of metalloproteinases by chelating metal ions (3). In addition, viperid snake venoms also contain tri-peptides which inhibit metalloproteinase activity (4, 89). Interestingly, these tri-peptides are encoded in the same mRNA of the BPP-CNP precursor and appear as short intervening sequences between the BPPs in the precursor (59). These protection mechanisms are no longer effective upon snake bite and venom injection into the prey because the venom is subjected to an environment suitable to the activity of SVMPs and SVSPs where the blood pH is slightly alkaline (7.35 to 7.45) and the concentrations of citrate ions.

**Fig. 3.** Heat maps of amino acid cleavage frequencies in positions P4-P4’ for all identified peptides in the venoms of BC, BF, and BJ venoms analyzed in the absence (A) or in the presence of proteinase inhibitors (B). Heat maps of the difference in amino acid cleavage frequencies in positions P4-P4’ for all identified peptides in the venoms of BC, BF and BJ analyzed in the presence and in the absence of proteinase inhibitors (C).
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### Table II

Peptides identified in the DVEs of BC, BF, and BJ analyzed in the absence of proteinase inhibitors by LC-MS/MS and database search

| Venom | Precursor Acc. number | Identification | Protein family | m/z | z Mascot score | Peptide sequence |
|-------|-----------------------|----------------|----------------|-----|----------------|-----------------|
| BF    | gi 82202072           | BPP-13a processed at G.G | BPP-CNP | 360.2 | +3 | 68 | SGSKAPAAPHHR |
| BF    | gi 82202072           | - | BPP-CNP | 426.9 | +3 | 54 | SGKAPAAPHRLS |
| BJ    | gi 189047126          | BPP-13a processed at Q.G | BPP-CNP | 601.8 | +2 | 50 | GWPRPQPEIPPP |
| BJ    | gi 189047126          | BPP-11c processed at Q.G + AP at C-terminus | BPP-CNP | 603.8 | +2 | 56 | GRAPAHPPFPAP |
| BJ    | gi 189047126          | BPP-13a processed at Q.G | BPP-CNP | 630.3 | +2 | 49 | GWPRAQPPGPEIP |
| BJ    | gi 189047126          | Bot.pH G 1 processed at H.D | BPP-CNP | 645.8 | +2 | 79 | DHHAALVGGGGGGGGGG |
| BJ    | gi 189047126          | BPP-13d (BPP 11c + AP at C-terminus) | BPP-CNP | 659.3 | +2 | 69 | <EGRAPAHPPFPAP |
| BJ    | gi 189047126          | Bot.pH G 1 processed at H.H | BPP-CNP | 476.6 | +3 | 65 | HDHHAAVGGGGGGGGGG |
| BJ    | gi 189047126          | Bot.pH G 1 | BPP-CNP | 522.2 | +3 | 66 | HHDDHAAVGGGGGGGGGG |
| BJ    | gi 189047126          | Bot.pH G 1 + V at N-terminus | BPP-CNP | 555.3 | +3 | 49 | VHDDHAAVGGGGGGGGGG |
| BJ    | gi 189047126          | BPP-10c + <EQWA at N-terminus | BPP-CNP | 855.4 | +2 | 54 | <EQWAQQWNPQDIPPE |
| BJ    | gi 189047126          | BPP-13a processed at Q.G | BPP-CNP | 942.4 | +2 | 40 | <EQWAQQWPRPQGPEIP |
| BJ    | gi 22234045           | BPP-13a processed at G.G | BPP-CNP | 593.5 | +2 | 52 | <EAPRPFP |
| BJ    | gi 22234045           | BPP-13a processed at G.G | BPP-CNP | 601.8 | +2 | 50 | GWPRPQPEIPPP |
| BJ    | gi 22234045           | BPP-13a processed at G.G | BPP-CNP | 630.3 | +2 | 49 | GWPRPQPEIPPP |
| BJ    | gi 22234045           | Bot.pH G 1 processed at H.D | BPP-CNP | 645.8 | +2 | 79 | DHHAALVGGGGGGGGGG |
| BJ    | gi 22234045           | Bot.pH G 1 processed at H.H | BPP-CNP | 476.6 | +3 | 65 | HDHHAAVGGGGGGGGGG |
| BJ    | gi 22234045           | Bot.pH G 1 + AP at C-terminus | BPP-CNP | 735.9 | +2 | 59 | <EAPRPFPAP |
| BJ    | gi 22234045           | Bot.pH G 1 | BPP-CNP | 522.2 | +3 | 66 | HHDDHAAVGGGGGGGGGG |
| BJ    | gi 22234045           | Bot.pH G 1 + V at N-terminus | BPP-CNP | 555.3 | +3 | 49 | VHDDHAAVGGGGGGGGGG |
| BJ    | gi 22234045           | BPP-10c + <EQWA at N-terminus | BPP-CNP | 855.4 | +2 | 54 | <EQWAQQWNPQDIPPE |
| BJ    | gi 22234045           | BPP-13a processed at Q.G | BPP-CNP | 942.4 | +2 | 40 | <EQWAQQWPRPQGPEIP |
| BJ    | gi 150438852          | BPP-14a | BPP-CNP | 842.9 | +2 | 53 | <EWAQQWPRPQ |
| BJ    | gi 150438852          | BPP-12c | BPP-CNP | 743.9 | +2 | 56 | <EWAQQWPRPQ |
| BC    | gi 231997             | Jarahgin (Bothrops jararaca) | SVM | 620.8 | +2 | 59 | S.VGQVOQVSINL |
| BC    | gi 231997             | Jarahgin (Bothrops jararaca) | SVM | 659.4 | +2 | 64 | S.VGQVOQVSINL |
| BC    | gi 231997             | Jarahgin (Bothrops jararaca) | SVM | 758.5 | +2 | 49 | S.VGQVOQVSINL |
| BC    | gi 231997             | Jarahgin (Bothrops jararaca) | SVM | 794.0 | +2 | 69 | S.VGQVOQVSINL |
| BC    | gi 22214994           | Metalloprotease BITM02A (Bothrops insularis) | SVM | 619.8 | +2 | 54 | P.QTLDSFGEWX |
| BC    | gi 6323638            | Metalloprotease (Echis ocellatus) | SVM | 619.8 | +2 | 54 | D.KTLDGSFGWR |
| BC    | gi 48427991           | Jerdotin (Trimeresurus jerdonii) | SVM | 813.4 | +2 | 51 | T.LSDATELDSFGEWX |
| BF    | gi 190411615          | Atroxylin-I (Bothrops atrox) | SVM | 1015.0 | +2 | 55 | L.INVPAAPOTLDSFGEWX |
| BF    | gi 2224846            | Acualycin-1 (Deinagkistrodon acutus) | SVM | 527.0 | +3 | 51 | H.LHEKKDLGFLSKDYS |
| BJ    | gi 190411615          | Atroxylin-I (Bothrops atrox) | SVM | 1015.0 | +2 | 55 | L.INVPAAPOTLDSFGEWX |
| BJ    | gi 231997             | KN BJ 2 (Bothrops jararaca) | SVM | 457.8 | +4 | 54 | K.IHLGVSHSKVPKN |
| BJ    | gi 162416185          | BmoomPafla-1 (Bothrops moojeni) | SVM | 559.3 | +2 | 52 | N.LEWSKSKDLI |
| BJ    | gi 38492529           | Bab1 (Bothrops asper) | SVM | 559.3 | +2 | 52 | N.LEWSKSKDLI |

and tri-peptides are lowered because of the venom spread and dilution process in the prey tissue. It is possible that during manipulation, the dilution of venom significantly changed the venom environment, causing the activation of proteolytic enzymes.

Therefore, the concept behind these experiments was to attempt to evaluate whether the observed complexity of the venom peptides was generated by the proteolysis of venom components upon the processes of lyophilization and resolubilization prior to mass spectrometric analyses. For this purpose, lyophilized venoms of BC, BF, and BJ were directly dissolved in a solution containing an inhibitor of SVSPs (2 mM PMSF) and 5 mM EDTA, for the inhibition of SVMPs, before the solid-phase extraction procedure. Peptides from the same toxin classes were identified in the inhibited venoms in comparison to those analyzed in the absence of inhibitors (supplemental Table 5). Interestingly, the SVMP insularinase A (gi 82197476), which was the most significant hit in the venom of BJ with 19 identified peptides (three unique peptides), was not identified in the venom analyzed in the absence of inhibitors. One possible reason for this finding is the fact that in the noninhibited BJ venom the protein may have been degraded by SVMPs and SVSPs to very short peptides that were not detected by the mass spectrometric analysis. The insularinase A 37-residue peptide, SVNVDASLANLEVWSKKDDSLIKVEKDSKTLTSFGEWR, which was identified 17 times in the inhibited BJ venom (Fig. 4; supplemental Table 6), has several cleavage sites compatible with SVMP and SVSP hydrolytic specificities (Supplemental Table 4), and other smaller peptides may have given nonsignificant hits in the database search of experiment carried out with the noninhibited venom.

As a general observation, a significantly lower number of peptides was identified in the inhibited venoms. The different number of total peptides identified in the venoms analyzed in the absence of inhibitor (331) as compared with the inhibited venoms (179) points out to a crucial role of SVMPs and SVSPs.
Moreover, the average peptide length of 18 amino acids in the noninhibited venoms increased to 21 in the inhibited venoms. In the case of LAAO, there were reductions of 68 and 82% in the number of total and unique identified peptides, respectively, and the average peptide size increased from 19 to 25 amino acids.

Similarly to the peptides of the noninhibited venoms, those detected in the inhibited venoms (179 peptides; supplemental Table 6) have amino acid sequences that suggest their origin is mainly the degradation of LAAO by SVSPs and SVMPs; nevertheless, the frequencies of these peptides have diminished in the inhibited venoms, as a result of the lower proteolytic activity. This observation is shown in the heat maps where the relative differences in occurrences of amino acids across P4-P4/H11032 are represented (Fig. 3). The decrease in frequencies of amino acids Asp at P4, Ser at P3, Gly at P2, Tyr at P1, Ile at P1’, Asn at P2’, Leu at P3’, and Gly at P4’, may be related to reduction of SVMP activity, according to their known specificities (Fig. 3; supplemental Table 4). It is worth to mention that the high frequencies of the amino acids Asn, Arg, Ala and Ser at positions P4, P3, P2, and P1 (Fig. 3), respectively, result from the large number of identified peptides derived from the C-terminal region of the LAAO from B. pauloensis (Bp-LAAO), which shows the sequence N-R-A-S.

To extend our analysis of the sequences of identified LAAO peptides detected in the noninhibited and inhibited venoms, all unique peptides of Bp-LAAO were selected and highlighted in the three-dimensional structure of Cr-LAAO (47). Some peptide sequences in Cr-LAAO are identical and others are homologous to the ones we found in the Bothrops venoms. In the Cr-LAAO structures shown in Fig. 5 it is possible to clearly visualize the differences in the regions of the LAAO structure that are affected by the venom proteinases in the venoms analyzed in the absence or in the presence of inhibitors. The lower number of peptides identified and lower sequence coverages from LAAO in the inhibited venoms are additional evidences that LAAO may be more susceptible to
the activity of SVSPs and SVMPs than the other venom components.

**Protein Identifications in the Venom of BC Collected and Fractionated in the Presence of Proteinase Inhibitors**—In another attempt to confirm the findings on the imbalance of venom proteome caused by the processes of venom lyophilization and resolubilization we performed another set of experiments in which the venoms of six specimens of *B. cotiara* were collected directly from the fangs into plastic tubes containing concentrated solutions of EDTA, PMSF, or both, so that the venom solutions contained final concentrations of 5 mM EDTA, or 2 mM PMSF, or both, and were minimally diluted. Interestingly, the LC-MS/MS analysis of BC fresh venom samples resulted in the identification of peptides derived only from Bp-LAAO from *B. pauloensis* (Table III). We observed that treatment of the venoms with EDTA or EDTA plus PMSF resulted in the identification of the same single LAAO peptide, whereas treatment with only PMSF or water (control) resulted in the identification of four and two peptides, respectively. The superposition of peptides identified in the fresh venom of BC in the crystallographic structure of Cr-LAAO (supplemental Figs. 2 and 3), clearly showed that the inhibition of BC venom proteinases immediately after milking resulted in less cleavage of LAAO as compared with the inhibition after lyophilization and to no inhibition (Fig. 5).

The sequence of the identified peptides showing the presence of Ala, Phe, Ile, Leu, and Tyr at their N termini (Table III) are compatible with SVMP activity (supplemental Table 4).

Interestingly, the LAAO peptide AGEYTAQAHGWIDSTIKSGL-TAARDVNRAS was detected in all venom samples, which suggests it may be an endogenous peptide. In the venom samples which did not contain EDTA more LAAO peptides were found (Table III) indicating that SVMPs play a key role in the proteolytic degradation of LAAO when the venom solution is submitted to chemical conditions different from that of the venom gland.

To extend the identification of peptides in the fresh BC venom samples we assembled a peptide database with all manually sequenced peptides, including BPPs and venom protein fragments (this study) and all known *B. jararaca* BPPs (Supplemental peptide database). The database contains 224 sequences and includes all putative peptide variants resulting from the presence of indistinguishable residues of I/L or Q/K. The search of LC-MS/MS spectra using this database resulted in the identification of additional peptides including the 39-residue LAAO peptide of BC venom and other fragments derived from it (Table IV). Interestingly, two of the new BPPs (BPP-10e and BPP-12d) identified in the BC venom were also detected in all fresh venom samples (Table IV). As observed in the results obtained by the automated NCBInr database search (Table III), the peptide sequences identified using the in-house peptide database indicate that the SVSPs do not contribute to the indiscriminate proteolysis of BC venom proteins. Hence, the overall analysis of the effects of proteinase inhibitors on the protection against degradation of venom components during sample processing points out the main
role that SVMPs play in the generation of protein fragments that increase the complexity of venom peptidomes.

Potentiation of the Hypotensive Effect of Bradykinin by New BPPs—To verify whether the new identified BPPs were biologically active, the synthetic replicates of five of them were tested for the ability to potentiate the effect of bradykinin (BK) upon mean arterial pressure (MAP). As a positive control in these experiments we used BPP-10c (\textit{<ENWPHPPMPPA}}, where \textit{<E} represents pyroglutamic acid), which is the most selective inhibitor of the active site at the C-domain of sACE (53), and displays a strong and sustained antihypertensive effect in spontaneously hypertensive rats (SHR) independently of the inhibition of sACE (90). The evaluation of the effects of the new BPPs upon the hypotension induced by 0.5 μg of BK (Figs. 6A, 6C, and 6D). Despite the slight reduction on blood pressure caused by BPP-10d, BPP-11f, and BPP-11h, the amplitude of these effects was not similar to that found for BK (Figs. 6B, 6E, and 6F). When evaluating the

| Venom treatment | Acc. number | Protein | Snake | Mascot score | Total peptides | Unique peptides | Sequence |
|-----------------|-------------|---------|-------|--------------|----------------|----------------|----------|
| EDTA gi 195927838 | LAAO Bothrops pauloensis | 59 | 1 | F.AGEYTAQAHGWIDSTIKSGLTAARDVNRAS.E |
| PMSF gi 195927838 | LAAO Bothrops pauloensis | 219 | 4 | F.AGEYTAQAHGWIDSTIKSGLTAARDVNRAS.E |
| EDTA+PMSF gi 195927838 | LAAO Bothrops pauloensis | 66 | 1 | F.AGEYTAQAHGWIDSTIKSGLTAARDVNRAS.E |

### TABLE III

Proteins from which peptides were identified by LC-MS/MS analysis and database search in the venom of BC collected in the presence of proteinase inhibitors

| Venom treatment | Peptide | Protein | m/z | Charge | Peptide score | Sequence |
|-----------------|---------|---------|-----|--------|---------------|----------|
| EDTA BPP-12d | 60 | 627.9 | 2 | 18 | <ENWPHPPMPPA |
| PSSF | 40 | 592.4 | 2 | 22 | <ENWPHPPMPPA |
| EDTA+PMSF | 46 | 627.9 | 2 | 15 | <ENWPHPPMPPA |
| Water | 33 | 620.9 | 2 | 14 | <ENWPHPPMPPA |

### TABLE IV

Peptides identified by LC-MS/MS analysis and database search in the venom of BC collected in the presence of proteinase inhibitors using a database compiled from de novo sequenced peptides (supplemental Peptide Database)

| Venom treatment | Peptide | Protein | m/z | Charge | Peptide score | Sequence |
|-----------------|---------|---------|-----|--------|---------------|----------|
| EDTA | BPP-12d | 60 | 627.9 | 2 | 18 | <ENWPHPPMPPA |
| PSSF | 40 | 592.4 | 2 | 22 | <ENWPHPPMPPA |
| EDTA+PMSF | 46 | 627.9 | 2 | 15 | <ENWPHPPMPPA |
| Water | 33 | 620.9 | 2 | 14 | <ENWPHPPMPPA |

\textit{a} MASCOT score for the identification considering the whole peptide entry listed in the database.

\textit{b} MASCOT score for the single peptide or peptide fragment identified.
potentiation of BK hypotensive effect we observed that 60 nmoles of BPP-10e and BPP-10f potentiated BK effect above 100%. It is worth to emphasize that the time for achieving maximal BK potentiation was different for each active peptide (Table V). BPP-10e and BPP-10f caused BK potentiation equivalent to BPP-10c (113% ± 15%, 101% ± 13, and 120% ± 23%, respectively). When compared with the structure of BPP-10c, BPP-10e, and BPP-10f show 3 and 4 amino acid substitutions, respectively (Table V). However, these differences do not seem to be important for the potentiation of

Fig. 6. Evaluation of BK hypotensive effects in anesthetized Wistar rats before and after the administration of BPPs. Intravenous bolus injection of each BPP was made at the dose of 60 nmol. A, BPP-10c (n = 4); B, BPP-10d (n = 5); C, BPP-10e (n = 4); D, BPP-10f (n = 5); E, BPP-11f (n = 5) and F, BPP-11h (n = 4). Data are expressed as mean ± S.E. * p < 0.05; ** p < 0.01 and *** p < 0.001 compared with the values obtained with 0.5 μg of BK before peptide injection.
BK hypotensive effect. In addition, the single difference between BPP-10e and BPP-10f sequences at position 2 did not affect the range of BK potentiation. Interestingly, all positions occupied by Pro residues in BPP-10c are preserved in BPP-10e and BPP-10f sequences (Table V). Therefore, it is important to consider that the restriction of peptide structural solution by Pro residues could be favorable to their biological activities, because Pro could play distinct roles in the structure of peptides/proteins (91). The BK potentiation by BPP-11f was lower than that of BPP-10c and other active peptides (75% ± 6%), although its C-terminal sequence also shows conserved Pro residues. Surprisingly, BPP-10d and BPP-11h did not potentiate the BK hypotensive activity at the dose of 60 nmol. The substitutions to Pro and Met residues at positions 7 and 8 of BPP-10c, respectively, are possibly the reason for the lack of BK potentiating activity by BPP-10d (Table V). In the case of BPP-11h, several sequence differences when compared with BPP-10c (length of peptide sequence; absence of Pro-Pro doublet at C terminus and several amino acid substitutions) may be related to its inability to potentiate BK hypotensive effect.

Inhibition of somatic angiotensin-converting enzyme (sACE) is a widely used approach in the treatment of hypertension and the first available competitive inhibitors of sACE were the naturally occurring BPPs from the venom of B. jararaca. Various BPPs have been shown to be able to inhibit the activity of sACE (53, 63). To further analyze the activities of the novel BPPs we tested their ability to inhibit the activity of sACE upon the peptide substrate Abz-Phe-Arg-Lys (2,4-dinitrophenol)-Pro-OH whereas BPP-10c was used as a positive control. The five new BPPs were able to inhibit sACE activity showing apparent $K_{\text{app}}$ values ranging from 2 to 87 $\mu$M (Table VI). BPP-10c was used as a control in the experiment and showed a $K_{\text{app}}$ value of 8.4 $\mu$M. Interestingly, BPP-10d and BPP-11h were not able to potentiate the effect of BK upon MAP (Fig. 6), however they inhibited sACE activity. These results are in line with previous findings showing that BPP-10c and other BPPs display a strong and sustained anti-hypertensive effect in SHR, by a pharmacological effect independent of ACE inhibition (90, 92, 93).

Taken together, our results indicate that the similar amino acid sequence features of new peptides identified by mass spectrometry are not enough to determine BK-potentiating activity. In light of previous reports on the structure and function relationship of BPPs (50, 60, 90, 94), our current data show that the presence of a pyroglutamic acid residue at the N-terminal position and a Pro-Pro doublet at the C terminus in BPP sequences are not enough requirements for the peptide to display BK potentiating activity.

**CONCLUSIONS**

This is the first in-depth study of sampling procedure-related proteomic degradation in snake venoms. In a previous study significant differences were observed in the venom proteomes of *B. cotiara* and *B. fonsecai*, and in this work we included *B. jararaca* and described differences in their venom peptidomes by MALDI-MS/MS and ESI-MS/MS followed by de novo sequencing and automated database search. Numerous variations in the BPPs sequences were found in each venom whereas no single BPP was simultaneously common to the three venoms. Five of the newly sequenced BPPs were synthesized and tested for their ability to potentiate bradykinin hypotensive effect and to inhibit sACE activity. There are still various new peptides to be synthesized and tested for their biological activities, as an illustration of the potential of snake venoms as sources of bioactive molecules. The presence of peptides from LAAO, SVMP and SVSP families in the venoms seems to be a result of the proteolysis of venom components after milking and lyophilization prior to fractionation and MS analysis. We suggest that upon lyophilization and resolubilization for fractionation processes the venom solution stability is disrupted by factors such as the dilution of venom proteins and of tri-peptides that inhibit SVMPs, the lowering of citrate concentration, and the pH imbalance, so that abundant venom proteinases can exert their catalytic activity upon other
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venom proteins. We conclude that sampling procedures can substantially influence the proteomic and peptidomic complexity of snake venoms. Although it is still true that lack of comprehensive snake venom sequence databases for analysis of MS data hampers to a certain degree proteomic/peptidomic studies, the use of complementary methodologies of MS analysis and spectra identification, especially extensive de novo sequencing, boosts the exploration of the largely underestimated complexity of venom peptidomes.

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This article contains supplemental Figs. 1 to 3 and Tables 1 to 6, Database and Spectra.

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