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# BJ46a, a snake venom metalloproteinase inhibitor

## Isolation, characterization, cloning and insights into its mechanism of action

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Fractionation of the serum of the venomous snake *Bothrops jararaca* with $(NH_4)_2SO_4$, followed by phenyl-Sepharose and $C_4$-reversed phase chromatographies, resulted in the isolation of the anti-hemorrhagic factor BJ46a. BJ46a is a potent inhibitor of the SVMPs atrolysin C (class P-I) and jararhagin (P-III) proteolytic activities and *B. jararaca* venom hemorrhagic activity. The single-chain, acidic (pI 4.55) glycoprotein has a molecular mass of 46 101 atomic mass units determined by MALDI-TOF MS and 79 kDa by gel filtration and dynamic laser light scattering, suggesting a homodimeric structure. mRNA was isolated from the liver of one specimen and transcribed into cDNA. The cDNA pool was amplified by PCR, cloned into a specific vector and used to transform competent cells. Clones containing the complete coding sequence for BJ46a were isolated. The deduced protein sequence was in complete agreement with peptide sequences obtained by Edman degradation. BJ46a is a 322-amino-acid protein containing four putative N-glycosylation sites. It is homologous to the proteinase inhibitor HSF (member of the fetuin family, cystatin superfamily) isolated from the serum of the snake *Trimeresurus flavoviridis*, having 85% sequence identity. This is the first report of a complete cDNA sequence for an endogenous inhibitor of snake venom metalloproteinases (SVMPs). The sequence reveals that the only proteolytic processing required to obtain the mature protein is the cleavage of the signal peptide. Gel filtration analyses of the inhibitory complexes indicate that inhibition occurs by formation of a noncovalent complex between BJ46a and the proteinases at their metalloproteinase domains. Furthermore, the data shows that the stoichiometry involved in this interaction is of one inhibitor monomer to two enzyme molecules, suggesting an interesting mechanism of metalloproteinase inhibition.

*Keywords*: inhibitor; metalloproteinase; reprolysin; snake venom.

Venoms of most members of the snake family *Viperidae* are known to contain complex mixtures of toxins that can cause hypotensive shock, intravascular clotting, systemic and local hemorrhage, as well as edema, pain and necrosis [1]. Among these snake venom toxins, the hemorrhagic proteinases cause disruption of the basement membrane of the vascular endothelium resulting in local bleeding [2] and, in severe cases of envenoming, contribute to systemic bleeding [3].

The reprolysin [4] protein family belongs to the superfamily metzincin of metalloproteinases [5] and comprises the snake venom metalloproteinase (SVMPs) and the a disintegrin and metalloproteinase (ADAMs) also known as the metalloproteinase, disintegrin, cysteine-rich (MDC) group. All venoms of snakes belonging to the Viperidae family have been shown to contain SVMPs, many of which cause hemorrhage [6]. The SVMPs are grouped, according to their domain composition, into four classes: P-I, contains the metalloproteinase domain only; P-II, metalloproteinase and disintegrin domains; P-III, metalloproteinase, disintegrin-like and cysteine-rich domains and P-IV, metalloproteinase, disintegrin-like, cysteine-rich and lectin-like domain [7].

Anti-hemorrhagic factors have been isolated from several species of snakes as well as from different mammals. They inhibit the proteolytic activity of the toxins, and consequently, hemorrhage [8–10]. Based on their molecular masses, these factors are grouped into two classes: high (700–1000 kDa) and low (52–90 kDa) molecular mass proteins. The low molecular mass class interacts with hemorrhagic metalloproteinases by a yet to be determined noncovalent mechanism [11–18].

To date, the best characterized of these low molecular mass factors are oprin (isolated from the opossum *Didelphis virginiana*), DM43 (isolated from *D. marsupialis*) and Habu serum factor (HSF; isolated from the venomous snake *Trimeresurus flavoviridis*). Data resulting from the partial sequence analysis of oprin and the complete sequence of DM43 revealed 36% identity with human α1B-glycoprotein, suggesting that they belong to the immunoglobulin supergene family [11,12]. On the other hand, the primary sequence of HSF is homologous to plasma glycoproteins (fetuins) and hence, the protein possesses a double-headed cystatin domain. Based on this, HSF was classified as a member of a novel family

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*Abbreviations*: ADAM, a disintegrin and metalloproteinase; a.m.u., atomic mass unit; MHD, minimum hemorrhagic dose; MMP, matrix metalloproteinase; SVMP, snake venom metalloproteinase.

*Enzymes*: atrolysin C (EC 3.4.24.42); jararhagin (EC 3.4.24.73).

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of the cystatin superfamily of proteinase inhibitors, although it failed to inhibit papain and cathepsin B activities and no other evidence of cysteine proteinase inhibition was demonstrated [19].

Although inhibitors of SVMPs from snake and mammalian sera have been previously isolated, little is known about their synthesis, structure and mechanism of action. The efforts of this study were directed at the structural and functional characterization of a metalloproteinase inhibitor isolated from the serum of the venomous snake *Bothrops jararaca*. This inhibitor was named BJ46a (46-kDa isoform a from *B. jararaca*). A less abundant inhibitor isoform (BJ46b) was isolated by the addition of one more chromatographic step during the purification procedure. It has a molecular mass of 46 389 a.m.u. by MALDI-TOF MS and identical N-termini through the first 20 residues (data not shown). It was not further characterized.

## EXPERIMENTAL PROCEDURES

### Materials

*B. jararaca* venom was a gift from C. R. Diniz (Ezequiel Dias Foundation, Belo Horizonte, MG, Brazil); jararhagin and jararhagin-C were a gift from A. G. C. Neves-Ferreira (Oswaldo Cruz Foundation, RJ, Brazil). Atrolysin C was isolated as previously described [20]. *N*-Isopropyliodoacetamide was from Molecular Probes, USA. Calibration standards for isoelectric focusing, gel filtration and electrophoresis, as well as phenyl-Sepharose CL-4B, Superdex 200 HR 10/30 and Sephadex G-25 Microspin columns were from Amershan Pharmacia Biotech, Sweden. Sequencing grade endoproteinases Asp-N and Lys-C were from Boehringer Mannheim, Germany. BSA, fetuin, 4-vinylpyridine and analytical grade or higher quality chemicals were from Sigma Chemical Company, USA. Mini Protean II and Mini IEF systems, $C_4$ Hipore RP-304 column and Bio-Lyte 3/10 were from Bio-Rad Laboratories.

### Serum from *Bothrops jararaca*

Serum was obtained from specimens at the Army Biology Institute, Rio de Janeiro, RJ, Brazil. Blood was collected by caudal vein puncture, left for 1 h at 37 °C, followed by incubation at 1.5 h at 4 °C, and centrifuged. Snake serum was heated for 30 min at 56 °C, centrifuged and the supernatant stored at −70 °C until use. All experiments with animals were in accordance with the ethical standards of the International Society on Toxinology [21].

### Protein determination

Protein contents were determined by the Folin–Lowry method [22] using BSA as standard.

### Isolation of BJ46a

*B. jararaca* serum (31.2 mL) was fractionated by ammonium sulfate precipitation. The pellet obtained in the 40–60% fraction was resuspended in 20 mL of 0.1 M sodium phosphate pH 7.0, 1 M $(NH_4)_2SO_4$ and applied, in two runs, on a phenyl-Sepharose column (1.6 × 18 cm) previously equilibrated with the same buffer. Elution was carried out isocratically at 0.5 mL·min$^{-1}$, followed by a decreasing linear salt gradient to 0.1 M sodium phosphate pH 7.0 buffer. The active fractions (anti-hemorrhagic activity) of both runs were pooled and precipitated with $(NH_4)_2SO_4$ at 80% saturation. The pellet was then resuspended in 22 mL of 25% acetonitrile in 0.1% trifluroacetic acid and applied, in six runs, at room temperature, onto a $C_4$ (1.0 × 25 cm) column previously equilibrated with the same solvent using a flow rate of 5 mL·min$^{-1}$. The solvent system used was: A (0.1% trifluroacetic acid in water) and B (0.08% trifluroacetic acid in acetonitrile) in a gradient program of 25% B for 11 min and 100% B at 100 min. The active fraction for each run was collected and immediately neutralized with 10% (v/v) 0.5 M Tris/HCl pH 8.0 and then pooled. The material was lyophilized and rechromatographed on the $C_4$ column under the same conditions described above. In this case, the resuspension volume was 9 mL and two runs were performed. The pool from these two runs (homogeneous BJ46a) was lyophilized and stored at −20 °C until use.

### Polyacrylamide gel electrophoresis

SDS/PAGE (12%) was performed on a Mini-Protean II system both under reducing and nonreducing conditions using 4% stacking gels [23]. Staining was carried out with Coomassie Blue R250.

### Minimum hemorrhagic dose determination and anti-hemorrhagic activity

Minimum hemorrhagic dose (MHD) was determined according to the method described by Theakston and Reid [24]. To evaluate the neutralization capacity of *B. jararaca* serum and BJ46a against hemorrhage induced by *B. jararaca* venom, rats were injected with two MHDs of venom plus increasing amounts of serum or purified inhibitor, previously incubated in NaCl/$P_i$ for 30 min at 37 °C. Hemorrhagic lesions were measured after 24 h. One anti-hemorrhagic activity unit is the quantity, in μg of protein, necessary to neutralize 1 MHD.

### Inhibition of proteolytic activity

The inhibition of the proteolytic activity of jararhagin or atrolysin C by BJ46a was tested using the small fluorogenic substrate Abz-Ala-Gly-Leu-Ala-Nba (Enzyme Systems Products, USA) [25]. Assays were performed, with stirring, in 0.02 M Tris/HCl pH 7.5, 0.02 M $CaCl_2$, 0.15 M NaCl buffer using 100 μM of fluorogenic substrate and 0.025 μM of jararhagin or 0.5 μM of atrolysin C as final concentrations. Proteinases and inhibitor were incubated for 5 min at 27 °C prior to substrate addition. Excitation and emission wavelengths were 340 and 415 nm, respectively. Results were represented as the mean of duplicate experiments.

### Isoelectric focusing

BJ46a was electrofocused using a Mini IEF system and thin-layer polyacrylamide gels prepared according to the manufacturer's instructions. Ampholites were in the 3.5–9.5 pH range (Bio-Lyte 3/10). Calibration standards used were between pH 3.5 and 9.3.

### Chemical deglycosylation

Carbohydrates were cleaved from BJ46a by anhydrous trifluoromethanesulfonic acid (Oxford GlycoSystems, UK) according to the manufacturer's instructions and then submitted to MALDI-TOF MS analysis. Glycoproteins were visualized using periodic acid/Schiff's reagent staining [26] after 12% SDS/PAGE under reducing conditions. Fetuin and BSA were used as controls.

### Amino-acid analysis

Amino-acid analyses were performed on a Biochrom 20 (Amershan Pharmacia Biotech, Sweden) automatic amino-acid analyzer based on ion-exchange chromatography separation and ninhydrin postderivatization [27]. Protein samples were vapor hydrolyzed in a Pico Tag workstation (Waters Corporation) under vacuum, after saturation of the atmosphere with nitrogen, using 200 μL of 5.7 M HCl for 24, 48 and 72 h at 108 °C. The amino-acid composition was calculated considering the highest value obtained for each amino acid, except for threonine and serine amounts, which were estimated extrapolating to zero time.

### Mass spectrometry by MALDI-TOF

Atrolysin C, BJ46a and deglycosylated BJ46a were analyzed on a Voyager DE-PRO (Perseptive Biosystems) using 3,5-dimethoxy-4-hydroxycinnamic acid as matrix. BJ46a peptides, obtained after digestion with Asp-N or Lys-C enzymes, were analyzed using cyano-4-hydroxycinnamic acid as matrix. The analyses were performed following the manufacturer's instructions.

### Dynamic laser light scattering

All studies were performed using a DynaPro molecular sizing instrument with a temperature controlled microsampler set to 20 °C (Protein Solutions). Protein samples were dissolved in 0.04 M sodium phosphate pH 7.4 and filtered through a 0.02-μm membrane upon injection into the molecular size detector sample cell. Typically, concentrations in the range 1–2 $mg \cdot mL^{-1}$ were used. Approximately 25 individual Stokes radius values were acquired for each sample and then statistically averaged. Based on this averaged value, the molecular mass was calculated assuming a globular conformation model using the software provided by the manufacturer.

### *S*-Pyridylethylation of BJ46a

Forty-six micrograms of protein were dissolved in 0.5 mL of 0.5 M Tris/HCl pH 8.3, 6 M guanidine HCl, 0.001 M EDTA, under nitrogen, and incubated for 2 h at 50 °C. A 50-fold molar excess of dithiothreitol (assuming 12 cysteine residues in BJ46a) was added to the reaction mixture. Following 30 min of incubation at 50 °C, 0.2 μL of 4-vinylpyridine were added and the alkylation mixture was allowed to incubate overnight at room temperature in the dark. Excess reagents were removed by RP-HPLC of the sample on a Jupiter $C_{18}$ (0.2 × 15 cm; Phenomenex) column, at 30 °C, using a flow rate of 200 $\mu L \cdot min^{-1}$. The eluent absorbance was monitored at 215 nm. The solvent system used was A (0.1% trifluroacetic acid in water) and B (0.09% trifluroacetic acid in acetonitrile) in a gradient program of 5% B for 5 min, 45% B at 50 min and 70% B at 55 min. Protein peaks were collected and lyophilized.

### Endoproteinase Asp-N digestion

The pyridylethylated protein was dissolved in 200 μL of 0.05 M sodium phosphate pH 8.0 and digested at an enzyme to substrate concentration of 1 : 200 (w/w) at 37 °C for 16 h. Asp-N digestion peptides were isolated by RP-HPLC on a Jupiter $C_{18}$ (0.2 × 15 cm; Phenomenex) column using the conditions described for *S*-pyridylethylation. Isolated peptides were submitted to MALDI-TOF MS and N-terminal amino-acid sequence analyses.

### Alkylation with *N*-isopropyliodoacetamide of BJ46a

Ten micrograms of protein were dissolved in 50 μL of a 0.64-M Tris/HCl pH 8.0, 8 M urea, 0.16 M methylamine HCl solution. Following the addition of 5 μL of a 2-mercaptoethanol solution (7 μL in 1 mL of water), the reaction mixtures were incubated for 1 h at 60 °C. Finally, 7 μL of an *N*-isopropyliodoacetamide solution (2.3 mg in 20 μL of methanol, 80 μL of water) were added to the protein solution and allowed to incubate for 30 min at room temperature before digestion with endoproteinase Lys-C.

### Endoproteinase Lys-C digestion

The *N*-isopropylcarboxyamidomethylated protein was digested with endoproteinase Lys-C (1 $\mu g \cdot \mu L^{-1}$ in 20% glycerol, 1 M Tris/HCl pH 8.0) without prior removal of alkylating reagents. Enzyme solution (0.5 μL) was added and allowed to react at 37 °C for 18 h. Lys-C digested peptides were isolated by RP-HPLC as described for *S*-pyridylethylation. Isolated peptides were submitted to MALDI-TOF MS and N-terminal amino-acid sequence analyses.

### Protein sequence analysis

N-Terminal Edman sequencing of pyridylethylated BJ46a as well as of the digestion peptides was performed on a 494 Procise protein sequencer (PerkinElmer, USA) according to the manufacturer's instructions.

### Complex formation

The inhibitor BJ46a was tested for complex formation with atrolysin C, jararhagin and jararhagin-C. Inhibitor and metalloproteinases were mixed at varying molar ratios while inhibitor and jararhagin-C were incubated at a 3 : 1 (BJ46a monomer: jararhagin-C) molar ratio. All incubations were performed for 5 min at 37 °C and then submitted to gel filtration chromatography on a Superdex 200 column, previously equilibrated in 0.02 M Tris/HCl pH 7.5, 0.02 M $CaCl_2$, 0.15 M NaCl buffer and eluted at a flow rate of 30 $mL \cdot h^{-1}$. Gel filtration standards used were dimeric-BSA (134 kDa), BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa). Fractions were collected and analyzed by SDS/PAGE under reducing and nonreducing conditions followed by Coomassie Blue staining.

### *Bothrops jararaca* liver extraction and storage

One snake, kindly donated by the Army Biology Institute, was sacrificed by decapitation and its liver was immediately removed, weighed (4.8 g) and transferred to a 50-mL tube containing 25 mL of RNA*later* (Ambion, USA). The material was incubated overnight at 4 °C and then stored at −20 °C until use.

### Isolation of mRNA from liver

From 115 mg of *B. jararaca* liver, a poly A mRNA pool was obtained using the Oligotex kit (Qiagen Inc.) following manufacturer's instructions. The integrity of the RNA was assessed by running 1% agarose gels in 0.04 M Tris/acetate, 0.001 M EDTA buffer. RNA concentration was estimated by measurement of the absorbance at 260 nm, assuming that one absorbance unit corresponds to 40 μg of RNA per mL.

### Transcription of mRNA to ds cDNA with adaptors

In this step, the Marathon cDNA Amplification Kit (Clontech Laboratories Inc., USA) was used and the conditions were those described by the manufacturer. One microgram of the mRNA isolated in the previous step was used as starting material to obtain the adaptor-ligated ds cDNA library in solution. Finally 25, 50 and 100 × dilutions in 0.1 M EDTA, 0.01 M tricine pH 8.5 buffer were made from this solution for further use.

### DNA synthesis

All oligonucleotides used as primers for PCR and DNA sequencing were synthesized on an Expedite 8900 Nucleic Acid Synthesis System (PerSeptive Biosystems) at a scale of 50 nmol. The products were then purified using Sephadex G-25 Microspin columns and diluted to 10 μM for PCR amplifications or 2 pmol·μL$^{-1}$ for DNA cycle sequencing.

### DNA Sequencing

Cycle sequencing of DNA was performed on PerkinElmer 377, ABI Prism DNA Sequencer using the BigDye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq DNA polymerase according to the manufacturer's instructions.

### Amplification of the *BJ46a* cDNA by PCR with degenerate and gene specific primers

The liver ds cDNA library was used as a template for PCR amplification of the BJ46a cDNA. In order to identify the BJ46a DNA sequence, a pair of degenerate oligonucleotides corresponding to partial amino-acid sequences were designed: DF1 (5′-GAYGTNTTYGCNAARTGYCA-3′) and DR1 (5′-ACYTTNCCNGGRCANCCYTT-3′). A PCR reaction was prepared using 3 μL of 50X diluted ds cDNA library solution and 1 μg of each degenerate primer per reaction, together with Advantage cDNA Polymerase Mix and reagents provided by Clontech, USA. The amplification was carried out in PTC-200 DNA Engine (M.J. Research, USA) under the following program: 94 °C for 30 s followed by 30 cycles (94 °C for 5 s and 60 °C for 4 min). The PCR product was electrophoresed in 1% agarose gel and stained with ethidium bromide. The main band of approximately 650 bp was excised and ligated into pCR II TA cloning vector (Invitrogen, USA). After overnight incubation at 14 °C, half of the ligation reaction was used for transformation of *Escherichia coli* competent cells provided with the kit. Mini prep plasmid DNA from individual subclones were obtained using the Concert System (GibcoBRL) and then cut with *Eco*RI and analyzed on agarose gel. Two clones that carried inserts corresponding by size to the PCR product were sequenced with vector specific primers in both directions. Based on the sequence obtained two complementary gene specific primers were synthesized: F1 (5′-CACGCTCACTCCCATTTGATTCAGCAG-3′) and R1 (5′-CTGCTGAATCAAATGGGAGTGAGCGTG-3′). Both primers were paired with adaptor specific primer (5′-CTAATACGACTCACTATAGGG-3′) and the two PCR reactions were completed under the previously described conditions. The two PCR products (approximate lengths 800 and 950 bp) were cloned into the TA cloning system described above and the complete sequences of each of them were determined for both strands. Next, the translation region of BJ46a gene was cloned and sequenced following PCR amplification using the oligonucleotides F2 (5′-ATGAATTCCCTGGTAGCTCTC-3′) and R2 (5′-CTACAGCTCGAAGTGATGTAC-3′).

## RESULTS

### BJ46a purification procedure

*B. jararaca* serum was fractionated by ammonium sulfate precipitation and the 40–60% saturation fraction (showing anti-hemorrhagic activity) was further fractionated through hydrophobic interaction and reversed phase chromatographies. A final reversed phase rechromatography step was necessary to ensure homogeneity of BJ46a (Fig. 1A–C). From 1.88 g of serum protein, 10.40 mg of BJ46a were isolated. Based on the anti-hemorrhagic activity, there was a 10-fold increase in the specific inhibitory activity (data not shown). Homogeneity was confirmed by SDS/PAGE under reducing (Fig. 2A) and nonreducing conditions (Fig. 3B,D) and IEF (Fig. 2B). N-Terminal Edman sequencing indicated a homogeneous protein.

### Physico-chemical and chemical characterization

BJ46a is an acidic protein of pI = 4.55 (Fig. 2B) that has a molecular mass of 55.4 kDa by SDS/PAGE (Fig. 2A) and 46 101 a.m.u. by MALDI-TOF MS. Gel filtration and dynamic laser light scattering values for molecular mass were both 79 kDa (Table 1) indicating that BJ46a is a dimer in its native state. BJ46a is glycosylated, as seen with positive staining by periodic acid-Schiff's reagent (data not shown). Following chemical deglycosylation, BJ46a was shown to have a mass of 38 166 a.m.u. by MALDI-TOF MS (Table 1) or, 17% of carbohydrate by weight. Amino-acid analysis data was in good agreement with the deduced protein sequence data (data not shown). The protein content of samples before acid hydrolysis was determined by the Folin–Lowry method [22]. A protein recovery of 80.40 ± 3.91% was obtained after amino-acid analysis.

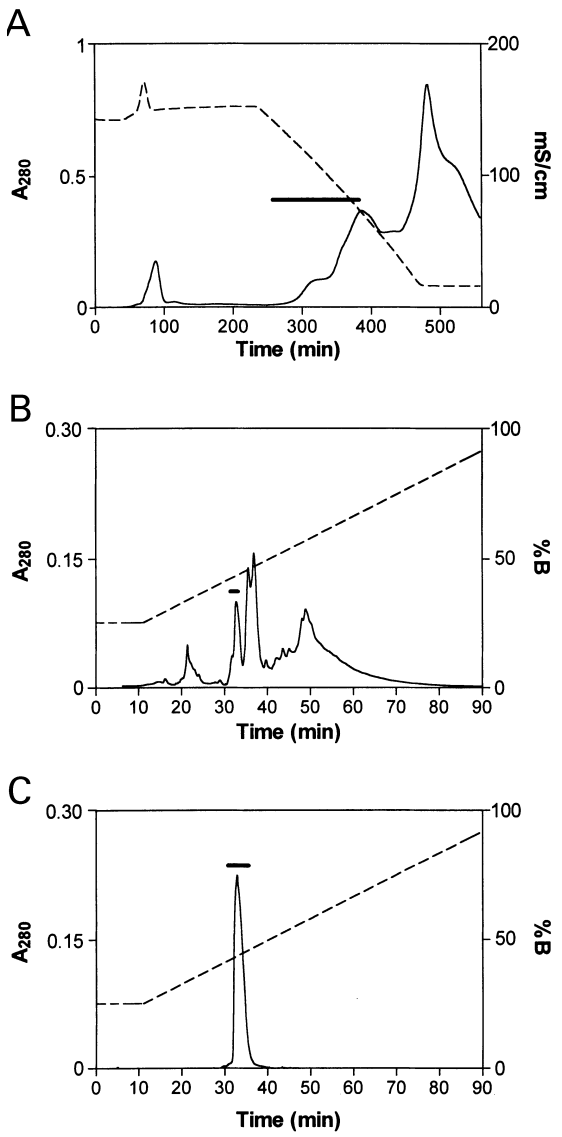


**Fig. 1. Isolation of the SVMP inhibitor BJ46a.** Following $(NH_4)_2SO_4$ fractionation, the anti-hemorrhagic fraction (40–60% saturation) was applied to a phenyl-Sepharose column (A) followed by $C_4$ reversed-phase chromatography (B), and a final reversed-phase rechromatography yielding homogeneous BJ46a (C). Bars indicate the fractions showing anti-hemorrhagic activity that were pooled in each step. Chromatographic conditions are described in Experimental procedures.

Considering that BJ46a is 17% glycosylated, we assumed that the method used for protein determination adequately estimates the protein content of BJ46a.

## Inhibition studies

BJ46a was assayed for its inhibitory activity against jararhagin (a class P-III SVMP) and atrolysin C (a class P-I SVMP) using the fluorogenic substrate Abz-Ala-Gly-Leu-Ala-Nbz. At the concentrations used in the assay and at an equimolar ratio (enzyme/BJ46a monomer), the inhibition of atrolysin C and jararhagin proteolytic activities were 100 and 91%, respectively (Table 2).

**Table 1. Masses of isolated SVMPs, BJ46a and inhibitor:SVMP complexes determined by different methods.** ND, not determined. DLLS, dynamic laser light scattering.

| Sample | MALDI-TOF MS [a] (a.m.u.) | Gel filtration [b] (kDa) | DLLS [c] (kDa) |
|---|---|---|---|
| BJ46a | 46 101 | 79 | 79 |
| BJ46a deglycosylated | 38 166 | ND | ND |
| Atrolysin C | 23 062 | ND | 25 |
| BJ46a: atrolysin C | ND | 87 | 105 |
| Jararhagin | ND | 57 | ND |
| BJ46a: jararhagin | ND | 128 | ND |

[a] Measurements were performed on a PerkinElmer Voyager DE-PRO instrument, using 3,5-dimethoxy-4-hydroxy cinnamic acid as matrix. [b] Chromatographies were performed on a Superdex 200 HR 10/30 column (Pharmacia) equilibrated with 0.02 M Tris/HCl pH 7.5, 0.02 M $CaCl_2$, 0.15 M NaCl buffer. Calibration curve standards were: di-BSA, BSA, ovalbumin and chymotrypsinogen A. [c] Dynamic laser light scattering. Molecular mass values were obtained, using the DYNALS software, from hydrodynamic radius values (Rh) assuming the globular protein model.

## Interaction studies

Figure 3A illustrates the results obtained from the titration experiments of BJ46a and atrolysin C. The peak areas in the chromatograms were integrated and plotted. Because the retention times for BJ46a alone and the complex BJ46a/atrolysin C are very similar, they were plotted as the sum of free BJ46a and complex peaks. There is a clear increase in the peak area of the complex + free BJ46a until a 2 : 1 atrolysin C/BJ46a ratio is reached. At a 3 : 1 ratio, the increase in the complex + free BJ46a peak area is not so prominent; moreover, the appearance of 90.7% of free atrolysin C at this ratio relatively to its control (1 : 0 ratio) indicates that complex saturation was attained at a 2 : 1 ratio. Complex peaks collected and analysed by SDS/PAGE under nonreducing (Fig. 3B) and reducing (data not shown) conditions revealed a noncovalent interaction and showed that the enzyme did not cleave the inhibitor polypeptide chain. Calculated molecular mass, from gel filtration experiments, for the complex (at saturation concentration, no free BJ46a) was 87 kDa (Table 1). However, the molecular mass determined for atrolysin C was far lower

**Table 2. Inhibition assay of the isolated inhibitor, BJ46a, against the SVMPs atrolysin C (class P-I) and jararhagin (class P-III).** ND, not determined.

| Mol/Mol | Atrolysin C [b] | Jararhagin [b] |
|---|---|---|
| [E]: [I][a] | | |
| 1 : 1 | 100 | 91 |
| 2 : 1 | 100 | 60 |
| 3 : 1 | ND | 28 |
| 4 : 1 | 68 | ND |

[a] [I] = [BJ46a monomer]. [b] Data presented as percentage inhibiton. Molecular masses assumed for the calculations were 46 101, 23 062 and 52 000 Da for BJ46a, atrolysin C and jararhagin, respectively.

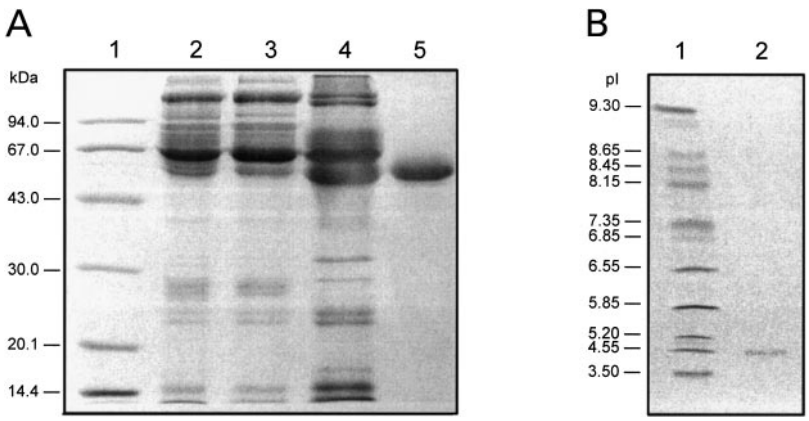


**Fig. 2. SDS/PAGE under reducing conditions of samples from BJ46a purification steps (A) and determination of isoelectric point (B).** (A) Lane 1, molecular mass markers (kDa): phosphorylase *b* (94); albumin (67); ovalbumin (43); carbonic anhydrase (30); trypsin inhibitor (20.1) and α-lactalbumin (14.4). Lane 2, 18 μg of *B. jararaca* serum; lane 3, 18 μg of 40–60% saturation fraction obtained by ammonium sulfate precipitation; lane 4, 12 μg of phenyl-Sepharose CL-4B fraction; and lane 5, 5 μg of BJ46a. Stained with Coomassie blue R-250. (B) Lane 1, isoelectric point markers (pI values shown on left on figure); lane 2, 1 μg of BJ46a.

than the one determined by MALDI-TOF MS (23 062 a.m.u.). Hence, we have used the molecular mass values obtained by dynamic laser light scattering for the complex (105 kDa), BJ46a (79 kDa) and atrolysin C (25 kDa) (Table 1) to perform further calculations in order to establish the stoichiometry involved in this interaction. Figure 3C illustrates the results obtained from the titration experiments of BJ46a and jararhagin. In this particular case, the results are more easily interpretable as the complex peak (BJ46a/jararhagin) has a distinct retention time when compared to BJ46a alone. There is a clear increase in the complex peak area until the 2 : 1 jararhagin/BJ46a ratio. At a 3 : 1 ratio, the increase in the BJ46a/jararhagin peak area is not so prominent; moreover, the appearance of 94.7% of free jararhagin at this ratio relatively to its control (1 : 0 ratio) indicates that complex saturation was attained at a 2 : 1 ratio. Also, free BJ46a can be seen at 1 : 1 ratio but not at 2 : 1 or 3 : 1 ratios. Complex peaks collected and

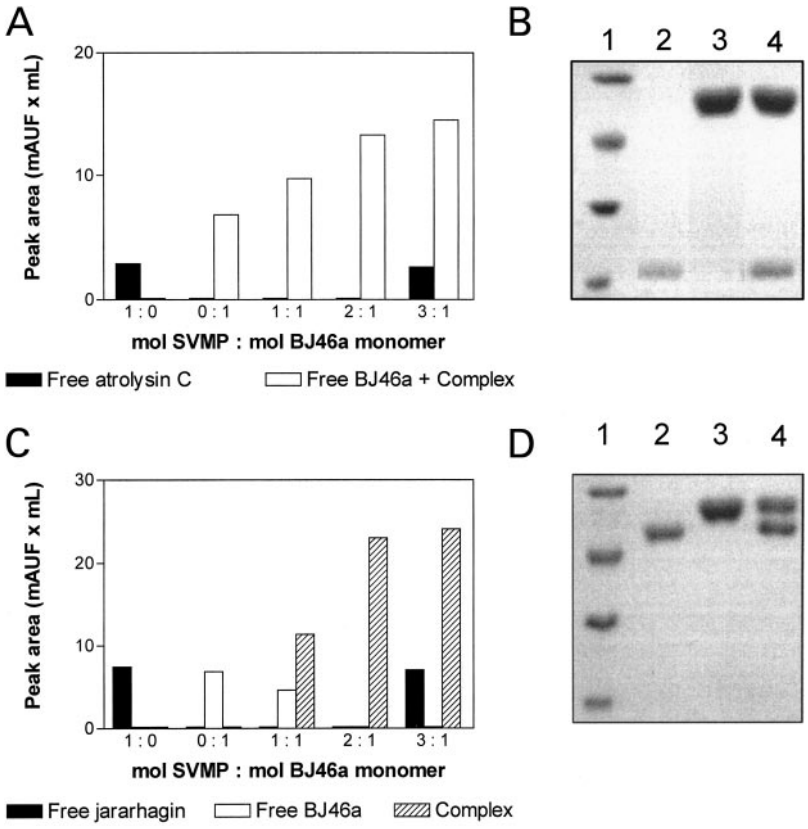


**Fig. 3. Complex formation between BJ46a and atrolysin C (A,B) or jararhagin (C,D).** Samples were subjected to gel filtration chromatography on Superdex 200 at different enzyme/inhibitor molar ratios. (B) and (D) show SDS/PAGE under nonreducing conditions of the fractions obtained by chromatography. Lane 1, molecular mass markers (kDa): bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (29) and β-lactoglobulin (18.4). (B) Lane 2, atrolysin C; lane 3, BJ46a; lane 4, atrolysin C/BJ46a complex. (D) Lane 2, jararhagin; lane 3, BJ46a; lane 4, jararhagin/BJ46a complex. Molar ratios were calculated using molecular masses of 46 101, 23 062 and 52 000 Da for BJ46a (monomer), atrolysin C and jararhagin, respectively.

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   1 CCAGCCATGAATTCCCTGGTAGCTCTCGTGCTCCTGGGTCAGATTATAGGATCTACGCTT  60
         M  N  S  L  V  A  L  V  L  L  G  Q  I  I  G  S  T  L
  61 AGCTCTCAAGTGAGGGGGGATTTAGAATGCGACGAGAAAGACGCTAAAGAGTGGACAGAT 120
 -1  S  S  Q  V  R  G  D  L  E  C  D  E  K  D  A  K  E  W  T  D   19
 121 ACAGGTGTGCGCTACATCAACGAGCATAAACTACATGGATACAAATATGCCCTCAATGTA 180
  20  T  G  V  R  Y  I  N  E  H  K  L  H  G  Y  K  Y  A  L  N  V   39
 181 ATTAAGAATATCGTTGTCGTTCCCTGGGATGGCGATTGGGTGGCAGTCTTCCTTAAATTA 240
  40  I  K  N  I  V  V  V  P  W  D  G  D  W  V  A  V  F  L  K  L   59
 241 AATCTTCTGGAGACAGAATGTCATGTGTTGGATCCAACTCCTGTCAAGAATTGTACTGTA 300
  60  N  L  L  E  T  E  C  H  V  L  D  P  T  P  V  K  N  C  T  V   79
 301 AGGCCACAGCATAATCATGCTGTTGAAATGGACTGTGATGTCAAGATAATGTTTAATGTT 360
  80  R  P  Q  H  N  H  A  V  E  M  D  C  D  V  K  I  M  F  N  V   99
 361 GATACTTTCAAAGAAGATGTTTTTGCAAAATGCCACTCCACTCCAGATTCTGTGGAAAAC 420
 100  D  T  F  K  E  D  V  F  A  K  C  H  S  T  P  D  S  V  E  N  119
 421 GTGCGGCGAAATTGTCCTAAATGTCCAATTCTGTTGCCTTCGAATAACCCTCAGGTGGTA 480
 120  V  R  R  N  C  P  K  C  P  I  L  L  P  S  N  N  P  Q  V  V  139
 481 GACTCTGTTGAATATGTGCTTAATAAACACAATGAAAAACTTTCCGACCACGTTTACGAA 540
 140  D  S  V  E  Y  V  L  N  K  H  N  E  K  L  S  D  H  V  Y  E  159
 541 GTTCTTGAGATTTCAAGAGGGCAGCACAAATATGAGCCTGAAGCTTACTATGTGGAGTTT 600
 160  V  L  E  I  S  R  G  Q  H  K  Y  E  P  E  A  Y  Y  V  E  F  179
 601 GCTATTGTGGAGGTTAACTGCACTGCTCAGGAACTCCACGATGACCATCATCACTGCCAT 660
 180  A  I  V  E  V  N  C  T  A  Q  E  L  H  D  D  H  H  H  C  H  199
 661 CCTAATACAGCAGGAGAAGACCATATTGGATTCTGCAGAGCAACTGTTTTCAGGTCGCAT 720
 200  P  N  T  A  G  E  D  H  I  G  F  C  R  A  T  V  F  R  S  H  219
 721 GCTAGCCTGGAAAAACCTAAAGATGAACAGTTTGAGTCGGACTGTGTCATCCTTCATGTC 780
 220  A  S  L  E  K  P  K  D  E  Q  F  E  S  D  C  V  I  L  H  V  239
 781 AAGGAGGGACACGCTCACTCCCATTTGATTCAGCAGCACGTTGAAAAAGACAGTATTTCC 840
 240  K  E  G  H  A  H  S  H  L  I  Q  Q  H  V  E  K  D  S  I  S  259
 841 CCAGAACACAACAATACTGCCCTCAACTTCGTCCATCCACACAATGATACAAGCACCTCA 900
 260  P  E  H  N  N  T  A  L  N  F  V  H  P  H  N  D  T  S  T  S  279
 901 CACGAGTCTCATGAACATTTGGCGGAAGTCCCAGTTGCTTTTGTCAAAAAAGAACTCCCC 960
 280  H  E  S  H  E  H  L  A  E  V  P  V  A  F  V  K  K  E  L  P  299
 961 AAAGATATATCAGATCGTCACACAACCCCTGTGAAAGGTTGTCCAGGAAAAGTACATCAC 1020
 300  K  D  I  S  D  R  H  T  T  P  V  K  G  C  P  G  K  V  H  H  319
1021 TTCGAGCTGTAGGCCAGCCATACTAGAGAGCATAGAGAAATCACCCAGTTAGTGCAAAAC 1080
 320  F  E  L  *                                                  322
1081 CATTTTCCAAATCTGAACTCAAAGCTTGAATCCAAAAAGGGTTTACCCTGACACATTACA 1140
1141 GTCTTTGCCAAGTGTTGTGATCACGCATGGTTGTTATAAGAAAGGGGCTATGTAAATGTG 1200
1201 CCTTTCTGCTTATAGAAATGCAGATTCTGATGCTCACTTAACATCTTCCTATCCCTCTTT 1260
1261 TTGTTCAGCCAAGGAAGGAGAAGAAAGTTGATCAGAAAGTTATATTAGTCCCAAAGTCAA 1320
1321 AATTCTTCCTTTTTTTTCTTTTAAAGAGAAAATGCATAGCATTTGACAACAATACCCCCA 1380
1381 AAGTCCCATCACCTCTATTCCTTATGAGATTTGCTTTCCCCAGAATGTTTCACCAGGCTA 1440
1441 CATTATTTGACAGGGGCAACGTTGAACAAAGTCCAATACACTGACAGAAAAAAGCAGAGG 1500
1501 GTACTCTCTAAATAAATTGTTCAATTCTGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1560
1561 A
```

**Fig. 4. Nucleotide and deduced amino-acid sequences of cDNA coding for BJ46a.** The nucleotide sequence was determined for both strands and is shown in the upper row with the deduced amino-acid sequence below. Sequence in bold corresponds to the signal peptide. Underlined residues were confirmed by Edman chemistry sequencing of BJ46a N-terminal and endoproteinase Asp-N and Lys-C derived peptides. Putative N-glycosylation sites are boxed and the polyadenylation signal is double underlined.

analysed by SDS/PAGE under nonreducing (Fig. 3D) and reducing (data not shown) conditions revealed a noncovalent interaction and, again, showed no cleavage of the polypeptide chain of the inhibitor. In this case, the molecular mass values used to perform further calculations were those obtained from gel filtration experiments: complex (128 kDa), BJ46a (79 kDa) and jararhagin (57 kDa) (Table 1).

In these gel filtration experiments, BJ46a was not able to form complexes with jararhagin-C even at a threefold molar excess of inhibitor (data not shown).

## cDNA and Edman sequencing

Following Edman sequencing of internal peptides from pyridylethylated BJ46a digestion with endoproteinase Asp-N, we designed a pair of degenerate PCR primers (DF1 and DR1; Experimental procedures) and successfully amplified a fragment from the ds cDNA library of *B. jararaca* liver. Specific primers (F1, R1, F2 and R2) were designed and the PCR products obtained were homogeneous and had the expected size as observed by agarose gel electrophoresis (data not shown). The complete cDNA sequence for BJ46a and the deduced protein sequence are shown in Fig. 4. To confirm the cDNA sequence as the genuine coding sequence for BJ46a, Edman sequencing of the pyridylethylated protein and peptides obtained from Asp-N and Lys-C digestion (data not shown) was performed. The sequenced amino acids obtained, underlined in Fig. 4, corresponded to 81% of BJ46a coding sequence. No discrepancy was found between DNA and protein sequencing data. The full length BJ46a has a signal sequence at the N-terminus that is processed in the mature protein purified from serum. Following the signal sequence is the BJ46a mature protein sequence. The schematic structure for BJ46a coding mRNA is shown in Fig. 5A.

The deduced amino-acid sequence of mature BJ46a was submitted to a homology search using BLASTP v. 2.0.12 software [28] and an 85% sequence identity was found when compared to HSF, an anti-hemorrhagic factor isolated from the serum of the venomous snake *T. flavoviridis*. HSF belongs to the family 4 of cystatin superfamily [19] or, more specifically, to the fetuin group. All of the fetuin sequences in this group contain 12 cysteine residues in positions identical to those in human and bovine fetuins. HSF (snake fetuin) is the one exception as it contains 13 cysteine residues. Interestingly, we found only 12 cysteine residues per molecule of BJ46a, as expected for the fetuin group [29]. The deduced BJ46a amino-acid sequence revealed that asparagine residues at positions 76, 185, 263 and 274 are putative N-glycosylation sites as the consensus sequence, NXS/T (where X can be any amino acid but proline) [30], was found in these regions. No PTH amino

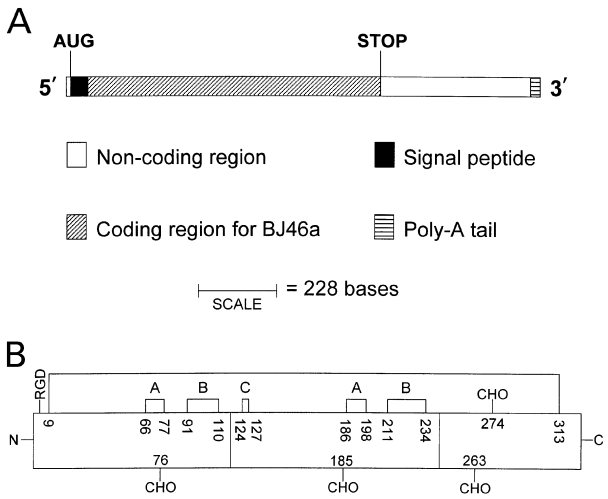


**Fig. 5. Schematic representation of *BJ46a* mRNA (A) and hypothetical arrangement of disulfide bridges in BJ46a protein (B).** Numbers identify the position of the amino-acid residues shown in Fig. 4. As in fetuin, each cystatin domain has an A- and B-type loop and the second domain also has the very narrow C-type loop. The N- and C-terminal regions are also connected by a disulfide bond [34]. Four positions (76, 185, 263 and 274) indicate putative N-glycosylation consensus sites (CHO). The N-terminal RGD sequence position is also shown.

acids were identified by Edman degradation at positions 76 and 263, corroborating the presence of glycosylation at these two positions. The other two putative N-glycosylation sites (asparagines 185 and 274) were in regions of the molecule for which no peptide was isolated for sequencing by Edman degradation.

## DISCUSSION

Isolation and characterization, to varying extents, of mammalian and reptilian endogenous inhibitors specific for snake venom metalloproteinases have been widely reported in the literature and have been the subject of review articles [15–17].

Among the high molecular mass inhibitors purified to date, erinacin, isolated from the muscle extracts of *E. europaeus*, is the best characterized. It is an oligomer ($\approx$ 1000 kDa) containing a noncovalent association of two types of subunits, α (37 kDa) and β (350 kDa). The α subunit exists as a monomer but the β subunit is a decamer of 35-kDa polypeptide chains crosslinked by disulfide bridges. The inhibitor's subunit composition, in its native state, is $\alpha_{10}{\cdot}2\beta_{10}$ and it is an effective metalloproteinase inhibitor when tested against jararhagin; an equimolar stoichiometry has been suggested, indicating one active hemorrhagic protein binding site per inhibitor molecule. Amino-acid sequence analysis indicated that the subunits are very similar and composed of common N-terminal, collagen- and fibrinogen-like domains homologous to proteins of the ficolin/opsonin p35 lectin family. The authors speculate on the possible role of the collagen- and fibrinogen-like domains in the mechanism of inhibition, but more data are necessary to support their claim [18].

Among the isolated low molecular mass inhibitors, only two have had their complete amino-acid sequence determined: HSF from the snake *T. flavoviridis* [19] and DM43 from the South American opossum *D. marsupialis* [12]. The former is classified as belonging to family 4 of the cystatin superfamily of proteinase inhibitors, while the latter belongs to the immunoglobulin supergene family. The mechanism of action of these low molecular mass inhibitors with isolated SVMPs seems to be by noncovalent complex formation between enzyme and inhibitor but no further details are available in the literature.

In the present study, we have isolated from the serum of the snake *B. jararaca* an anti-hemorrhagic factor we termed BJ46a. The homogeneous protein has a molecular mass of 55.4 kDa by SDS/PAGE under denaturing conditions (Fig. 2A) and 46 101 a.m.u. by MALDI-TOF MS (Table 1). The anomalous higher value obtained by electrophoresis is probably an artefact as a result of the glycosylated state of BJ46a (17% carbohydrate, Table 1) and has also been observed in the opossum serum inhibitor DM43 [31]. Gel filtration experiments in conjunction with dynamic laser light scattering revealed a molecular mass of 79 kDa for the native inhibitor in solution (Table 1). The above results clearly indicate that the inhibitor is an homodimer in its native state; this is the first time that a nonmonomeric structure for these low molecular mass SVMP inhibitors has been demonstrated.

The calculated pI for BJ46a, based on its deduced protein sequence (Fig. 4), is 5.74 (PROTEINPROSPECTOR Software v.3.2.1, MSDIGEST option, UCSF, CA, USA). However, the experimental pI obtained was 4.55 (Fig. 2B) suggesting that the carbohydrate portion of BJ46a has an acidic nature, which could be due to the presence of *N*-acetylneuraminic acid, as previously observed for other members of the low molecular mass class of inhibitors [10,11].

The inhibitor BJ46a was effective in inhibiting *B. jararaca* venom hemorrhagic activity as tested by intradermic injection in rat skin (data not shown). Also, when the inhibitor was tested against isolated SVMPs, specifically atrolysin C (class P-I) and jararhagin (class P-III), it inhibited their metalloproteinase activity (Table 2). Studies of the interaction of these isolated SVMPs and the inhibitor clearly indicate that BJ46a interacts with the metalloproteinases by the formation of a noncovalent complex (Fig. 3A–D). As atrolysin C contains only a metalloproteinase domain whereas jararhagin contains metalloproteinase, disintegrin-like and cysteine-rich domains, our results indicate that the interaction between BJ46a and proteinases occurs between the metalloproteinase domain and the inhibitor. Corroborating this result, is the data showing that the incubation of a threefold molar excess of BJ46a with jararhagin-C [32], a protein with identical sequence to jararhagin but lacking the metalloproteinase domain, did not lead to complex formation. Moreover, by analyzing the results presented in Table 1 and Fig. 3A–D, we concluded that BJ46a does not interact as a dimer with either of the enzymes tested. Our proposed mechanism infers that there is a dissociation of the inhibitor dimer during complex formation and that the final stoichiometry is of one inhibitor monomer to two metalloproteinases molecules. This result suggests that two binding sites for a SVMP metalloproteinase domain are present in one monomer (subunit) of BJ46a.

Of significance is that this is the first reported full-length cDNA sequence for an endogenous SVMP inhibitor (Fig. 4). The sequence is comprised of a 5′ noncoding region, a signal sequence, a coding region for BJ46a, a 3′ noncoding region and the poly A tail (Fig. 5A). The cleavage site [Ser(−1) to Ser1 in Fig. 4] between the signal peptide and the coding region for BJ46a was predicted by the SIGNALP software v.1.1, a neural network software designed to predict theorethical cleavage sites for signal peptides in eukaryotes and prokaryotes [33]. The predicted cleavage site was in agreement with the one determined experimentally by N-terminal Edman sequencing of the isolated protein. Hence, there is no pro-region in BJ46a structure and no C-terminal processing required after translation of the coding region into the mature BJ46a subunit.

The homologous HSF contains 13 cysteines; the disulfide bond pattern observed in fetuins suggests that 12 of these are found in disulfide bonds. The thirteenth cysteine (at position 44) is considered to exist in a mixed disulfide with a thiol compound since no free SH group could be detected [19]. BJ46a has only 12 cysteines, all at corresponding cysteine positions in HSF, typical of the cysteine pattern observed for the fetuin group of proteins. However, at position 44 of the amino-acid sequence, BJ46a has a valine instead of the thirteenth cysteine as in HSF. As BJ46a, like HSF, is a potent SVMP inhibitor, this suggests that the 'thirteenth cysteine' is not involved in the inhibition process. We postulated the disulfide bridge arrangement for BJ46a is identical to that of fetuin. As in fetuin, each cystatin domain has an A- and B-type loop and the second domain also has the very narrow C-type loop where two amino acids separate the cysteines. Also, like in fetuins, the N- and C-terminal regions of BJ46a are connected by an additional disulfide bond [34] (Fig. 5B). Fetuin is involved in inhibition of the insulin receptor tyrosine activity [29], calcium homeostasis [35,36], osteogenesis [37] and serine proteinase inhibitory activity [38,39]. Although classified as members of the cystatin superfamily, the fetuins (including HSF and BJ46a) are unlikely to be viable cysteine proteinase inhibitors because the consensus sequence for the cystatin active site, QXVXG, has been extensively altered in these molecules [29]. Corroborating this view, HSF failed to inhibit papain and cathepsin B activities and no other evidence of cysteine proteinase inhibition was demonstrated [19]. Nevertheless, Brown and Dziegielewska [34] propose that it is most probable that the fetuins (containing two inactive cystatin domains) have evolved from a common cysteine proteinase inhibitor containing two active cystatin domains and that thus, in the cystatin superfamily, the common building blocks (cystatin domains) have been used to create functionally diverse proteins.

In the N-terminal portion, BJ46a contains the tripeptide sequence arginine-glycine-aspartic acid (RGD) which represents a common integrin recognition site [40]. The RGD sequence is present in the disintegrins, a family of integrin inhibitory proteins from viper venoms, and is responsible for the inhibition of platelet aggregation [41]. HSF (*T. flavoviridis* fetuin) also contains the RGD sequence in its N-terminal region and was shown to inhibit platelet aggregation rather weakly (as stated by the authors) when compared to members of the disintegrin family [19].

The mechanism of action for BJ46a does not appear to be like that of cystatins, where there is no direct interaction with the proteinase catalytic residues [42], because our results (Table 2) indicated that even a small substrate (tetrapeptide) was not cleaved by the SVMPs (atrolysin C or jararhagin) when the inhibitor was present. Hence, as in the tissue inhibitor of metalloproteinases [43], it is possible that the mechanism of action involves a direct interaction of the inhibitor with the metalloproteinase catalytic zinc, leading to enzymatic inhibition.

Snake bite represents a significant public health problem throughout the world, particularly in tropical countries where mortality and morbidity rates are high [44]. In a recent global survey carried out on snake-bite epidemiology, the incidence exceeded 5 million per year, with a 50% envenomation rate. The associated mortality level was found to be of 125 000 persons per year and probably more than 100 000 persons suffer from severe sequelae, despite anti-venom treatment [45]. Intravenous administration of equine or ovine-derived anti-venoms constitutes the primary approach in treating snakebite envenomation [46], but local tissue damage induced by snake venoms is difficult to prevent by serotherapy, even in a situation where high amounts of antibodies are present before venom entry [47]. Hence, the endogenous SVMP inhibitor presented in this work, BJ46a, could be used to develop new and more effective anti-snake-venom therapy.

Moreover, BJ46a could be used in the study of other important related metalloproteinases such as the ADAMs and the MMPs. The ADAMs are involved in spermatogenesis, signal transduction, sheddase (a group of enzymes sometimes called secretases or convertases, that proteolytically process membrane proteins leading to release of the physiologically active forms of the proteins; reviewed in [47a]) activity, cell adhesion and migration as well as apoptosis, inflammatory processes and Alzheimer's disease [48]. They belong to the reprolysin family (as do the SVMPs) as their domain structure is similar to that of the SVMPs to a certain extent and they share a 30% sequence identity in the metalloproteinase domain [4]. The MMPs also play a relevant role in a variety of normal cellular processes as well as pathologies such as cancer invasion and metastasis, arthritis, autoimmune diseases, periodontitis, tissue ulceration, atherosclerosis, aneurysm and heart failure [49]. They share with the SVMPs identical zinc-binding environment and topology [5]. Recently, a synthetic peptidomimetic MMP inhibitor (batimastat or BB-94) was shown to neutralize BaP1 (a class P-I SVMP from *Bothrops asper*) hemorrhagic, dermonecrotic and edema-forming activities [50]. This suggests that the two families of enzymes (SVMPs and MMPs) may share a related mechanism for inhibition. Thus, BJ46a could be used as molecular tool to provide new insights into the mechanism of inhibition of ADAMs and MMPs, leading to new therapeutical approaches against the pathologies associated with these enzymes.

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## SUPPLEMENTARY MATERIAL

The following material is available from
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Fig. S1. Amino-acid composition of BJ46a.
Table S1. Reversed-phase HPLC isolation of peptides obtained by endoproteinase Asp-N and Lys-C digestions of BJ46a.