Proteoforms of the platelet-aggregating enzyme PA-BJ, a serine proteinase from Bothrops jararaca venom

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A B S T R A C T
Snake venoms contain serine proteinases that are functionally similar to thrombin and specifically cleave fibrinogen to convert it into fibrin or activate platelets to aggregation. PA-BJ is a serine proteinase from Bothrops jararaca venom that promotes platelet aggregation and this effect is mediated by the G-coupled protein receptors PAR1 and PAR4. In this study we describe an improved procedure to obtain PA-BJ from B. jararaca venom that uses less chromatographic steps, and, interestingly, results in the isolation of eight proteoforms showing slightly different pl values and molecular masses due to variations in their glycosylation levels. The identity of the isolated PA-BJ forms (1−8) was confirmed by mass spectrometry, and they showed similar platelet-activating activity on washed platelet suspensions. N- and O-deglycosylation of PA-BJ 1−8 under denaturing conditions generated variable electrophoretic profiles and showed that some forms were resistant to complete deglycosylation. Furthermore, N- and O-deglycosylation under non-denaturing conditions also showed different electrophoretic profiles between the PA-BJ forms and caused partial loss of their ability to cleave a recombinant exodomain of PAR1 receptor. In parallel, three cDNAs encoding PA-BJ-like enzymes were identified by pyrosequencing of a B. jararaca venom gland library constructed with RNA from a single specimen. Taken together, our results suggest that PA-BJ occurs in the B. jararaca venom in multiple proteoforms displaying similar properties upon platelets regardless of their variable isoelectric points, molecular masses, carbohydrate moieties and susceptibility to the activity of glycosidases, and highlight that variability of specific venom components contributes to venom proteome complexity.

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1. Introduction
Snake venom proteomes are complex mixtures comprised of distinct protein families and peptides with biological activity. The composition of snake venoms is a result of multiple factors, and its inherent variability is an intriguing aspect of the evolution of venomous snakes, which is often related to environmental and ecological traits, and may change from species to species. Although there is not a general pattern, the causes of variability are often related to the snake diet in addition to geographical and biological factors, such as sexual dimorphism, and ontogenetic changes in diet habits [1–7]. In a recent investigation of the mechanisms governing venom variation in six related medically important viperid snakes (Echis ocellatus, Echis coloratus, Echis pyramidum leakeyi, Echis carinatus sochureki, Bitis arietans and Cerastes cerastes) it has been demonstrated that different levels of toxin transcription, translation, and posttranslational modification have a significant impact on the resulting proteome [8].

Variability of specific venom components has frequently been observed. Phospholipases A2 and serine proteinases are among the most abundant enzymes found in snake venoms and diverse proteoforms of these enzymes have been identified which vary in their biophysical and biochemical properties [9–12]. These enzymes have diversified amino acid sequences and display a variety of biological activities. Protein glycosylation is a key post-translational modification important to a range of biological phenomena. Glycosylation is a frequent post-translational modification of snake venom proteins, which significantly contributes to venom proteome complexity [4,13–17]. Members of a given toxin family may be differentially glycosylated or not glycosylated, while some specific toxins may display variable glycosylation levels and are recognized as isozymes or proteoforms, however, very little is known about the structure and size of carbohydrate moieties present in venom glycoproteins [18–21].

Snake venom serine proteinases (SVSPs) may affect hemostatic pathways by limited proteolysis that activates components involved in coagulation, fibrinolysis and platelet aggregation or by unspecific
proteolytic degradation [11,22]. A comparison of the exon/intron organ-
nization of the proteinase regions of serine proteinase genes, and of the organization of the gene encoding the fibrinogen-clotting enzyme batroxobin, indicated that SVSPs evolved from glandular kallikrein, a tryptsin-like serine proteinase [23]. In addition, there are evidences at the transcriptomic level that the SVSPs originated from kallikrein [24, 25]. Despite the fact that most SVSPs show some specific activity on components of the coagulation cascade, various lines of evidence suggest that the SVSPs, which share a common tertiary structure with trypsin, do not belong to the thrombin gene family and evolved to serve other functions. Most SVSPs are single-chain glycoproteins showing around 233 amino acid residues and a variable number of putative N- or O-glycosylation sites in sequence positions that are not conserved. Bothrops venoms contain various serine proteinases that function similarly to enzymes of the coagulation/fibrinolyis system resulting in an imbalance of hemostasis that is an important part of the envenomation process [11,26,27]. In the venom transcriptome/proteome of Bothrops jararaca the abundance of serine proteinases increases from −3% to −8% upon newborn to adult transition [14,28].

PA-BJ is a basic serine proteinase of B. jararaca venom that has been extensively characterized. PA-BJ causes 95% platelet aggregation in platelet-rich plasma at a concentration of 10−7 M. On washed platelet suspensions it causes 40% aggregation at a concentration of 10−7 M [29,30]. Its amino acid sequence was determined by Edman degradation and shows one N-glycosylation (Asn20) and one O-glycosylation site (Ser23). The effect of PA-BJ on platelets is mediated by the thrombin receptors PAR1 and PAR4, which are platelet receptors attached to G protein [31]. In vitro, PA-BJ cleaves the recombinant exodomain of human receptor PAR1 on the Arg41–Ser42 and Arg46–Asn47 bonds, resulting in the inactivation of the tethered ligand. Moreover, PA-BJ promotes the release of calcium in fibroblasts transfected with PAR4 and makes these cells resistant to the action of thrombin [31]. PA-BJ cleaves p-nitroanilide from several synthetic substrates containing Arg at the scissile bond. The benzamidine derivative N-[2-naphthylsulfonyl]-γ-lycidyl]-4-aminophenylalanine lidipider inhibits the amidolytic activity of PA-BJ with a Ki value of 15 μM. The platelet-aggregating activity of PA-BJ is also inhibited by heparin and by monoclonal antibodies recognizing PAR1 but not by hirudin and thrombomodulin [31].

PA-BJ has been isolated from the venom of B. jararaca by a multi-step procedure composed of precipitation with 50–90% ammonium sulfate followed by chromatographic steps on DEAE-Sephacel, SP-Sephadex C-50 and Mono-S, including a recombinant chromatography on the latter, which results in a homogeneous preparation of the enzyme [29]. The goal of this study was to establish a less complex method to purify PA-BJ and, interestingly, the procedure we used enabled us to identify eight different proteoforms of PA-BJ (PA-BJ 1–8) showing variable isoelectric points and molecular masses, however, displaying similar activity upon human platelets. To further elucidate the relationship between structure and function of PA-BJ we explored its carbohydrate moieties by submitting the proteoforms to deglycosylation with N- and O-glycosidase under non-denaturing and denaturing conditions, and compared their activities upon the recombinant exodomain of receptor PAR1. Furthermore, we examined the possibility of the existence of transcripts encoding SVSPs similar to PA-BJ in the venom gland of B. jararaca.

2. Materials and methods

2.1. Isolation of PA-BJ

Lyophilized B. jararaca venom (100 mg) (Institute Butantan, São Paulo, Brazil) was dissolved in 2 mL of 50 mM sodium phosphate buffer pH 7.0 (Buffer A), and the turbid solution was clarified by centrifugation (1600 g; 20 min; 4 °C) and the supernatant was submitted to cationic exchange chromatography on a HiTrap 16/10 SP XL column (GE Healthcare) using Buffer A and 50 mM sodium phosphate buffer pH 7.0 plus 1 M NaCl (Buffer B) for elution in an Äkta purifier FPLC system (GE Healthcare) (Fig. 1). Fractions containing PA-BJ were submitted to cationic exchange chromatography on a Mono S HR 5/5 column (GE Healthcare) using the same buffers of the previous chromatography. The resulting fractions eluted with 20–35 mL were combined in three pools (Fig. 2). To improve the separation of PA-BJ different forms, these three pools were separately rechromatographed on the Mono S HR 5/5 column using a decreased concentration of the NaCl gradient or a higher volume of the NaCl gradient (Fig. 3). After each chromatography the electrophoretic profile of some fractions was visualized by SDS-PAGE (12% polyacrylamide gel). Before submitting each protein pool to the next chromatographic step they were lyophilized, redissolved in ultra-purified water, dialyzed in Buffer A and the concentration of proteins was determined.

2.2. Analytical procedures

Protein concentrations were determined by the Bradford protein assay using bovine serum albumin as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to [32] and gels were stained with silver.

2.3. Mass spectrometric identification of PA-BJ

Protein bands containing PA-BJ 1–8 were excised from the SDS-polyacrylamide gel and submitted to in-gel trypsin digestion [33] and mass spectrometric analysis by LC–MS/MS. For the mass spectrometric analysis 5 μL of the resulting peptide mixture was separated by reversed-phase chromatography in 5 cm Jupiter column (Phenomenex) packed with 10 μm C18 resin in tandem with a 10 cm Aqua column (Phenomenex) packed with 5 μm C18 resin at a flow rate of 200 μL/min in an Easy-nLC II (Thermo Scientific). The elution gradient was 5–40% acetonitrile in 0.1% formic acid over 25 min. The eluate was applied to 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 positive mode, and spectra were acquired in the m/z range of 200–2000 and resolution R = 30,000. The top 10 method was used to proceed with collision-induced dissociation. For the MS2 the following parameters were used: dynamic exclusion duration of 15 s; repeat duration of 30 s; exclusion list size of 500. Resulting fragment spectra were searched using Mascot (version 2.4.1) against the NCBI NR database restricted to the taxa Serpentes (25 211 entries; downloaded on June 20th, 2011), with a peptide mass tolerance of 10 ppm and fragment mass tolerance of 0.5 Da. Lodoacetamide derivative of cysteine and oxidation of methionine were specified in Mascot as fixed and variable modifications, respectively. Alternatively, the search of spectra was carried out against the sequences of cDNAs encoding PA-BJ-like proteins (BJARSVSP14, BJARSVSP16 and BJARSVSP22), using the same criteria described above.

2.4. LC–MS analysis of PA-BJ 1–8

LC–MS profiling of intact PA-BJ 1–8 was performed using an Easy-nLC II (Thermo Scientific) nanoLC system coupled on-line with a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). Samples of PA-BJ 1–8 (5 μg/5 μL) were loaded onto a 5 cm Jupiter column (Phenomenex) packed with 10 μm C18 resin in tandem with a 10 cm Aqua column (Phenomenex) packed with 5 μm C18 resin at a flow rate of 200 μL/min in an Easy-nLC II (Thermo Scientific). Chromatography was performed with solvent A (0.1% formic acid in deionized water) and B (0.1% formic acid in acetonitrile) at a flow rate of 200 μL/min using the following gradient: to 3% B in 1 min; to 60% B in 60 min; to 85% B in 3 min; hold at 85% B for 5 min; then back to 3% B in 1 min; hold at 3% B for 21 min. Spray voltage was set at 2.1 kV and the instrument was operated in full scan (MS1) mode only (in the m/z range of 300–2000) with a resolution at 400 m/z 100,000. Spectra deconvolution was carried out using the software Protein Deconvolution v. 3.0 (Thermo Scientific).
2.5. Sequencing and identification of PA-BJ-like cDNAs

Total RNA from the venom glands of a single specimen of *B. jararaca* was extracted with TRIZOL Reagent (Life Technologies) and mRNA was prepared with Dynabeads® mRNA DIRECT kit (Life Technologies). mRNA was quantified by Quant-iT™ RiboGreen® RNA reagent and Kit (Life Technologies). A cDNA library was constructed from 500 ng of mRNA using cDNA Synthesis System kit (Roche Diagnostics). The library was pyrosequenced in a GS Junior 454 Sequencing System (Roche Diagnostics) following the manufacturer’s protocols. Sequences were assembled with Newbler 2.7 (Roche Diagnostics), which removed adaptors and ribosomal RNA sequences in an initial step. A minimum overlap length of 80% of the read and a minimum identity of 98% in the overlap were set, with the other parameters set as the software default. Isotigs were subjected to a Blast search against serine proteinases downloaded from GenBank and UniProt databases with the BlastX algorithm, and those showing a high degree of similarity to PA-BJ were selected and checked by remapping reads to consensus and verifying each base position to confirm differences between the isoforms using CLC Genomics Workbench (CLC, Qiagen).

2.6. Deglycosylation of PA-BJ proteoforms

For deglycosylation under denaturing conditions, samples of PA-BJ 1–8 (1 μg) were incubated in 10% SDS for 1 min at 95 °C. After adding 0.02 M sodium phosphate, 0.08% sodium azide, 0.01 M EDTA, 2% Triton X-100, pH 7.0, and the incubation was prolonged for 2 min at 95 °C. After cooling, 0.1 U of N-glycosidase F or 0.2 mU of O-glycosidase (Roche) was added, and the mixture was incubated for 18 h at 37 °C.

For deglycosylation under non-denaturing conditions PA-BJ 1–8 samples (1 μg) were incubated in 0.02 M sodium phosphate buffer, pH 7.2, 0.01 M EDTA, with 0.1 U of N-glycosidase F or 0.2 mU of O-
Rechromatography on Mono S of fractions from *B. jararaca* venom containing platelet-aggregating activity. The pools 1–3 from the chromatography on Mono S (indicated by double-headed arrows on Fig. 2) were equilibrated in 50 mM phosphate buffer pH 7.0 and submitted to rechromatography on a Mono S HR 5/5 using 50 mM phosphate buffer pH 7.0 (Buffer A) and 50 mM phosphate buffer pH 7.0 containing 1 M NaCl (Buffer B) for elution in an Äkta purifier FPLC system. The arrows indicate the peaks containing the isolated proteoforms of PA-Bj. (A) rechromatography of pool 1; (B) rechromatography of pool 2; (C) rechromatography of pool 3.
glycosidase (Roche), containing 1% Triton X-100® (Sigma) for 18 h at 37 °C.

After incubation with the glycosidases the mixtures were submitted to SDS-PAGE (12% polyacrylamide gel) and proteins were stained with silver.

2.7. Homology modeling of PA-BJ

A tridimensional model of native PA-BJ [29] was generated using the Modeller 9v12 program [34] and the crystal structure of TSV-PA, a SVSP which is a plasminogen activator from the venom of Trimeresurus stejnegeri (residues 25–258; PDB entry 1BQY) and shows 74% sequence identity to PA-BJ, as a template. The modeled region includes 232 residues (6–237) of PA-BJ (Uniprot entry P81824). The best model was chosen according to the MODELLER objective function and stereo chemical analyses using PROCHECK [35], Verify 3D [36] and WhatIf [37]. Angle distortions and rotamers were corrected using COOT [38]. The figures were prepared using ‘The PyMOL Molecular Graphics System’, Version 1.2.3pre, Schrödinger, LLC. (http://www.pymol.org).

2.8. Platelet aggregation assays

Platelets were isolated from fresh blood donations. The washed platelet suspensions (WPS) were prepared as described by Antunes et al. [39], and adjusted to 5 × 10^11/L in Tyrode buffer pH 7.4 containing 2 mM CaCl2. Aliquots of 0.4 mL of WPS were kept at 37 °C for 1 min before the addition of 0.01 mL 50 mM phosphate buffer, pH 7.0 (control) or 0.01 mL PA-BJ proteoforms (1–8) (1 μg in 50 mM phosphate buffer, pH 7.0) with bar stirring speed set at 1200 rpm at 37 °C in the aggregometer Chrono-log 490 for 10 min. The aggregometer was calibrated by setting WPS to 0% and PBS to 100% aggregation.

2.9. Hydrolysis of PAR1

The recombinant soluble PAR1 exodomain, TR78 (residues 26–103, molecular mass 8925 Da), was produced in Escherichia coli at the C-terminal portion of a fusion protein, ketosteroid isomerase, and purified by RP-HPLC using a C-18 column and eluted at 35% acetonitrile as described by Kuliopulos et al. [40]. TR78 (2 μg) was incubated with PA-BJ 1–8 (20 ng) (enzyme to substrate ratio of 1:100 (w/w)) in 0.1 M Tris-HCl, pH 8.0 for 2 h at 37 °C. The proteolysis profile was analyzed by SDS-PAGE (15% SDS-polyacrylamide gel).

3. Results and discussion

3.1. Isolation of PA-BJ

In order to achieve a less complex method to isolate PA-BJ, the venom of B. jararaca was submitted to cationic exchange chromatography on a HiPrep 16/10 column and fractions containing PA-BJ (Fig. 1) were submitted to cationic exchange chromatography on a Mono S HR 5/5 column. All resulting fractions from the Mono S column contained PA-BJ and showed slightly variable electrophoretic profiles and molecular masses by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and increasing isoelectric points, as they were eluted by increasing concentrations of NaCl (Fig. 2). To improve the separation of PA-BJ different forms, the protein fractions of the Mono S column were combined in three pools which were separately rechromatographed and eluted using a decreased concentration of the NaCl gradient and using a higher volume for the NaCl gradient compared to the previous chromatography (Fig. 3). The rechromatography of pool 1 resulted in the separation into PA-BJ forms 1–4, whereas pool 2 resulted in PA-BJ forms 5–7, which eluted in partially separated peaks (Fig. 3). Rechromatography of pool 3 resulted in the separation of PA-BJ 8 from a minor contaminant (Fig. 3).

The determination of the molecular mass of PA-BJ forms 1–4 by SDS-PAGE under reducing conditions showed values that varied between 30 and 32 kDa, while PA-BJ forms 5–8 are more basic and showed higher molecular masses (33–38 kDa) (Fig. 4A; Supplemental Table 1). The molecular masses of PA-BJ 1–8 were also determined by submitting the intact samples to liquid chromatography coupled to electrospray-ionization (ESI) mass spectrometry. Deconvolution of the multiple-charge spectra yielded isotope-averaged molecular masses for the most intense signal of each PA-BJ form that ranged from ~25 kDa to ~30 kDa (Supplemental Fig. 1), however, in all samples, besides the most intense signal, other low signals corresponding to close molecular masses were detected indicating the microheterogeneity of the isolated PA-BJ forms. As PA-BJ is a glycosylated protein, the LC–MS profiles showing microheterogeneity suggest the possible presence of different glycan structures at the glycosylation sites of the isolated PA-BJ proteoforms. Nevertheless, the molecular masses of intact PA-BJ 1–8 obtained by LC-MS are lower than those obtained by SDS-PAGE analysis of reduced samples possibly due to the fact that under reducing conditions the absence of disulfide bonds makes proteins less compact and thereby might influence their mobility on the gel.

Protein bands containing PA-BJ 1–8 (Fig. 4A) were excised and submitted to in-gel trypsin digestion and mass spectrometric analysis by LC–MS/MS. The analysis resulted in the identification of PA-BJ forms by the sequencing of various fragments with high score (Supplemental Table 1), which matched the sequence of PA-BJ obtained previously by Edman degradation [29] (Uniprot entry P81824), however, the putative tryptic peptide that contains the N- and O-glycosylation sites of PA-BJ (KSLVLVNSLSLCSSGTVVVLAAHCS.K) was not detected among the spectra due to its long sequence. The identity of the eight PA-BJ proteoforms was further confirmed by testing their ability to activate platelets. PA-BJ 1–8 (1 μg) showed similar potencies to aggregate washed human platelet suspensions (Supplemental Fig. 2), indicating that their different glycosylation levels do not play a role in their ability to trigger the aggregation process. Two other SVSPs, KN-BJ and TL-BJ, are present in the venom of B. jararaca in different proteoforms (KN-BJ 1-2 and TL-BJ 1-3) that also differ in isoelectric point and molecular mass [18,41] however display similar kinin-releasing and fibrinogen-coagulant activities, respectively.

3.2. Deglycosylation of PA-BJ proteoforms

We next assessed the presence of carbohydrate moieties in the PA-BJ proteoforms by submitting them to N- and O-deglycosylation under non-denaturing conditions. N-glycosidase F is an enzyme able to remove Asn-linked oligosaccharides (Asn-GlcNAc-glycans) from the protein backbone while O-glycosidase is used to release the Gal-beta(1,3)GalNAc unit from O-glycans (attached to Ser or Thr residues). As shown in Fig. 4B, only a very small fraction of the PA-BJ 1–4 forms was susceptible to N-glycosidase F and O-glycosidase deglycosylation when the protein samples were not denatured prior to incubation with the glycosidases, as detected by the generation of a very faint protein band of ~25 kDa which corresponds to the deglycosylated form. On the other hand, PA-BJ 5–8 forms likely contain larger carbohydrate moieties than PA-BJ 1–4, as judged by their higher molecular masses (Fig. 4A), and showed different electrophoretic profiles after N- and O-deglycosylation under non-denaturing conditions (Fig. 4B). The glycosidases promoted only partial deglycosylation of PA-BJ 5–8, as observed by shifts of their apparent molecular masses. Interestingly, these forms of PA-BJ showed variable profiles of protein bands under non-denaturing deglycosylation conditions indicating that they display different susceptibilities to the activity of the glycosidases. Indeed, only a small amount of PA-BJ 5–6 was apparently fully deglycosylated while PA-BJ 7–8 were not (Fig. 4B).

Denaturation of proteins increases the deglycosylation rate considerably. However, when PA-BJ 1–8 were denatured with SDS prior to the incubation with the glycosidases they showed variable deglycosylation profiles and not all forms were fully deglycosylated (Fig. 4C and D). The electrophoretic profiles of PA-BJ 1–8 after N-deglycosylation under denaturing conditions revealed the apparent complete deglycosylation
of forms 1, 2, 5, 6 and 7, and the resistance of PA-BJ forms 3, 4 and 8, which underwent only partial deglycosylation (Fig. 4C). Moreover, in the case of PA-BJ forms 3, 5, 6 and 7, multiple protein bands of molecular masses of \(-17\) kDa and lower have been generated, which indicates a certain degree of instability of these proteins after N-deglycosylation (Fig. 4C). O-Deglycosylation under denaturing conditions also revealed variable electrophoretic profiles between the PA-BJ forms. Only PA-BJ 4 was nearly fully O-deglycosylated and showed a molecular mass of \(-26\) kDa. PA-BJ 1–3 and 5–8 were partially O-deglycosylated and showed variable molecular masses above 25 kDa, which correspond to their partially deglycosylated polypeptide chains (Fig. 4D). Moreover, although at different degrees, after O-deglycosylation PA-BJ forms showed additional bands of molecular mass of \(-17\) kDa and below, as a result of the possible structure instability induced by deglycosylation (Fig. 4D). Glycosylation is one of the major post-translational modifications in viperid venoms, and, as in other eukaryotic proteomes, should significantly affect protein folding, conformation, and activity. Likewise, glycosylation confers stability on secreted glycoproteins and although it is not a strict requisite for correct folding, unglycosylated proteins are unstable. Taken together, our results indicate that the carbohydrate
moieties of the isolated PA-BJ forms are variable regarding the size, access to the activity of N- and O-glycosidase. Moreover, removal of oligosaccharide chains likely affects the thermodynamic stability of some of the PA-BJ proteoforms and makes them prone to degradation in solution, however, these different structural features do not seem to affect their ability to activate platelets, which is similar (Supplemental Fig. 2).

With the purpose of investigating the spatial location of the N- and O-glycosylation sites of PA-BJ, a tridimensional model of the protein was predicted by homology modeling using the crystal structure of TSV-Pa, a plasmaminogen activator from T. stejnegeri venom, as a template. As shown in Fig. 4E, PA-BJ belongs to the typical α/β-hydrolase fold of serine proteinases where the residues of catalytic triad are located at the junction of the two six-stranded beta-barrels. PA-BJ has two glycosylated sites at Asn20 and Ser23, but the proportions of the carbohydrates within the isolated eight forms may be different, as judged by their variable molecular masses. Nevertheless, the location of these carbohydrate chains in the modeled structure seems to be near the entrance to the catalytic cleft (Fig. 4E) and even if they protruded into the cleft or into the space in front of the entrance to it, they would apparently not hinder the interaction between PA-BJ and its ligands, which likely occurs via exosites I and II located quite distant from the active center [42]. On the other hand, the presence of carbohydrate chains near the access to active site could hinder the interaction of PA-BJ with serpins, and this seems to be the case, since all forms are capable of activating platelets in platelet rich plasma (not shown) and hence escape inhibition by plasma proteins.

3.3. Glycosylation and the activity of PA-BJ proteoforms on PAR1

In order to assess the effect of the variable carbohydrate chains in the proteolytic activity of PA-BJ 1–8 these were incubated with the recombinant exodomain of receptor PAR1 (TR78; 8925 Da) at a 1:100 enzyme-to-substrate ratio for 2 h at 37 °C. As shown in Fig. 5A, under these conditions, PA-BJ 1–7 cleaved the protein and generated a main product of ~6 kDa while PA-BJ 8 was only capable of degrading it partially. We next tested the ability of the PA-BJ forms submitted to N- and O-deglycosylation under non-denaturing conditions to cleave PAR 1 (Fig. 4B). Fig. 5B shows that apparently, except for PA-BJ 2, all other partially deglycosylated enzymes had their ability to cleave PAR 1 impaired, suggesting that the removed carbohydrate chains might play a role in the interaction of PA-BJ with the recombinant exodomain of receptor PAR1 in vitro. Alternatively, the partially removed carbohydrate moieties could be important to keep the tertiary structure of the PA-BJ forms and hence their proteolytic activity.

The presence of PA-BJ in different proteoforms showing variable isolectric points and molecular masses in the venom of B. jararaca is intriguing and likely occurs as a result of molecular fine-tuning via accelerated evolution [9] to diversify the toxin arsenal upon evolution [40]–[43] to deal with different types of prey. The role of structural N- and O-glycans in the function of SVSPs is poorly known and studies aimed at identifying their carbohydrate units as well as the mutation of sequons of these enzymes with their ligands.

3.4. Identification of PA-BJ-like cDNAs in the venom gland of B. jararaca

The presence of multiple isoform forms of serine proteinases in snake venom glands has been reported in the Viperidae family. We next examined the possibility of the existence of transcripts encoding SVSPs similar to PA-BJ in the venom gland of B. jararaca, using a next generation sequence approach. Hence, RNA from the venom glands of a single specimen of B. jararaca was extracted and used to construct a cDNA library, which was submitted to pyrosequencing. As a result, three full-length cDNAs encoding PA-BJ-like serine proteinases (BJARSVP14, BJARSVP16 and BJARSVP22) were identified (Fig. 6A). An alignment of the sequence region corresponding to the mature form of these serine proteinases and PA-BJ showed 83% of amino acid sequence identity. The theoretical isoelectric points of the mature forms of these PA-BJ-like proteins range from 9.37 to 9.59 and are slightly above that of PA-BJ (9.08) (Fig. 6B). In addition to the N-glycosylation site at Asn20, which has been determined by Edman degradation sequencing [29], the three PA-BJ-like enzymes show one putative N-glycosylation site at the C-terminal region that is absent in PA-BJ (Fig. 6A). Although the transcripts of PA-BJ-like proteins also contain a conserved Ser residue at position 23, which is occupied by a carbohydrate chain in PA-BJ [29], this was not considered as a potential O-glycosylation site since it is not part of any consensus sequence for glycosylation and it is not predicted as being glycosylated by the NetOGlyc 4.0 Server (at www.expasy.org). The number of amino acid substitutions in the amino acid sequence of BJARSVP14, BJARSVP16 and BJARSVP22 compared to PA-BJ is variable (20, 40 and 12, respectively), however, these substitutions did not significantly affect their theoretical isoelectric points and molecular masses, and putative glycosylation sites (Fig. 6B).

We next carried out a new search of the fragment spectra obtained by the in gel trypsin digestion of protein bands containing PA-BJ 1–8 (Fig. 4A) against the sequences of BJARSVP14, BJARSVP16 and BJARSVP22 which resulted in the identification of tryptic peptides belonging to these sequences in the PA-BJ forms 5–8 but not in PA-BJ forms 1–4 (Supplemental Table 2). These results are in agreement
with the fact that the isolated proteoforms 5–8 are more basic than 1–4 and the sequences of the PA-BJ-like proteins show theoretical isoelectric points somewhat higher than that of PA-BJ (Uniprot entry P81824) (Fig. 6B). Thus, these results suggest that in the protein bands corresponding to proteoforms 5–8 (Fig. 4A), a mixture of the PA-BJ (Uniprot entry P81824) and PA-BJ-like proteins is present. The lack of a transcript encoding a sequence identical to PA-BJ in the cDNA library of the venom glands of B. jararaca is due to the fact that this library was constructed with RNA isolated from one specimen, which likely lacked an mRNA that matched that of PA-BJ, and is related to the individual variation occurring in B. jararaca venom [2,43].

Deshimaru and colleagues observed that the SVSP genes form a multigene family, and showed that nonsynonymous nucleotide substitutions have accumulated at a high rate in the mature protein-coding regions to cause amino acid changes that diversified their enzymatic activities [9]. In this study, pyrosequencing of a cDNA library of the venom gland of B. jararaca resulted in the identification of 13 full-length sequences encoding SVSPs (not shown) among which three are similar to PA-BJ. Interestingly, no cDNA encoding a SVSP showing 100% identity to PA-BJ (Uniprot entry P81824) was identified in the library likely due to the fact that it was constructed using RNA isolated from the venom glands of a single B. jararaca specimen. These results indicate that in agreement with the isolation of eight proteoforms of PA-BJ from the venom of B. jararaca, different mRNAs encoding PA-BJ-like enzymes may be present in the venom glands and contribute to the proteome complexity. Taken together, the findings of this study support the concept that the characterization of snake venom proteomes requires hypothesis-driven research and technology-driven approaches integrated in an experimental strategy that includes decomplexation of the venom proteome prior to LC–MS/MS and a species-specific transcriptome [44]. In this regard, our study emphasizes the validity of this concept to the characterization of proteoforms of a specific toxin.

4. Conclusions

Variation in snake venom proteomes is considered a consequence of gene duplication and accelerated molecular evolution to provide the venom with a dynamic repertoire of toxins and increase the diversity of molecules to deal with prey targets. In this study we isolated eight proteoforms of the serine proteinase PA-BJ in B. jararaca venom that clearly differ in their isoelectric points, glycosylation levels, and susceptibilities to enzymatic deglycosylation. The reason for the presence of multiple proteoforms of this serine proteinase in the venom is unknown but it might be related to its ability to interact with prey platelets as

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<th>PA-BJ</th>
<th>BJARVSVP14</th>
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<td>234</td>
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</tr>
<tr>
<td>Theoretical molecular mass</td>
<td>25215.1 Da</td>
<td>25575.9 Da</td>
<td>25659.0 Da</td>
<td>25288.4 Da</td>
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<td>Theoretical isoelectric point</td>
<td>9.08</td>
<td>9.59</td>
<td>9.52</td>
<td>9.37</td>
</tr>
<tr>
<td>Number of glycosylation sites</td>
<td>1 N- and O- glycosylation site indicated by Edman degradation</td>
<td>2 putative N-glycosylation sites</td>
<td>2 putative N-glycosylation sites</td>
<td>2 putative N-glycosylation sites</td>
</tr>
<tr>
<td>Number of amino acid substitutions compared to PA-BJ</td>
<td>-</td>
<td>20</td>
<td>40</td>
<td>12</td>
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</tbody>
</table>

Fig. 6. Sequencing of cDNAs encoding PA-BJ-like serine proteinases in the venom gland of B. jararaca. (A) Amino acid sequence comparison of PA-BJ (SwissProt entry P81824) with three cDNAs encoding serine proteinases similar to PA-BJ. The sequences of the putative mature proteins are shown. The numbering starts from the N-terminal residue of PA-BJ. Gaps were introduced to improve alignment. Residues of the catalytic triad are indicated by asterisks. Residues different from PA-BJ are indicated in red letters. Potential glycosylation sites of the cDNA encoding serine proteinase sequences and the experimentally determined glycosylation sites of PA-BJ [29] are shown in underlined green letters. (B) Comparison of protein parameters of PA-BJ and PA-BJ-like serine proteinases.
B. jararaca may feed on different small mammals during adult life. Besides the variation regarding the presence or absence of typical toxin families and the relative amount of members within a toxin family, the variability of proteoforms of a specific toxin adds one more level to snake venom proteome complexity.

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References


