Biochemical and structural characterization of a protein complex containing a hyaluronidase and a CRISP-like protein isolated from the venom of the spider Acanthoscurria natalensis

Barth, Tania; Mandacaru, Samuel Coelho; Charneau, Sébastien; Souza, Marcelo Valle de; Ricart, Carlos André Orinelas; Noronha, Eliane Ferreira; Souza, Amanda Araújo; Freitas, Sonia Maria de; Roepstorff, Peter; Fontes, Wagner; Castro, Mariana S.; Pires Júnior, Osmindo Rodrigues

Published in: Journal of Proteomics

DOI: 10.1016/j.jprot.2018.08.012

Publication date: 2019

Document version: Accepted manuscript

Document license: CC BY-NC-ND

Citation for published version (APA):

Terms of use:
This work is brought to you by the University of Southern Denmark through the SDU Research Portal. Unless otherwise specified it has been shared according to the terms for self-archiving. If no other license is stated, these terms apply:

- You may download this work for personal use only.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying this open access version

If you believe that this document breaches copyright please contact us providing details and we will investigate your claim. Please direct all enquiries to puresupport@bib.sdu.dk
Biochemical and structural characterization of a protein complex containing a hyaluronidase and a CRISP-like protein isolated from the venom of the spider Acanthoscurria natalensis

Tania Barth, Samuel Coelho Mandacaru, Sébastien Charneau, Marcelo Valle de Sousa, Carlos André Ornelas Ricart, Eliane Ferreira Noronha, Amanda Araújo Souza, Sonia Maria de Freitas, Peter Roepstorff, Wagner Fontes, Mariana S. Castro, Osmindo Rodrigues Pires Júnior

PII: S1874-3919(18)30324-5
DOI: doi:10.1016/j.jprot.2018.08.012
Reference: JPROT 3198
To appear in: Journal of Proteomics
Received date: 18 May 2018
Revised date: 23 July 2018
Accepted date: 19 August 2018

Please cite this article as: Tania Barth, Samuel Coelho Mandacaru, Sébastien Charneau, Marcelo Valle de Sousa, Carlos André Ornelas Ricart, Eliane Ferreira Noronha, Amanda Araújo Souza, Sonia Maria de Freitas, Peter Roepstorff, Wagner Fontes, Mariana S. Castro, Osmindo Rodrigues Pires Júnior, Biochemical and structural characterization of a protein complex containing a hyaluronidase and a CRISP-like protein isolated from the venom of the spider Acanthoscurria natalensis. Jprot (2018), doi:10.1016/j.jprot.2018.08.012

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Biochemical and structural characterization of a protein complex containing a hyaluronidase and a CRISP-like protein isolated from the venom of the spider *Acanthoscurria natalensis*.

Tania Barth\(^1,2,\ast\), Samuel Coelho Mandacaru\(^3\), Sébastien Charneau\(^3\), Marcelo Valle de Sousa\(^3\), Carlos André Ornelas Ricart\(^3\), Eliane Ferreira Noronha\(^4\), Amanda Araújo Souza\(^5\), Sonia Maria de Freitas\(^5\), Peter Roepstorff\(^6\), Wagner Fontes\(^3\), Mariana S. Castro\(^1,3\), Osmindo Rodrigues Pires Júnior\(^1,\ast\)

\(^1\)Laboratory of Toxinology, Department of Physiological Sciences/IB, University of Brasilia, Brasilia-DF, Brazil, 70910-900; E-mails: tanbarth@uesc.br (T.B.); mscastro@unb.br (M.S.C.); osmindo@unb.br (O.R.P.Jr.)

\(^2\)Laboratory of Animal Histology, Department of Biological Sciences, State University of Santa Cruz, Ilhéus-Bahia, Brazil, 45662-900; E-mail: tanbarth@uesc.br (T.B.)

\(^3\)Laboratory of Protein Chemistry and Biochemistry, Department of Cell Biology/IB, University of Brasilia, Brasilia-DF, Brazil, 70910-900; E-mails: wagnerf@unb.br (W.F.); mscastro@unb.br (M.S.C.); samueldtna@gmail.com (S.C.M.); charneau@unb.br (S.C.); mvsousa@unb.br (M.V.S.); ricart@unb.br (C.A.O.R.)

\(^4\)Laboratory of Enzymology, Department of Cellular Biology/IB, University of Brasília, Brasilia-DF, Brazil, 70910-900; E-mail: enoronha@unb.br (N.E.F.)

\(^5\)Laboratory of Molecular Biophysics, Department of Cellular Biology/IB, University of Brasília, Brasilia-DF, Brazil, 70910-900; E-mail: amandhacoelho@gmail.com (A.A.S.), nina@unb.br (S.M.F.)
6Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense M, Denmark; E-mail: roe@bmb.sdu.dk (P.R.)

(*) Corresponding author address:

E-mail: tanbarth@uesc.br (T.B.), Tel.: +55-61-984310464

State University of Santa Cruz, Ilhéus-BA, Soane Nazaré de Andrade Campus, Department of Biological Sciences, Laboratory of Animal Histology. Ilhéus-BA. CEP: 45662-900. Brazil.

E-mail: osmindo@unb.br (O.R.P.Jr.), Tel.: +55-61-3107-3110

University of Brasília, Brasilia-DF, Darcy Ribeiro University Campus, Institute of Biology-IB, Department of Physiological Sciences, Laboratory of Toxinology, Building G - Ground Floor. Brasilia-DF. CEP: 70910-900. Brazil.
Biochemical and structural characterization of a protein complex containing a hyaluronidase and a CRISP-like protein isolated from the venom of the spider *Acanthoscurria natalensis*.

Abstract

Spider venoms are composed of a complex mixture of bioactive molecules. The structural and functional characterization of these molecules in the venom of the Brazilian spider *Acanthoscurria natalensis*, has been little explored. The venom was fractionated using reversed-phase liquid chromatography. The fraction with hyaluronidase activity was named AnHyal. The partial sequencing of AnHyal revealed the presence of a CRISP-like protein, in addition to hyaluronidase, comprising 67% coverage for hyaluronidase from *Brachypelma vagans* and 82% for CRISP-like protein from *Grammostola rosea*. 1D BN-PAGE zymogram assays of AnHyal confirmed the presence of enzymatically active 53 kDa monomer and 124 and 178 kDa oligomers. The decomposition of the complexes by 2D BN/SDS-PAGE zymogram assays showed two subunits, 53 (AnHyalH) and 44 kDa (AnHyalC), with sequence similarity to hyaluronidase and CRISP proteins, respectively. The secondary structure of AnHyal is composed by 36% of α-helix. AnHyal presented maximum activity at pH between 4.0 and 6.0 and 30 and 60°C, showed specificity to hyaluronic acid substrate and presented a $K_M$ of 617.9 μg/mL. Our results showed that hyaluronidase and CRISP proteins can form a complex and the CRISP protein may contribute to the enzymatic activity of AnHyalH.
Significance

Spider venoms represent an important source of bioactive molecules, such as acylpolyamines, neurotoxic peptides, hyaluronidase and CRISP-like proteins. Currently, there are 46,778 described species of spiders, however, total or partial sequencing of hyaluronidases was established for only seven species, six of which are available in the databases Uniprot and NCBI (accessed 28.08.2017). Although the number of CRISP-like sequences deposited in the databases (from 10 different species) is higher in relation to hyaluronidases, a large part of these was not evidenced experimentally or functionally characterized. The present work led to the detection of a new complex involving a hyaluronidase and a CRISP-like protein from *Acanthoscurria natalensis* venom, as well as, the partial sequencing of its components by LC-MS/MS analysis of the sample digested with trypsin, Lys-C and Glu-C combined to Edman degradation using the intact sample. Based on these results, we propose the formation of a complex between hyaluronidase and CRISP-like protein from *A. natalensis* venom with hyaluronidase activity.

Keywords: spider venom, *Acanthoscurria natalensis*, hyaluronidase/CRISP complex, de novo sequencing, enzymatic characterization.
1. Introduction

Spider venoms contain different biologically active compounds, including high molecular mass enzymatic and non-enzymatic activities proteins. Hyaluronidases (Hyal) constitute a well-conserved enzyme class, produced by unicellular organisms up to vertebrates [1]. This class of enzymes preferably catalyzes the hydrolysis of hyaluronic acid, one of the main constituents of the extracellular matrix present in connective tissues [2]. This action facilitates the diffusion of other toxins present in the venom and is therefore known as a "spreading factor" [3]. This property has been commonly employed to facilitate the absorption and dispersion of injected drugs. However, its potential in the medical and biotechnology areas is even broader, being demonstrated by the use of hyaluronidase in antitumor therapies and immunotherapy [2,4–6].

The presence of hyaluronidase activity in spider venoms was previously described for species of several families such as Lycosidae, Ctenidae, Scicardiidae, Theridiidae and Theraphosidae [1]. However, its primary structure was described only for few species, through the venom gland RNA-seq or partially by Edman degradation [2]. Recently, these strategies have been associated with mass spectrometry, enabling the complete sequencing of a hyaluronidase from *Cupenius salei* venom [7].

CRISPs represent an extensive family of highly conserved cysteine-rich secretory proteins, comprising three main groups, named CRISP-1, CRISP-2 and CRISP-3 [8]. The main biological functions related to CRISP-1 and CRISP-2 include ion channels blocking and involvement in the processes of gametes maturation and fusion, being in this case mediated by a motif named signature 2 [9]. CRISP-3 is found in blood plasma and exocrine secretions and its role in
innate immunity has been proposed [10]. Additionally, it has been shown that CRISP-3 forms complexes with α1B-glycoprotein in blood plasma [11] and PSP94 in seminal plasma [12,13]. More recently, CRISPs were identified in the venom of several animals, presenting different functions. In the snake venoms, CRISPs are widely distributed and act primarily as ion channel blockers [14]. In Bothrops jararaca snake, Bj-CRP, the first CRISP isolated from the venom of this species, presented activities on the complement system and induced inflammatory responses [15]. On the other hand, in the venom of other animals, CRISPs have been poorly reported. In the cone snail Conus textile [16] and Grammostola iheringi spider [17], the CRISPs identified showed protease activity, but in Grammostola rosea (M5AWW7, from UniProtKB database) and Trittame loki spider, no activity was reported [18]. These evidences show that the biological functions related to CRISPs can be quite diverse.

Proteomics has different methodologies that make it an important ally for new discoveries. Among these, the combined use of different proteases (such as trypsin, Lys-C etc.) for the hydrolysis of the target protein and mass spectrometry fragmentation techniques (such as CID/ETD/HCD), can promote complete sequencing of high molecular mass proteins [19].

The knowledge about biologically active molecules in species of Acanthoscurria genus is limited, being represented mainly by antimicrobial peptides and polyamines isolated from the venom and hemocytes, respectively [20–22]. The genus Acanthoscurria (Theraphosidae) comprises 28 species of tarantula (caranguejeira) spiders, distributed predominantly throughout countries in South America [23]. Acanthoscurria natalensis is a non-aggressive species with wide distribution in the Brazilian Caatinga and Cerrado biomes [24]. For this
species, only μ-theraphotoxin-An1, a neurotoxic peptide isolated from the venom, was described [25], and there is no current information in the literature about other components, such as hyaluronidases or CRISP-like proteins. Moreover, the formation of a complex between these proteins has not been reported in the literature.

In the present study the purification, structural and enzymatic characterization of a complex involving a hyaluronidase and CRISP-like protein from A. natalensis venom are presented.

2. Material and Methods

2.1. Spiders and venom extraction

Female spiders of A. natalensis (n=30) were collected (SISBIO license number 51803-1) from Fazenda Nossa Senhora Aparecida (GO, Brazil). The venom (~ 30 μL/animal) was extracted by electrostimulation (75 V for 3 s) between 1 and 2 times for each animal [26]. The samples were lyophilized and stored at -20 °C until use.

2.2. Enzymatic activity

Hyaluronidase activity was determined by turbidimetry, according to di Ferrante [27], with some modifications. The reaction mixture in triplicates was prepared in a 96-well plate using 57 μL of sodium acetate buffer 200 mM, pH 5.8 containing 150 mM of NaCl, 12 μL of hyaluronic acid (0.5 mg/mL) and 5 μL of enzymatic sample (corresponding to 50 μg of venom or 5 μg of polled chromatographic fractions). Bovine testis hyaluronidase (5 μg) (400-1000 U/mg, Sigma Aldrich) was used as positive control. Then the plate was incubated for 15
min at 37 °C and the enzymatic reaction was stopped by adding 125 μL of cetyltrimethylammonium bromide (CTAB) 2.5% (w/v) in NaOH 2% (w/v). After 10 min, the absorbance was measured at 405 nm against a blank containing buffer and CTAB. Wells containing only buffer or hyaluronic acid in buffer, were used as controls of 0% and 100% of hyaluronic acid (no activity), respectively. The hyaluronidase activity was expressed as a percentage of substrate hydrolyzed relative to the control in which no enzyme was added.

2.3. Crude venom fractionation by reversed-phase liquid chromatography (RP-HPLC)

The crude venom was solubilized (25 mg/mL) in solvent A (0.12% trifluoroacetic acid (TFA) (v/v) in water) and centrifuged (10,000 rpm). Aliquots of 200 μL of the supernatant were applied to a C₁₈ reversed-phase column (Vydac 218TP54, 4.5 mm x 250 mm, 5 μm), previously equilibrated with the same solvent, using a flow rate of 1 mL/min. The elution of fractions was obtained using a linear gradient of 0 to 60% of solvent B (0.12% TFA (v/v) in acetonitrile (ACN)) in 60 min and detected simultaneously at 216 nm and 280 nm. The chromatographic fractions were manually collected, lyophilized and analyzed for enzymatic activity. The fraction that presented hyaluronidase activity was denominated AnHyal. The AnHyal fraction was accumulated from several chromatographic runs and the enzymatic activity was tested for each run. Fractions eluted at the expected retention time that did not show activity were solubilized in water, kept overnight at 37 °C and tested again.

2.4. SDS-PAGE and zymogram
Electrophoresis was carried out using AnHyal (4 μg) as samples, on 12% SDS-PAGE as previously described [28]. The run was performed in a mini-gel system under voltage of 150 V and current of 40 mA and the gel was stained with silver nitrate method [29].

Zymogram was performed as above mentioned for SDS-PAGE gels, with exception to the addition of hyaluronic acid (1 mg/mL) for copolymerization with polyacrylamide gel [30]. Aiming to detect the enzymatic activity the gel was washed with TritonX-100 2.5% (v/v) and incubated in Tris-HCl 20 mM buffer, pH 7.4 containing 0.5 mM of CaCl₂, for 20 h at 37 °C, and then stained with Alcian Blue 0.5% (w/v) in methanol 25% (v/v) and acetic acid 10% (v/v) and Coomassie Brilliant Blue (CBB) G250 0.25% (w/v) in methanol 25% (v/v) and acetic acid 10% (v/v). The formation of clear zones in the gel in contrast to the blue background indicated enzyme activity. A molecular mass marker (Low Molecular Weight Calibration Kit for Electrophoresis, GE Healthcare, U.K.) containing proteins presenting molecular masses from 97 to 14.4 kDa were used in both analyses. The apparent molecular masses were determined by interpolation from the standard curve constructed from molecular markers, using distance of migration versus log (MW) plots.

2.5. Blue native (BN)-PAGE, 2D BN/SDS-PAGE and zymogram

BN-PAGE [31,32] was performed using an SE 600 electrophoresis system (Hoefer, San Francisco, CA), with some modifications. AnHyal samples were solubilized into 50 mM bis-Tris-HCl buffer containing 15% (v/v) glycerol and loaded in duplicate on 5 to 18% (w/v) polyacrylamide gradient gels copolymerized with hyaluronic acid (1 mg/mL). The run was performed at 15 mA and 4 °C for 4
h. The anode buffer consisted of 50 mM bis-Tris-HCl, while the cathode buffer consisted of 50 mM Tricine, 15 mM bis-Tris, and 0.02% (w/v) CBB G-250. After the electrophoretic run, one half of the gel was stained with CBB G-250 0.25% (w/v) in methanol 50% (v/v) and acetic acid 10% (v/v) and the mirror half was used to detect the enzymatic activity as described in Section 2.4. Proteins presenting molecular masses from 669 to 66 kDa were used as molecular mass marker. The apparent molecular masses were determined as described above (Section 2.4).

For SDS-PAGE (second dimension), the band of interest was excised from the BN-PAGE (first dimension) before staining and placed into a well on 12% (w/v) SDS-PAGE gels copolymerized with hyaluronic acid (1 mg/mL). The run was performed at 25 mA and 15 °C for 6 h and enzymatic activity was detected as described in Section 2.3. Proteins presenting molecular masses from 97 to 14.4 kDa (Low Molecular Weight Calibration Kit for Electrophoresis, GE Healthcare, U.K.) were used as molecular weight marker.

2.6. In-gel Digestion

For in-gel digestion [33], gel spots from zymogram and 2D BN/SDS-PAGE zymogram were excised, washed in ACN and lyophilized. The samples were reduced with 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate buffer for 1 h at 52 °C and alkylated by the addition of 55 mM iodoacetamide (IAA) in the same buffer for 45 min at 25 °C. The samples were then washed with ACN and buffer, lyophilized and hydrated for trypsin (12.5 ng/µL in buffer) digestion overnight at 37 °C. Peptides were extracted with 0.1% TFA (v/v) and 0.1% TFA (v/v) in 50% ACN (v/v), lyophilized and desalted on a pipette tip packed with C18.
membrane (Empore, Supelco). The sample was washed 3 times with 0.1% TFA (v/v) and the peptides eluted with 40%, 70% and 100% ACN solutions, all containing 0.1% TFA (v/v), were lyophilized for mass spectrometry analysis.

2.7. Mass spectrometry

Native AnHyal was analyzed using an AB SCIEX TOF/TOF™ 5800 mass spectrometer (AB Sciex, Framingham, MA, USA) with sinapinic acid as the matrix. The ions were detected in linear positive mode between 10-150 kDa and the mass spectra were converted to the “.mzXML” format for data analysis using the mMass 5.5.0 software.

For analysis by LC-MS/MS using an Orbitrap Elite™ hybrid ion trap-orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), four aliquots containing 20 μg of AnHyal were used. One aliquot was previously acetylated by the addition of 40 μL of acetic anhydride in 1.5 mL of 100 mM Tris-HCl buffer, pH 8.0. This mixture was incubated for 1 h at 25 °C and desalted on a pipette tip packed with C8 membrane (Empore, Supelco). The sample was washed 3 times with TFA 0.1% (v/v) and the proteins eluted with 40%, 70% and 100% acetonitrile solutions, all containing TFA 0.1% (v/v), were lyophilized.

The four aliquots of AnHyal were reduced with 10 mM dithiothreitol (DTT) in 6 M guanidine buffer, 0.25 M Tris, pH 8.6 for 1 h at 52 °C and alkylated by the addition of 50 mM iodoacetamide for 1 h at 25 °C. The acetylated aliquot was digested overnight with trypsin (Promega, Madison, WI, USA) at 37 °C in 100 mM ammonium bicarbonate buffer, pH 8.1. The other three aliquots were digested with trypsin, Lys-C or Glu-C (Promega, Madison, WI, USA) under the same conditions, all with an enzyme:substrate ratio of 1:50. The samples were desalted
on a pipette tip packed with C\textsubscript{18} membrane, as described above [34] and lyophilized. The digests (from native AnHyal and in-gel spots) were resuspended in 0.1\% (v/v) formic acid and loaded into a nano-UPLC-Dionex 3000 system (Thermo Fisher Scientific Inc., Waltham, MA, USA) equipped with a trap type C\textsubscript{18} column (100 μm x 3 cm with particles of 5 μm/100 Å) and a C\textsubscript{18} analytical column (75 μm x 35 cm with particles of 3 μm/100 Å). The peptides were eluted from the analytical column with a gradient of 2\% to 35\% of solvent B (0.1\% (v/v) formic acid in acetonitrile) during 50 min directly into the mass spectrometer under ESI ionization. Molecular mass spectra were acquired in Data Dependent Acquisition (DDA) mode and controlled by Xcalibur 2.0 software (Thermo Fisher Scientific Inc., Waltham, MA, USA). The DDA acquisition cycle comprised the range of m/z 350 to 1650 and resolution of 120,000 for MS1. The ten most abundant precursor ions were fragmented by collision-induced dissociation (CID), with dynamic exclusion for 90 s, collision energy normalized to 35 \% and the fragments were detected using an ion trap system. The same samples were injected again to the LC-MS/MS system for high energy collision dissociation (HCD) and for electron transfer dissociation (ETD) fragmentation, when the MS2 detection was performed at 60,000 resolution in the orbitrap analyzer.

2.8. N-terminal sequencing by Edman degradation

The Edman degradation method using an automatic peptide and protein sequencer (PPSQ-31A / 33A, Shimadzu) was performed for the determination of the N-terminal sequence of the native AnHyal.

2.9. Data processing and sequence analysis
The "raw" files generated by the spectrometer were imported by PEAKS Studio 7.0 (Bioinformatics Solution Inc., Waterloo, Canada) software to perform peptide de novo sequencing from the MS/MS spectra and to search for similarities in databases. The databases were generated in FASTA format containing peptide sequences corresponding to subsets of the UniprotKB. The first subset contained only proteins presenting the search term “Hyaluronidase” (1,721 sequences, downloaded in February 25, 2016). The second subset was assembled by merging the results from first subset and proteins presenting the term “Allergen” (30,211 sequences, UniprotKB, downloaded in May 12, 2017). Finally, a third subset containing proteins presenting the search term “CRISP” (9,620 sequences, UniprotKB, downloaded in January 10, 2018) was used for a complementary search. De novo sequenced peptides with average local confidence (ALC) scores ≥50% were selected for database searches. The search parameters included specification of the enzyme for each sample, cysteine carbamidomethylation as fixed modification and oxidation of methionine and acetylation of lysine as variable modifications, maximum of 2 missed cleavages by trypsin, Lys-C and Glu-C, and a mass error tolerance of 10 ppm for precursor ions, 0.5 Da for fragment ions in CID and 0.05 Da for fragment ions in HCD and ETD. A false discovery rate (FDR) <1% and a minimum of one unique peptide were used as criteria to consider the identifications as positive. Peptide sequences were first searched against the first subset (hyaluronidases). Peptides that generated de novo sequences not matched to the database (named de novo only peptides) and N-terminal sequences obtained by Edman degradation were subjected to BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) homology searches against the non-redundant protein sequences (nr) and UniProtKB/Swiss-Prot.
(swissprot) database. BLAST results suggested the inclusion of allergens, thus the second subset was assembled, and the PEAKS search repeated. *De novo* only sequences from the second Peaks search were also subjected to BLAST homology searches against the same databases and aligned to the sequences of hyaluronidase and CRISP-like proteins from the spiders *Brachypelma vagans* and *Grammostola rosea*, respectively (identified by PEAKS) using the Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The search for conserved domains was performed using InterPro (https://www.ebi.ac.uk/interpro/) and Prosite (http://prosite.expasy.org/) programs. For multiple sequence alignment, Hyaluronidase and CRISP *de novo* sequences, *de novo* only sequences and N-terminal sequence were aligned by Clustal Omega with known hyaluronidase and CRISP sequences from other arthropods obtained from UniprotKB. The alignments and *de novo* sequences were manually verified.

2.10. Circular Dichroism (CD) assays

To investigate the secondary structure content of AnHyal (0.18 mg/mL in 2 mM sodium acetate buffer, pH 5.8), circular dichroism spectra were recorded using the Jasco J-815 CD Spectropolarimeter (Jasco Corporation, Tokyo, Japan) with a peltier cell holder and temperature controller (Jasco Analytical Instruments, Japan). All measurements were carried out at 25 °C using the 0.1 cm quartz cuvettes in the Far-UV regions (190–260 nm) with data acquisition interval of 0.2 nm. Three successive scans were accumulated, and the mean spectrum was recorded. The CD signal contribution of the buffer was subtracted and the mean ellipticities were converted to molar ellipticities \([\theta]\) (deg.cm2.dmole−1) based on a mean molecular mass per residue of 115 Da. The secondary structures content
of protein was estimated by deconvolution of the CD data using CDNN version 2.1 software [35].

2.11. Enzymatic characterization

Temperature and pH effect

The effect of pH and temperature on AnHyal was evaluated by the method described above (Section 2.2), varying the pH from 3.0 to 8.0 using citrate-phosphate buffer 100 mM (pH 3.0 to 6.0) and Tris-HCl buffer 100 mM (pH 7.0 and 8.0) and temperature from 10 to 70°C, respectively. The pH and temperature conditions at which the enzyme showed maximum activity were used in subsequent enzymatic assays.

Kinetic characterization

The substrates hyaluronic acid and chondroitin sulfate-A (both at 0.5 mg/mL) were used to evaluate the substrate specificity. The enzymatic assay was performed as described above using hyaluronic acid or chondroitin sulfate-A as substrate.

Kinetic parameters $K_M$ and $V_{max}$ were obtained by incubating the AnHyal (5 μg) with different amounts of hyaluronic acid (0 to 40 μg) in sodium acetate buffer 200 mM, pH 5.8 containing 150 mM of NaCl. The rate of reaction (Vo) was expressed as μg of hyaluronic acid consumed per minute. The values of $K_M$ and $V_{max}$ were calculated using non-linear regression from the data in technical triplicates, using the software GraphPad Prism 5 (GraphPad Software, Inc, USA).

3. Results
3.1. Purification of AnHyal

The venom of *A. natalensis* was fractionated by RP-HPLC and fractions were tested for hyaluronidase activity by the *in vitro* turbidimetric assay. Only the fraction eluted between 50 to 55 min exhibited hyaluronidase activity (data not shown), being named AnHyal (Fig. 1A). MALDI-TOF analysis showed only one singly charged ion at 42.55 kDa, suggesting the homogeneity of AnHyal (Fig. 1B). Multi-charged ions suggested the presence of oligomeric forms, like that observed in 12% SDS-PAGE (Fig. 1C) and zymogram (Fig. 1D), that showed enzymatically active bands in addition to the 53 kDa band, estimated from the standard curve. There was also a non-active band visualized at approximately 44 kDa.

3.2. Structural characterization of AnHyal

The partial sequence of AnHyal was obtained by *de novo* MS sequencing and Edman degradation. For *de novo* sequencing, AnHyal was digested with multiple enzymes and the peptides were fragmented on the spectrometer by CID, HCD end ETD (see Supporting Information 1 and 2). A total of 5583 *de novo* sequenced peptides were obtained, and the generated spectra were analyzed using PEAKS software for database searches and compared against a subset from UniprotKB hyaluronidase database. This analysis confidently identified one hyaluronidase (from the spider *Brachypelma vagans*) with protein coverage of 54%. Since many confidently sequenced peptides did not match any protein from the database (named *de novo* only peptides), these were submitted to a BLAST search against non-redundant protein sequences (nr) and UniProtKB/Swiss-Prot database and suggested the presence of allergens. In this way and considering
that several hyaluronidases are described as potential allergens, a new database subset from UniprotKB with the search term allergen was created and merged to the hyaluronidase database for a new analysis by PEAKS. In this analysis, 489 de novo sequences presented statistically significant (FDR <1%) matches to proteins in this database. Among these, the main proteins reported were hyaluronidase from *Brachypelma vagans* (47.52 kDa) and GTx-VA1 (CRISP-2/Allergen) (45.31 kDa) from *Grammostola rosea* spider, with 67% and 59% sequence coverage, respectively (Fig. 2 and Fig. 3). The identification of *B. vagans* hyaluronidase was based on 60 MS/MS spectra, 59 of them corresponding to unique peptides, whereas for the CRISP, of the 93 MS/MS spectra employed, 80 corresponded to unique peptides. The amino acid sequences of the matching peptides to the respective proteins are shown in Table 1 and 2. The complementary search using the subset with the term CRISP confirmed the GTx-VA1 as the main homologous protein.

In addition to the AnHyal peptides used to match database sequences, a total of 4908 de novo only peptides were generated by PEAKS and those with ALC > 50% were tested for alignment to *B. vagans* hyaluronidase and GTx-VA1 from *G. rosea*. Of these, three peptides showed similarity to regions of GTx-VA1 not covered by PEAKS. (Fig. 3, Table 2). As for *B. vagans* hyaluronidase, only one of these peptides was aligned with relevant similarity (Fig. 2, Table 1).

The N-terminal sequence of native AnHyal was obtained by Edman degradation, showing a single sequence comprising 26 amino acid residues. Of these, 12 residues were also identified in MS/MS de novo only peptides sequenced by PEAKS and similar to GTx-VA1 from *G. rosea*, although the first N-terminal residue was different between the two sequencing methods (Fig. 3).
The N-terminal residue obtained by Edman and MS/MS corresponded to glycine (G) and glutamic acid (E), respectively. The MS/MS spectrum was then manually verified, and an acetylation in the glutamic acid was identified, preventing its sequencing by Edman degradation. These combined results indicated two N-terminal sequences similar to CRISP in the sample, suggesting the presence of protein species. Possibly the sequence obtained by Edman represents a low abundance protein species in AnHyal, since no corresponding peptide was identified by de novo sequencing.
Table 1. *De novo* sequencing of AnHyalH peptides obtained by digestion with different enzymes and fragmentation modes with similarity to hyaluronidase from *Brachypelma vagans*.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
<th>Enzyme/fragmentation</th>
<th>ALC (%)</th>
<th>-10lgP</th>
<th>Number of hits</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLQQYV</td>
<td>DN-only</td>
<td>Glu-C/HCD</td>
<td>97</td>
<td>31.48</td>
<td>1</td>
<td>Clustal</td>
</tr>
<tr>
<td>KDPOVFAVRWNVPITOCR</td>
<td>PTM</td>
<td>Trypsin/HC/D</td>
<td>72.59</td>
<td>6 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WNVPTOCR</td>
<td>DB</td>
<td>Trypsin/HC/D</td>
<td>68.43</td>
<td>2 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KYGMDFVPLLK</td>
<td>PTM</td>
<td>Trypsin/CID</td>
<td>96.32</td>
<td>2 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SYGILVNSDEFKGE</td>
<td>PTM</td>
<td>Glu-C/CID</td>
<td>45.61</td>
<td>1 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GQRVNGIPQLGDLPE</td>
<td>PTM</td>
<td>Trypsin/OD</td>
<td>52.93</td>
<td>3 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KYQDESFEEALL</td>
<td>PTM</td>
<td>Lys-C/CID</td>
<td>62.67</td>
<td>6 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAKNFMQLTR</td>
<td>PTM</td>
<td>Trypsin/HC/D</td>
<td>58.78</td>
<td>4 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAQTRPNSLWCYLFDPDCYNYNQTPQEFR</td>
<td>DB</td>
<td>Trypsin/CID</td>
<td>70.48</td>
<td>1 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPSIVTGNQLSWLWHESK</td>
<td>DB</td>
<td>Trypsin/HC/D</td>
<td>128.5/3</td>
<td>11 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVCPSLYVADGYLQKYTFEQR</td>
<td>DB</td>
<td>Trypsin/HC/D</td>
<td>127.3/3</td>
<td>11 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TWYVDGRLKEALR</td>
<td>DB</td>
<td>Trypsin/HC/D</td>
<td>74.82</td>
<td>4 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ILAQVASAGSSGTVWAGASATLR</td>
<td>PTM</td>
<td>Trypsin/HC/D</td>
<td>98.03</td>
<td>6 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YVKDILGPSVTTVKENAEAR</td>
<td>SPIDER</td>
<td>Trypsin/HC/D</td>
<td>48.73</td>
<td>1 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DILGPSVTTVKENAECK</td>
<td>PTM</td>
<td>Lys-C/HCD</td>
<td>65.56</td>
<td>1 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRCWPNPDNPVVARVLYLDRNKHPFK</td>
<td>PTM</td>
<td>Lys-C/HCD</td>
<td>49.23</td>
<td>2 PEAKS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SEITCHCVEGYSGRYCDVK</td>
<td>PTM</td>
<td>Trypsin/HC/D</td>
<td>50.88</td>
<td>5 PEAKS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Source of the sequence information: DN-only: peptide *de novo* only. PEAKS tools: DB: for database search, PTM: for unspecified PTM search and SPIDER: for similarity search depending on the *de novo* sequencing result

2 ALC: local confidence interval of the *de novo* only peptide, given by PEAKS

3 -10lgP: score attributed to the *de novo* peptide that supports the identification of the protein

4 Alignment method used to match the peptide (as shown in fig. 4). Clustal: Clustal Omega (multiple sequence alignment program). PEAKS: peptides automatically assigned by PEAKS software
Bold K in the sequence: Lysin acetylation identified by PEAKS
Table 2. N-terminal sequence and *de novo* sequencing of AnHyalC peptides obtained by digestion with different enzymes and fragmentation modes with similarity for GTx-VA1 from *Grammostola rosea*.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Source</th>
<th>Enzyme/fragmentation</th>
<th>ALC (％)</th>
<th>-10lgP</th>
<th>Number of hits</th>
<th>Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSCPALYLRYSKDHTFCLPRKSSCTI</td>
<td>Edman</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1</td>
<td>Clustal</td>
</tr>
<tr>
<td>ESCPALYRYSK</td>
<td>DN-only</td>
<td>Lys-C/HCD</td>
<td>94</td>
<td>–</td>
<td>2</td>
<td>Clustal</td>
</tr>
<tr>
<td>KVPTAPLCFKLDYR</td>
<td>DN-only</td>
<td>Trypsin/HCD</td>
<td>91</td>
<td>–</td>
<td>1</td>
<td>Clustal</td>
</tr>
<tr>
<td>TGPOVDOQAVAGTANAIFK</td>
<td>DN-only</td>
<td>Trypsin/CID</td>
<td>78</td>
<td>–</td>
<td>1</td>
<td>Clustal</td>
</tr>
<tr>
<td>SSCTILQSGVTKSDIEIIVREHNLLR</td>
<td>PTM</td>
<td>Trypsin/HCD</td>
<td>41.73</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>LRSKVATGKETEYMPKASNMR</td>
<td>SPIDER</td>
<td>Glu-C/HCD</td>
<td>39.80</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>QMVWDELALAAVKQ</td>
<td>DB</td>
<td>Trypsin/CID</td>
<td>88.10</td>
<td>–</td>
<td>7</td>
<td>PEAKS</td>
</tr>
<tr>
<td>LAAVQKHAQDVQFEEHDCNCRR</td>
<td>PTM</td>
<td>Trypsin/HCD</td>
<td>45.97</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>RVKNFQVQNLFQR</td>
<td>DB</td>
<td>Trypsin/HCD</td>
<td>128.4</td>
<td>3</td>
<td>15</td>
<td>PEAKS</td>
</tr>
<tr>
<td>VKNGFGQONLFQRT</td>
<td>PTM</td>
<td>Trypsin/CID</td>
<td>60.92</td>
<td>–</td>
<td>2</td>
<td>PEAKS</td>
</tr>
<tr>
<td>DWYSEIKDFDK</td>
<td>SPIDER</td>
<td>Trypsin/HCD</td>
<td>41.51</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>KIQDFIDGK</td>
<td>PTM</td>
<td>Trypsin/HCD</td>
<td>37.22</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>QIDGFIDGKGPPOTGHFETQIEW ADWR</td>
<td>PTM</td>
<td>Trypsin/CID</td>
<td>68.91</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>VGGGYSA YK</td>
<td>PTM</td>
<td>Trypsin/CID</td>
<td>54.47</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>KGSFEELTNCNYGPQGNIK</td>
<td>PTM</td>
<td>Trypsin/HCD</td>
<td>43.34</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>CPLNCSGNGCSCGTRYPGLCR</td>
<td>PTM</td>
<td>Trypsin/HCD</td>
<td>56.25</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>ISGENAPYRNE</td>
<td>PTM</td>
<td>Glu-C/HCD</td>
<td>56.51</td>
<td>–</td>
<td>2</td>
<td>PEAKS</td>
</tr>
<tr>
<td>RPEGLTFYCSFNEPDCAAATTGADK</td>
<td>DB</td>
<td>Trypsin/HCD</td>
<td>33.85</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>CAATTGADKWEVSK</td>
<td>PTM</td>
<td>Trypsin/CID</td>
<td>40.59</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>VS KTLSGSYMGI L NAGE</td>
<td>SPIDER</td>
<td>Glu-C/CID</td>
<td>58.25</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>GGE SSSLFTLPPK</td>
<td>SPIDER</td>
<td>Trypsin/HCD</td>
<td>42.13</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>VPTAPLCFK</td>
<td>PTM</td>
<td>Trypsin/HCD</td>
<td>54.36</td>
<td>–</td>
<td>7</td>
<td>PEAKS</td>
</tr>
<tr>
<td>KLGWTMETFSVSESVPK</td>
<td>PTM</td>
<td>Trypsin/CID</td>
<td>33.17</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>PKGKPAQYLE</td>
<td>DB</td>
<td>Glu-C/CID</td>
<td>44.43</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
<tr>
<td>GKPQAYLELTDLSARAGPC</td>
<td>PTM</td>
<td>Trypsin/HCD</td>
<td>52.95</td>
<td>–</td>
<td>1</td>
<td>PEAKS</td>
</tr>
</tbody>
</table>

1 Source of the sequence information: Edman degradation. DN-only: peptide *de novo* only. PEAKS tools: DB: for database search, PTM: for unspecified PTM search and SPIDER: for homology search depending on the *de novo* sequencing result

2 ALC: local confidence interval of the *de novo* only peptide, given by PEAKS

3 -10lgP: score attributed to the *de novo* peptide that supports the identification of the protein.
4 Alignment method used to match the peptide (as shown in fig. 5). Clustal: Clustal Omega (multiple sequence alignment program). Manual: manually aligned peptide for alignment optimization. PEAKS: peptides automatically assigned by PEAKS software.
Regarding the peptides assigned to hyaluronidase, manual verification of the MS/MS spectra showed that the automatically assigned sequences were correct, and the first residue acetylated, preventing its chemical sequencing. These results suggested that both proteins, CRISP and Hyaluronidase, are present in AnHyal.

The N-terminal sequence together with de novo only peptides increased the protein coverage to 82% for the CRISP component (Fig. 3, Table 2).

These results suggested the presence of two protein sequences contained in the AnHyal fraction, differently from what was observed in the MALDI-TOF analysis that showed only one singly charged ion (42.55 kDa) (Section 3.1, Fig. 1B). However, the peaks observed at m/z 28394.21, 64040.77, 85273.53 and 127932.68 could represent oligomeric forms either consequent to samples in high concentration or to complexes in the form of homo or hetero oligomers of the identified proteins. The direct infusion of the native AnHyal to the mass spectrometer as well as the injection of undigested AnHyal to the LC-MS did not reveal any ions apart from the solvent noise.

The comparative analysis between the experimental peptide sequences of the two AnHyal components (now called AnHyalH and AnHyalC for peptides from AnHyal fraction with similarity to B. vagans hyaluronidase and G. rosea GTx-VA1, respectively) showed that these comprised non-redundant peptides, discarding the possibility of being protein species, despite the supposed proximity of their molecular masses, based on the corresponding homologous proteins.

To further study the sequence similarity of AnHyalH and AnHyalC with literature data and BLAST searches, we performed InterPro and Prosite searches. Two domains were identified in AnHyalH: Glyco_hydro_56 (Pfam
PF01630) and epidermal growth factor (EGF)-like domains (Fig. 4), and two motifs of the CRISP family named Signature 1 and Signature 2 (Fig. 5) in AnHyalC, corroborating the presence of both proteins in AnHyal.

Amino acid sequences of AnHyalH and AnHyalC were also aligned with other hyaluronidases and CRISP/Allergen proteins, respectively.

Sequence alignment of AnHyalH showed high similarity to hyaluronidases of other arthropods (Fig. 4). Ten of the twelve conserved cysteine residues among the other aligned species were identified in AnHyalH peptides. The other two cysteins are in regions where no sequence was obtained. Other conserved residues are indicated in figure 4.

Sequence alignment of AnHyalC showed high similarity to CRISPs of other spiders (Fig. 5). Seventeen of the eighteen conserved cysteine residues among the other aligned species were identified in AnHyalC peptides. The other one is in a region where no sequence was found. Other conserved residues are indicated in figure 5.

3.3. Analysis of active protein complexes by BN-PAGE

Considering the above results, AnHyal was analyzed by 1D and 2D BN-PAGE to verify the possible formation of a complex between AnHyalH and AnHyalC. 1D BN-PAGE and its mirror zymogram showed that AnHyal contains enzymatically active protein bands at 53 kDa, 124 and 178 kDa (Fig. 6A and B). The 2D SDS-PAGE zymogram from the 1D BN-PAGE showed that the 178 kDa band was separated in two subunits with 53 and 44 kDa and enzymatic activity only in the 53 kDa band (Fig. 6C). Subunits were not detected in the other bands from AnHyal possibly due to the small amount of sample. De novo sequencing of 53 kDa (AnHyalH) and 44 kDa (AnHyalC) subunits (see Supporting Information


3) showed sequence similarity to the hyaluronidase and CRISP proteins, respectively, indicating the formation of complex between both proteins. In view of this, an additional zymogram experiment was performed (data not shown) and bands corresponding to 53 and 44 kDa were sequenced, displaying similarity to hyaluronidase and CRISP, respectively. Based on the molecular mass of the monomeric form of CRISP (44 kDa) and hyaluronidase (53 kDa) proteins estimated by SDS-PAGE and BN-PAGE, we can suggest that the apparent mass of the 124 kDa and 178 kDa oligomers corresponds to an arrangement stoichiometric ratio of 2:1 and 3:1 (CRISP: hyaluronidase), respectively.

3.4. Circular dichroism and stability thermal analysis

In order to check the secondary structure arrangement compatible with AnHyal containing 53 kDa (AnHyalH) and 44 kDa (AnHyalC) proteins, the CD experiment was performed. The secondary structure of AnHyal at pH 5.8 was estimated from Far-UV CD spectrum at 25 °C (Fig. 7). The deconvoluted spectrum resulted in about 36% of α-helix and 16% of parallel and antiparallel β-sheet (inset Fig. 7).

3.5. Enzymatic characterization of AnHyal

AnHyal showed activity at pH values ranging from 3.5 to 7.0, reaching its maximum activity between pH 4.0 and 6.0 (Fig. 8A). The complex was also active in a wide temperature range (20 °C to 60 °C), with maximum activity between 30 and 60°C (Fig. 8B). AnHyal hydrolyzed hyaluronic acid substrate at a rate of 97% but was not able to degrade the substrate chondroitin sulfate A (Fig. 8C).
AnHyal fits on Michaelis-Menten kinetics, presenting $V_{\text{max}}$ of 1.53 μg/mL/min and $K_M$ of 617.9 μg/mL (Fig. 8D).

4. Discussion

4.1. Purification of AnHyal

AnHyal was obtained in a single chromatographic step, similarly to hyaluronidase from *Tityus stigmurus* and *Lasiodora* sp. [36,37]. Hyaluronidases from the spiders *Hippasa partita*, *Vitalius dubius* and *Cupiennius salei* [7,38,39] have already been purified, but the purification process involved several chromatographic steps.

One of the AnHyal components presented molecular mass of 42.55 kDa by MALDI-TOF analyses, a result in agreement with values described for spider hyaluronidases which typically have molecular masses ranging from 33 to 47 kDa [2]. Multiply-charged ions were also identified in the spectrum obtained by MALDI-TOF, suggesting that AnHyal can form dimers and trimers. Oligomeric forms have been identified for human hyaluronidases and different species of sand flies [40–42], among others. This phenomenon was also reported in *Loxosceles reclusa* [43], where a hyaluronidase and its dimer presented molecular mass estimated by SDS-PAGE of 33 kDa and 63 kDa, respectively.

4.2. Sequencing of AnHyal

Hyaluronidases present in the venom of spiders are commonly described as a single component, although the presence of protein species was identified in *Loxosceles intermedia* (41 and 43 kDa) [44], as well as in the venom of other animals, such as *Tityus serrulatus* (44.5 and 44.9 kDa) [45] and *Polybia paulista*
(four protein species with 44.3 kDa) [46]. In these cases, the protein species presented similar molecular masses, unlike *Naja naja*, which has a protein form of 70.4 kDa and another of 52 kDa [47]. In the case of *T. serrulatus* and *P. paulista*, the sequences of the respective protein species were determined and, as expected, showed high sequence similarity. Regarding AnHyal, however, the difference between the experimental peptide sequences would not support this hypothesis. Moreover, similarity searches on sequence databases showed that AnHyalH and AnHyalC are different proteins, that belong to hyaluronidase and CRISP/Allergen families, respectively.

In relation to the N-terminal sequence of AnHyalC obtained by Edman degradation, this showed 86% of identity to the N-terminal sequence of a hyaluronidase (UniProtKB-P0C8X3) from *T. stigmurus*, also obtained by Edman degradation. The *T. stigmurus* hyaluronidase was purified by RP-HPLC and showed high activity against hyaluronan [36]. Thus, similarly to AnHyal, it is possible that the enzymatically active fraction presented in *T. stigmurus* venom, contained both proteins, hyaluronidase and CRISP, being only the latter sequenced by Edman degradation, since the hyaluronidase N-terminal could also be blocked.

In a recent study on the content of hyaluronidase-like enzymes in the venom of *Poecilotheria regalis*, *in-gel* digestion and MS analysis of a 42 kDa band revealed *de novo* sequences with similarity to *B. vagans* hyaluronidase or allergens of some arthropods [48]. *P. regalis* peptides with similarity to allergens were then compared with those of AnHyalC, showing high similarity. In AnHyal, the hyaluronidase and CRISP proteins correspond to the 53 and 44 kDa bands, respectively (SDS-PAGE and 2D BN-PAGE) and the sequences of each protein
were confirmed by MS analyses from in-gel digests. Apparently (by SDS-PAGE), the AnHyalC is more abundant than AnHyalH, what could cause a signal suppression of AnHyalH, explaining the presence of only one singly-charged ion in the MALDI-TOF analysis. In *Acanthoscurria geniculata*, the electrophoretic profile of venom showed a main band at 45 kDa, containing proteins with similarity to hyaluronidase and CRISP, and it is suggested that the latter is present in a higher proportion [49].

These results showed for the first time that hyaluronidase and CRISP are complexed and may form hetero-oligomers in spider venom. However, future studies are needed to precisely determine the stoichiometric arrangement of these oligomeric forms. On the other hand, some reports have shown that CRISP-3 from human plasma can form complexes with human blood plasma proteins (α1B-glycoprotein-A1BG) [11] and seminal proteins (prostate secretory protein of 94 amino acids-PSP94) [12,13]. In these cases, the proteins complexed with CRISP-3 would act as protectors of the harmful effects of free CRISP-3.

Regarding AnHyal, it is still not possible to attribute some specific function to the complex hyaluronidase/CRISP, however, it was possible to verify that the enzymatic activity of hyaluronidase was maintained in both hetero-oligomeric and monomeric components of AnHyal.

Although hyaluronidases are commonly found in spider venoms [2], few protein sequences are available in databases. The alignment of AnHyalH sequences with hyaluronidases from different arthropods showed a high similarity between them, including the domains Glyco_hydro_56 (Pfam PF01630) and epidermal growth factor (EGF)-like and, highly conserved cysteine residues, which represent one of the main structural features of hyaluronidases [50].
CRISP-like proteins were first reported in the venom of mygalomorph spiders, through a transcriptomic and proteomic study of *Trittame loki* venom [18], although the GTx-VA1 from *G. rosea* sequence was deposited in UniProtKB database before that. Afterwards, CRISP proteins were also identified in *Latrodectus hesperus* [51], *Stegodyphus mimosarum* and *Acanthoscurria geniculata* [49] and in *Grammostola iheringi* [17] spiders. The evident sequence similarity between AnHyalC and other CRISPs, including the sequences of signatures 1 and 2, confirmed the presence of this protein in *A. natalensis* venom, demonstrating that the distribution of CRISP proteins is wider than previously known.

Although the presence of two proteins in AnHyal was found, the only one N-terminal sequence obtained by Edman degradation (AnHyalC) and the identification of an N-terminal lysine acetylation (AnHyalH) by MS sequencing indicated that the N-terminal of AnHyalH is blocked. As shown in previous reports, hyaluronidase from *Apis mellifera* [52] and bovine testis [53] were blocked, the latter being due to the presence of an N-terminal acetyl group.

4.3. Circular Dichroism analyze

The secondary structures content of α-helix (36%) and β-sheets (16%) obtained from the deconvoluted spectrum of AnHyal agrees with a higher contribution of the dichroic signal of the CRISP (AnHyalC), specifically those from snake venom (Protein data bank code 1WVR, 1XTA, 1RC9 and 2GIZ) that present about 33% of α-helix and 16% of β-sheets.
4.4. Enzymatic characterization of AnHyal

AnHyal showed maximum activity at pH values between pH 4.0 and 6.0, being active in pH acid and neutral [2]. A narrower range of activity has been demonstrated for venom hyaluronidase from other species of spiders, such as Dugesiella hentzi (3.5 – 4.0) [54], V. dubius (4.0 – 5.0) [39] and C. salei (4.0 – 6.0) [7].

The enzyme was active in a wide temperature range (20 °C to 60 °C) and showed maximum activity between 30 and 60 °C. Hyaluronidases from C. salei and V. dubius also showed activity at the same temperature range, but their maximum activity occurred at 50 °C e 35-40 °C, respectively [7,39].

AnHyal showed specificity to the hyaluronic acid substrate, like the one produced by Brachypelma vagans [55] and H. partita [38]. Conversely, hyaluronidase from L. intermedia [44], V. dubius [39], and several other species of spiders [7], were also able to degrade chondroitin sulfate. Hyaluronic acid, heparan and chondroitin sulfate are the main components of the extracellular matrix of vertebrates [56] and invertebrates [57], respectively. In general, spiders feed mainly on invertebrates, however, it has been shown that large spiders, such as tarantulas, often feed on small vertebrates such as rodents, amphibians, birds and lizards [58], the latter being also reported as relatively common preys for A. natalensis [59]. Thus, the specificity of AnHyal on hyaluronic acid may represent an advantage in obtaining its prey.

AnHyal fits on Michaelis-Menten kinetics presenting $K_M$ of 617.9 μg/mL, similar to the hyaluronidase from V. dubius (677 μg/mL) [39] suggesting a low affinity for the substrate. However, that is not a common feature among spider
venoms hyaluronidases, since lower values have already been described, as *C. salei* hyaluronidase that presented a $K_m$ of 80.8 μg/mL [7].

5. Conclusions

The data presented here describe the enzymatic and structural characterization of AnHyal, the major protein component of the venom from *A. natalensis*, presenting hyaluronidase activity. AnHyal has two components (named AnHyalH and AnHyalC), highly similar to hyaluronidases and CRISPs, respectively, from other arthropods. Moreover, the combination of chromatography (RP-HPLC), mass spectrometry, SDS-PAGE, BN-PAGE and zymogram methods, allowed the identification of the complex between hyaluronidase and CRISP and that it retains hyaluronidase action. Further studies must be done to confirm and clarify the function of CRISP in the *A. natalensis*’ venom. This may help to elucidate the possible relationship between these two proteins in the venom. The discovery and characterization of new types of hyaluronidases and the presence of such enzymes in a complex with CRISP may further expand the possibilities for biological interpretations of these enzymes.

Acknowledgements

The authors thank the members of the Laboratory of Toxinology, MSc Ana Carolina M. Magalhães, MSc Andréia C. Pinheiro, MSc Carlos J. C. de Santana and Lucas R. Ferreira, Laboratory of Enzymology, MSc Alonso R. P. Ticona and, Dra. Marta R. Magalhães of the Pontifical Catholic University of Goiás, for the valuable technical and intellectual contribution. We also thank Nuno M. F. M. Domingos and Dr. Jaques M. F. de Souza for the relevant technical support with
mass spectrometer experiments and the Dr. Diego Madureira for making feasible the experiments in MALDI-TOF. We are grateful for the Prime Scientifica that kindly granted the revision of English. This work was supported by CNPq (Grants 563972/2010-6 and 407386/2013-0 conceded to Mariana S. Castro), FAPDF (Grant 193.000461/2011 conceded to Mariana S. Castro) and Financiadora de Estudos e Projetos – FINEP (Grants 0439/11 and 0694/13 conceded to Marcelo V. de Sousa through PROINFRA program).

The authors confirm that this work is original and is not currently under consideration for publication elsewhere.

The authors confirm that there are no known conflicts of interest associated with this publication.
References


doi:10.1016/j.bbrc.2005.05.139.


Legends

Figure 1. Purification of AnHyal from *A. natalensis* crude venom and eletrophoreetic analysis. (A) Typical chromatography profile of fractionation of the crude venom (5 mg) by RP-HPLC on a C18 column, under linear gradient from 0 to 60% solvent B (0.12% v/v TFA in ACN) in 60 min and flow 1 mL/min. AnHyal: fraction exhibiting hyaluronidase activity. (B) MALDI-TOF/MS spectrum from native AnHyal (42.55 kDa) and its multi-charged ions. Spectrum obtained in positive linear mode using sinapinic acid as matrix. (C) SDS-PAGE (12%) and (D) Zymogram. M: molecular markers (kDa), phosphorylase b (97), albumin (66), ovoalbumin (45), carbonic anhydrase (30), trypsin inhibitor (20) and α-lactalbumin (14,4). AnHyal: chromatographic fraction.

Figure 2. AnHyalH peptides obtained by *de novo* sequencing matching hyaluronidase sequence of the *Brachypelma vagans* venom (UniProtKB - J9XYC6), identified with 67% protein coverage (in grey). #: *de novo* only sequence identified by PEAKS and with similarity to hyaluronidase from *B. vagans*.

Figure 3. AnHyalC peptides obtained by Edman degradation and *de novo* sequencing matching GTx-VA1 sequence of the *Grammostola rosea* venom (UniProtKB - M5AWW7), identified with 82% protein coverage (in grey). Underlined: Sequence matches of AnHyalC peptides identified by PEAKS. ♦: N-terminal sequence obtained by Edman degradation. #: *de novo* only sequences identified by PEAKS and with similarity to GTx-VA1. ♠: *de novo* sequences
identified by PEAKS and with similarity to CRISPs from *H. infensa* (UniProtKB - A0A1D0BZZ0) and *T. loki* (UniProtKB - W4VS53).

Figure 4. Multiple alignments amongst the partial primary structure of the AnHyalH from the venom of the *A. natalensis*, with the sequences of hyaluronidases from other arthropods: *B. vagans* (J9XYC6), *L. intermedia* (R4J7Z9) and *T. serrulatus* (W0HFN9). Sequences were aligned using Clustal Omega. Highly conserved cysteine residues are marked in grey. The sequences of AnHyalH corresponding to Glyco_hydro_56 (Pfam PF01630) and epidermal growth factor (EGF)-like domains are underlined with lines and dots respectively. Gaps (·) have been inserted to maximize alignment.

Figure 5. Multiple alignments amongst the partial primary structure of the AnHyalC from the venom of the *A. natalensis*, with the sequences of CRISP proteins from other spiders: *G. rosea* (M5AWW7) and *T. loki* (W4VS53). Sequences were aligned using Clustal Omega. Highly conserved cysteine residues are marked in grey. Sequences of AnHyalC corresponding to Signature1 and Signature2 domains are underlined with line and dots respectively. Gaps (·) have been inserted to maximize alignment.

Figure 6. Electrophoretic patterns of AnHyal on BN-PAGE, 2D BN/SDS-PAGE and zymogram. (A) 1D BN-PAGE CBB staining and (B) mirror zymogram Alcian Blue staining. Arrow: monomer (53 kDa) and oligomers (124 and 178 kDa). M: molecular markers (kDa): thyroglobulin (669), ferritin (440), catalase (232), lactate dehydrogenase (158), and bovine serum albumin (66). AnHyal:
chromatographic fraction. (C): 2D SDS-PAGE/zymogram from 1D BN-PAGE. M: molecular markers (kDa): phosphorylase b (97), albumin (66), ovoalbumin (45), carbonic anhydrase (30), trypsin inhibitor (20) and α-lactalbumin (14.4). AnHyalH: subunit with 53 kDa corresponding to hyaluronidase. AnHyalC: subunit with 44 kDa corresponding to CRISP.

Figure 7. Far-UV Circular Dichroism (CD) spectrum of AnHyal at 25 °C in 2 mM sodium acetate buffer pH 5.8. Inset: The secondary structures content of AnHyal.

Figure 8. Enzymatic characterization of AnHyal. (A) Effect of pH on enzymatic activity. (B) Effect of temperature on enzymatic activity. (C) Specificity of the enzyme on the substrate. (D) Determination of kinetic parameters $V_{max}$ and $K_M$ using non-linear regression. The dots represent the mean ± SD ($n = 3$).
Highlights

- A hyaluronidase/CRISP complex was described for the first time in the literature.
- The AnHyal complex was purified using a single chromatographic step by RP-HPLC.
- The hyaluronidase showed activity in monomeric form and in association with the CRISP.
- Partial sequencing of hyaluronidase and CRISP was obtained by MS and Edman degradation.
- The secondary structure pattern of AnHyal is composed predominantly by α-helix.
Figure 1
Figure 2
Figure 3

MQIRVILMLS WFWLGVSAMET CPAIYLRYSR EHTYCLPRKST SCTLQSGVTS GS CPAIYLRYSK DHTFCLPRKST SCTI #ES CPAIYLRYSK
KSDIEIIIVRE HNLLRSKVFVAT GKTQYSMMPK ASNMLQMVWD DELAAVAQKH *LAAVAQKH
ADQCTFDHDC GDCRRVKNFQG VGQNLFQRTS PSGQPSPPTW AEAVKDWyKE ADQCVFEEHDC NDCRR
IKDFQKQQID GFIDGKGPPQ TGHFTQEIQWA DTWRVGCGLYS AYKKGSQGFEE
LYTCNYGPQG NIKTRPIYEQK GNPCTRCPQLN SCCGNSCSGG TSYPGLCRIS
GENAPQYKRP EGLTFYCSFNF NEPDCAATTG GADKWEVSKT LSGSYIGIVL #VSKT LSGSYMAGIVL
NGGESSTLSF TKSFKVPTAP LCFTSYYRTG PQVKGEKSAG IFTEIFKLPA NAGE #KVPTAP LCFKLDYR #TG PQVGDQAVAG TANAIFK
RPDKSFPTVVL TSSSMSFTKTF TKKLGYWTMET TFSVSFSVPPK GKPAQYLELT
DLSARAGPC
Figure 4
Figure 6
Figure 7

[Diagram of UV spectrum with wavelength (nm) on the x-axis and [\theta] (deg.cm^{-2}.dmol^{-1}) x 10^3 on the y-axis. A table to the right shows secondary structure percentages: Alpha helix 36.0%, \beta-antiparallel 7.0%, \beta-parallel 8.0%, \beta-turn 16.0%, Random coil 31.0% at pH 5.8.]
Figure 8

A. Relative activity (%) vs pH for Phosphate-citrate and Tris-HCl.

B. Relative activity (%) vs Temperature (°C).

C. Relative activity (%) for different substrates: Condroitin and Hyaluronic acid.

D. Michaelis-Menten kinetics for Hyaluronic acid with parameters:

- $V_{max} = 1.53 \mu g/min \pm 0.11 \mu g/min$
- $K_m = 617.9 \mu g/mL \pm 129.3 \mu g/mL$