Arch Toxicol
DOI 10.1007/s00204-010-0597-6

MOLECULAR TOXICOLOGY

Characterization of toxins from the broad-banded water snake *Helicops angulatus* (Linnaeus, 1758): isolation of a cysteine-rich secretory protein, Helicopsin

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Received: 7 April 2010 / Accepted: 21 September 2010

Abstract *Helicops angulatus* (broad-banded water snake) according to recent proposals is presently cited in the family Dipsadidae, subfamily *Xenodontinae*, forming the tribe *Hydropsini* along with the genera *Hydrops* and *Pseudoeryx*. The current work characterizes the proteolytic and neurotoxic activities of *H. angulatus* crude toxins from salivary excretion (SE) and describes the isolation and identification of a cysteine-rich secretory protein (CRISP) called helicopsin. The SE lethal dose (LD_{50}) was 5.3 mg/kg; however, the SE did not contain hemorrhagic activity. Helicopsin was purified using activity-guided, Superose 12 10/300 GL molecular exclusion, Mono Q10 ion exchange, and Protein Pak 60 molecular exclusion. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) showed a highly purified band of approximately 20 kDa. The minimal lethal dose for helicopsin was 0.4 mg/kg. Liquid chromatography mass spectrometry (LC-MS/MS) analysis identified 2 unique peptides MEWYPEAAANAER and YTQIVWYK, representing a protein sequence (deleted homology) belonging to cysteine-rich secretory proteins, which are conserved in snake venoms (CRISPs). CRISPs are a large family of cysteine-rich secretory proteins found in various organisms and participate in diverse biological processes. Helicopsin exhibited robust neurotoxic activity as evidenced by immediate death (~8 min) due to respiratory paralysis in NIH mice. These observations for helicopsin purified from *H. angulatus* provide further evidence of the extensive distribution of highly potent neurotoxins in the *Colubroidea* superfamily of snakes than previously described.

Keywords CRISP · Colubroidea · Dipsadidae salivary excretion · Neurotoxin · *Helicops angulatus* · Broad-banded water snake

Amalid Estrella and Elda E. Sánchez contributed equally to this work.

Ethical statement: The persons behind the experiments declare that all the experimental events concerning the use of live animals were done by specialized personnel following the Venezuelan pertinent regulations as well as institutional guidelines, according to protocols approved by the Tropical Medicine Institute of the Universidad Central de Venezuela and the norms from the guide for the care and use of laboratory animals, published by the US National Institute of Health (Anonymous 1985).

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Introduction

Dipsadidae family integrates the highly developed snake superfamily Colubroidea (Kraus and Brown 1998; Zaher et al. 2009), which comprises well over half of the snake species on earth, and yet have received very little attention. Representatives of this family had been believed to be non-venomous;

Published online: 08 October 2010

however, studies have confirmed that many of these snakes have toxins that cause local pain, edema, hemorrhage, and neurological alterations. Previous work in our laboratory exposed that a number of Colubridae venoms in Venezuela had in vivo neurotoxic activity (Lemoine et al. 2004a, b; Rodríguez-Acosta et al. 2006). *Helicops angulatus* (broad-banded water snake) was formerly considered as belonging to the family Colubridae, but now it belongs to the Dipsadidae family, subfamily Xenodontinae. The snake is nocturnally active, occurring in fishponds, watercourses, and rivers of northern South America (Dixon and Soini 1986; Ford and Ford 2002), and feeds mainly on fish, eels and frogs. The current work intends to complement information regarding cysteine-rich secretory proteins (CRISPs) purified from venom of the Colubroidea superfamily (Mackessy et al. 2006).

Cysteine-rich secretory proteins are only found in vertebrates, including snakes (Kierszenbaum et al. 1981; Hill and Mackessy 2000; Fry et al. 2006). CRISP is a subfamily of the CAP [*c*ysteine-rich secretory proteins (CRISPS), *a*ntigen 5 (Ag5), and *p*athogenesis-related 1 (Pr-1)] superfamily of proteins (Gibbs et al. 2008). CRISPs are found in the mammalian male reproductive tract and in the venom secretory ducts in numerous snakes, lizards, and other vertebrates (Gibbs and O'Bryan 2007; Yamazaki and Morita 2004). CRISPs have a distinct and structurally conserved N-terminal CAP domain weighing approximately 21 kDa and a C-terminal cysteine-rich domain (CRD) of approximately 6 kDa (Gibbs et al. 2008). The CRD contains two sub-regions consisting of a hinge region and a carboxyl ion channel regulatory region (ICR) similar to peptide toxins from the sea anemone (Dauplais et al. 1997). CRISPs have been known to have K^+, Ca^{2+}, cyclic nucleotide-gated (CNG), and ryanodine receptor (RyR) ion channel regulatory activities (Morrissette et al. 1995; Yamazaki et al. 2002; Yamazaki and Morita 2007). Some have been identified to induce paralysis of peripheral smooth muscle and induction of hypothermia as a result of the action upon voltage-gated Ca^{2+} channels and resultant blockage of K^+-induced contraction (Fry et al. 2008). This work represents, to our knowledge, the first report in the literature of an isolated and characterized neurotoxin belonging to the CRISP family in the salivary excretion of the Dipsadidae *H. angulatus*, from the Americas.

Materials and methods

Animals and venom

Female BALB/c mice (NIH strain) weighing 18–22 g maintained at a relative humidity of 45–70%, temperature of 22–24°C, and a 12-h light/dark cycle and obtained from the National Institute of Hygiene "Rafael Rangel", Caracas, Venezuela, were used. Animals were adapted for 1 week prior to each experiment and received water and food ad libitum. The Animal House authorities' surveillance reports established that mice were free of known pathogenic bacteria, viruses, mycoplasmas, and parasites. The investigation complied with the bioethical standards taken from "Principles of Laboratory Animal Care" (Anonymous 1985).

Helicops angulatus snake specimens were captured in Morichal Largo River, Monagas state in Venezuela. The salivary excretion (SE) (the analyzed fluid was a mixture of saliva from diverse salivary glands) was collected through a 50-mL plastic centrifuge cylinder transversely cut and covered on the top with parafilm. The snake was forced to bite the parafilm with its rear tooth (opisthodont with absenting groove and not associated with any salivary gland). The venom was collected by a capillary tube. Approximately 0.2 mL of SE was obtained from each extraction. All SE extractions were pooled, centrifuged, lyophilized, and frozen at −80°C until use (Rodríguez-Acosta et al. 2006).

Helicops angulatus venom fractionation

Isolation of helicopsin was accomplished by three chromatographic steps. First, samples of *H. angulatus* SE were run in a preparative process using molecular exclusion chromatography on a Superose 12 10/300 GL (GE Healthcare, USA) (column equilibrated at 4°C with 50 mM ammonium acetate buffer pH 6.9). The pool of SE was resuspended in the equilibrating buffer (10 mg/100 µL) and injected into the column. The elution was carried out with the same buffer at 0.2 mL/min and monitored at 280 nm. Second, the eluted fraction with neurotoxic activity (peak 3) was refractionated on an anionic exchange Mono-Q10 (Pharmacia, Sweden) column using a Biologic workstation (BIO-RAD, USA), equilibrated at 4°C with 50 mM Tris–HCl, pH 7.0. The proteins were eluted with the same equilibrating buffer at a flow rate of 1.5 mL/min with a NaCl gradient from 0 to 1 M. The eluted proteins were monitored at 280 nm. Finally, neurotoxic fraction 1 was run on a Waters™ Protein Pak 60 column, equilibrated at room temperature with 50 mM ammonium acetate buffer, pH 6.9. The proteins were eluted at 0.5 mL/min with the same buffer. The neurotoxic active fraction 3, helicopsin, was lyophilized and stored at −80°C until further use.

Protein quantification

Protein concentration was estimated by the method of Lowry et al. (1951).

Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out on SE and SE fractions by

the Laemmli method (1970), using 15% gels under reducing and non-reducing conditions with a 3.5% concentration of stacking gel. Molecular weight markers (BIO-RAD, USA) were run in parallel, and gels were stained with Coomassie blue. The relative masses were estimated by the Multi-Analyst PC version 1.1 program (BIO-RAD, USA).

Hemorrhagic activity

The SE hemorrhagic activity was assayed by the Gutiérrez et al. (1985) method. One hundred microliters of variable amounts of SE (10–82 µg) were intradermally inoculated into the abdominal skin of NIH BALB/c mice. The skins were isolated 4 h later, and the diameters of the hemorrhagic spots on the inside surfaces were measured (Huang and Perez 1980). *Bothrops colombiensis* venom (6.5 µg) and saline solution were used as positive and negative controls, respectively.

Lethal activity

The median lethal dose (LD_{50}) of the *H. angulatus* SE was tested in 18 to 22 g female NIH mice. A total of 0.1 mL of SE (five different concentrations) was intraperitoneally injected into five groups of eight mice. The endpoint of lethality was determined after 48 h. The LD_{50} was calculated according to the Spearman-Kärber method (1964).

Due to minimal amounts of the neurotoxic active fraction (helicopsin) obtained from the purification scheme, a minimal lethal dose assay was performed (Ozkan et al. 2007). Mice were injected intraperitoneally (i.p) with 100 µL of helicopsin at different concentrations (0.2, 0.4, 0.6, and 1.0 mg/kg), and deaths were registered within 24 h. A total of 2 mice were used for each concentration. The minimal lethal dose was determined as the minimum concentration that caused 100% deaths.

Gelatinase activity

A modified method (Lemoine et al. 2004b) was used to test the gelatinase activity of *H. angulatus* SE and chromatographic SE fractions from Superose 12 10/300 GL. An X-ray film (Kodak X-OMAT) was soaked with distilled water and incubated at 37°C for 45 min. After incubation, the film was completely dried, and 10 µL of SE (10 mg/mL) and SE fractions (10 mg/mL) were located on the X-ray scientific imaging film containing a gelatine coating. The hydrolysis of gelatine on the X-ray film was observed, after 2-h incubation at 37°C in a humid incubator, by rinsing the X-ray film with distilled water. Serial dilutions from 1 to 1/8 were carried out to determine the minimum amount of SE necessary to produce a clear spot on the X-ray film. The titer was defined as the reciprocal of the highest dilution that produced a clear spot on the X-ray film. The specific gelatinase activity was calculated by dividing the titer by the amount of protein (µg) applied on the film. The assay was repeated 3 times.

In vivo neurotoxic activity

To confirm the neurological symptomatology produced by the SE and all SE fractions collected from Superose 12/300, Mono Q10, and Protein Pak 60 columns, six mice were subcutaneously injected with 100 µL of each sample. Salivary excretion was injected at a concentration of 0.2 mg/mouse, while all other fractions were injected at concentrations of 0.016 mg/mouse. These concentrations were determined according to the LD_{50} and minimal lethal doses obtained from SE and helicopsin, respectively. The mice were observed for neurotoxic effects (Lemoine and Rodríguez-Acosta 2003) for 20-min postinjection or until death occurred (Table 1). Symptoms of envenoming and survival times were noticed and compared with previous mouse bioassay results from known neurotoxins (*Micrurus* sp.). Assays were done according to the Association of Analytical Communities (AOAC INTERNATIONAL 1995) method for paralytic neurotoxins.

Nanoflow LC-MS/MS

Twenty micrograms of helicopsin was reconstituted in 100 µL of NH_4HCO_3, pH 8.0. Eight molar urea was added for denaturation, and the sample was reduced with 5 mM DTT for 30 min at 37°C. The sample was alkylated with 15 mM iodoacetamide for 1 h in the dark at room temperature. Urea in the sample was diluted 4 times with 20 mM Tris–HCl, pH 7.5, and trypsin was added at protein/enzyme ratio of 50:1 (w/w) and incubated at 37°C for 16 h. Fifty picomoles of digest was taken out and dried using a Speed-Vac (Labconco, USA) and reconstituted in 8 µL of 0.1% formic acid.

Peptide samples were re-dissolved in 8 mL of 0.1% formic acid and introduced into an Agilent nanoflow 1100 HPLC system. The nanoflow LC capillary column with integrated electrospray emitter tip was constructed in-house using a procedure as described (Ficarro et al. 2009). The buffer used was 0.1% formic acid with the eluting buffer of 100% CH_3CN run over a shallow linear gradient over 90 min with a flow rate of 200 nL/min. The Agilent 1100 HPLC system was coupled online to a hybrid linear ion trap Orbitrap mass spectrometer (LTQ-Orbitrap XL; Thermo Fisher, San Jose, CA). The mass spectrometer was operated in the data-dependent mode, in which a full-scan MS was followed by MS/MS scans of the 3 most abundant ions with excluding +1 charge state. The mass exclusion time was 180 s.

Table 1 Neurotoxic signs and symptoms in mice injected intraperitoneally with *H. angulatus* SE and neurotoxic fractions from Superose 12 10/300 GL, Mono Q10 and Protein Pak 60

Time (min)	Hyper-excitability	Face washing	Involuntary trembling and fasciculation	Tachypnea	Exophthalmos	Convulsion	Flaccid paralysis	Urinary sphincter relaxation	Death
H. angulatus salivary excretion									
1	1–6*	1,2,4,5	–	–	–	–	–	–	–
3	–	–	3–6	1–6	1, 4–6	–	–	–	–
6	–	–	–	–	2,3	1,3	–	–	–
8	–	–	–	–	–	4,5	1–6	1–6	1–6
Superose 12 10/300 fraction									
1	1–6	1,2,4,5	1,2	–	–	–	–	–	–
3	–	–	3–5	1–6	1,4–6	–	–	–	–
6	–	–	–	–	2,3	1,3,4	1–6	1–3, 6	1–3, 6
8	–	–	–	–	–	4,5	–	4, 5	4, 5
Mono Q10 f raction 1									
1	1–6	–	–	–	–	–	–	–	–
4	–	1,3	2,3,5,6		–	–	–	–	–
6	–	–	–	1–6	4	4–6	5	–	–
8	–	–	–	–	–	–	1–5	1–6	1–6
Protein Pak 60 fraction 3-Helicopsin									
4	1–6	–	–	–	–	–	–	–	–
5		3,5	1–4,6	1–6	–	–	–	–	–
8	–	–	–	–	–	1–4	1–6	1–6	1–6

* Only six mice were used to test neurotoxic symptoms for the SE and for each SE fraction was analyzed. Each mouse is represented by a number from 1 to 6

Data analysis

The MS/MS data were searched against Swiss Protein database (Version 55.4 with 385721 entries) using the SEQUEST algorithm on the Sorcerer IDA server (Software Version 2.5.6; SageN, Inc, San Jose, CA). Peptide mass tolerance was set at 5.0 ppm, and MS/MS tolerance was set internally by the software with the values varying from 0 up to 1 amu. Search criteria included static modifications of cysteine alkylation (57.0214 Da). Searches were performed with semi-tryptic digestion and allowed a maximum 2 missed cleavages on the peptides analyzed from the sequence database. Peptide identifications were filtered using protein and peptide probability of 1 and 0.9 on the Sorcerer using the open-source Trans-Peptide Pipeline (TPP) software (Version 2.9.4).

Results

Electrophoretic profile

Helicops angulatus SE on a 15% SDS–PAGE revealed ~6 major protein bands with relative molecular masses (M_r) of approximately <13, 20, 28, 39, 47, and 70 kDa, under reducing conditions, and $M_r \sim 4$ bands of approximately 20, 23.5, 47, and 70 kDa, under non-reducing conditions (Fig. 1).

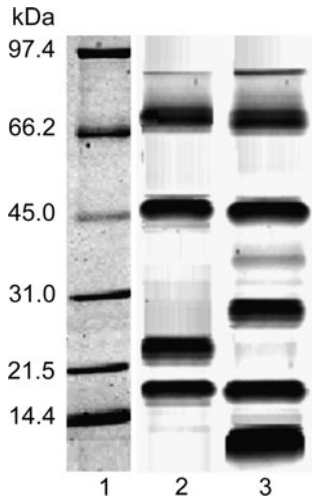

Fig. 1 Electrophoresis of crude *H. angulatus* SE. A total of 16 µg of SE was run on a 15% SDS–PAGE gel. *Lanes 1* Molecular weight markers, *2* non-reduced SE, and *3* reduced SE. The gel was stained with Coomassie blue

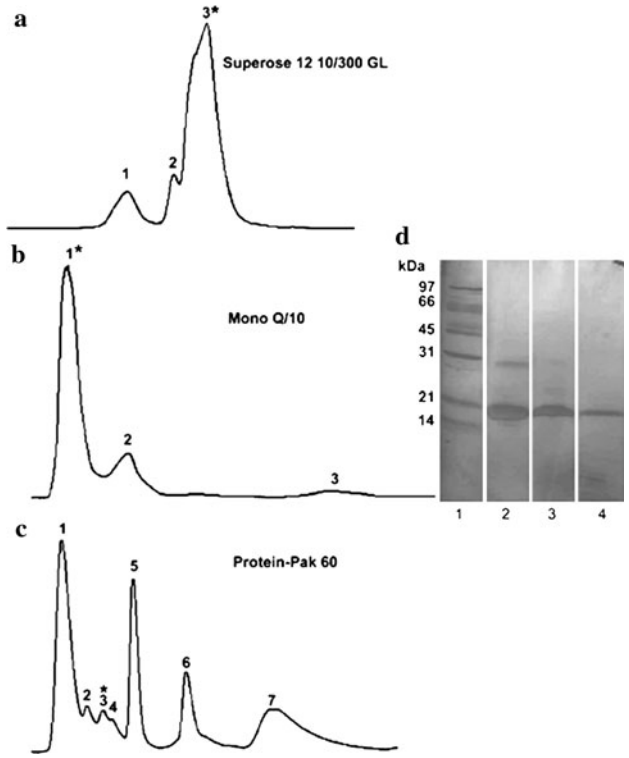

Fig. 2 Purification of helicopsin (CRISP) from *H. angulatus* SE. **a** Molecular exclusion Superose 12 10/300 GL; **b** Ion exchange, Mono Q/10; **c** Molecular exclusion, Protein Pak 60 columns; and **d** 15% SDS–PAGE under reducing conditions of each chromatographic fraction with neurotoxic activity. *Lanes 1* Molecular weight markers, *2* Superose 12 10/100 fraction 3, *3* Mono Q fraction 1, *4* Protein Pack fraction 3. The gel was stained with silver stain. The peaks with the *asterisk* contain neurotoxic activity

Helicops angulatus venom fractionation

The fractionation of *H. angulatus* SE was done on a Superose 12/300 GL column, resulting in three fractions (Fig. 2a). Fraction 3 contained the neurotoxic activity, which then was fractionated on a Mono Q column resulting in three fractions (Fig. 2b). Fraction 1 had neurotoxic activity. This fraction was chromatographed on a Protein Pak 60 column resulting in seven fractions (Fig. 2c). All fractions tested positive for neurotoxic activity, and fraction 3 was the most neurotoxic protein having a molecular weight of ~20 kDa by SDS–PAGE (Fig. 2d). This protein was designated as helicopsin.

Hemorrhagic activity

Helicops angulatus SE had no hemorrhagic activity, at a maximum of 82 µg/mouse, when tested by intradermal injections.

Lethal activity

The LD_{50} for *H. angulatus* SE was calculated to be 106 µg/20 g of mouse body weight (5.3 mg/kg). The minimal lethal dose of purified helicopsin that caused 100% deaths in mice (i.p) was 0.4 mg/kg.

Gelatinase activity

Helicops angulatus SE contained gelatinase activity up to dilutions of 1/2, which represents a minimal gelatinase activity of 50 µg. Only Superose 12/300 fraction 1 had gelatinase activity up to dilutions of 1/4 with a minimal gelatinase activity of 25 µg, and a specific gelatinase activity of 0.16, which indicated that this fraction was 4 times more potent than SE (0.04).

In vivo neurotoxic activities

Salivary excretion and all fractions from each chromatographic step (Fig. 2a–c) were analyzed for their activities on NIH mice (Table 1). Salivary excretion and fractions: 3 (Superose 12/300), 1 (Mono Q10), and 3 (Protein Pak 60) displayed signs of neurotoxicity. The mice injected with these fractions showed neurological symptoms typical of depolarizing neuromuscular blocking neurotoxins (Hawgood and Smith 1977), including edginess, ataxia, convulsions, flaccid paralysis of respiratory muscles, and death, which occurred from 6 to 8 min. On the basis of their short survival period and envenoming signs (effects of anticholinesterase signs and/or effects of depolarizing neuromuscular blocking agent signs of envenoming), it is safe to infer that neurotoxins existed in the *H. angulatus* SE and its fractions. All animals were subjected to immediate postmortem examination after their deaths and all presented signs of heart fibrillation.

Nanoflow LC-MS/MS

Mass spectrometry analysis resulted in the identification of single toxin protein. Three unique precursor ions were detected all having +2 charges. The peptides identified were MEWYPEAAANAER, oxidized M(ox)EWYPE AAANAER, and YTQIVWYK (m/z 1537.660, 1553.669 and 1100.270) totaling 21 amino acids and representing a neurotoxic, cysteine-rich venom protein precursor (CRISP) from *Protobothrops mucrosquamatus* (Accession: P79845), *Vipera berus* (Accession: CAP74089), and *Liophis poecilogyrus* (Accession: AAZ75601) in the National Center for Biotechnology Information (NCBI) database (Fig. 4a, b).

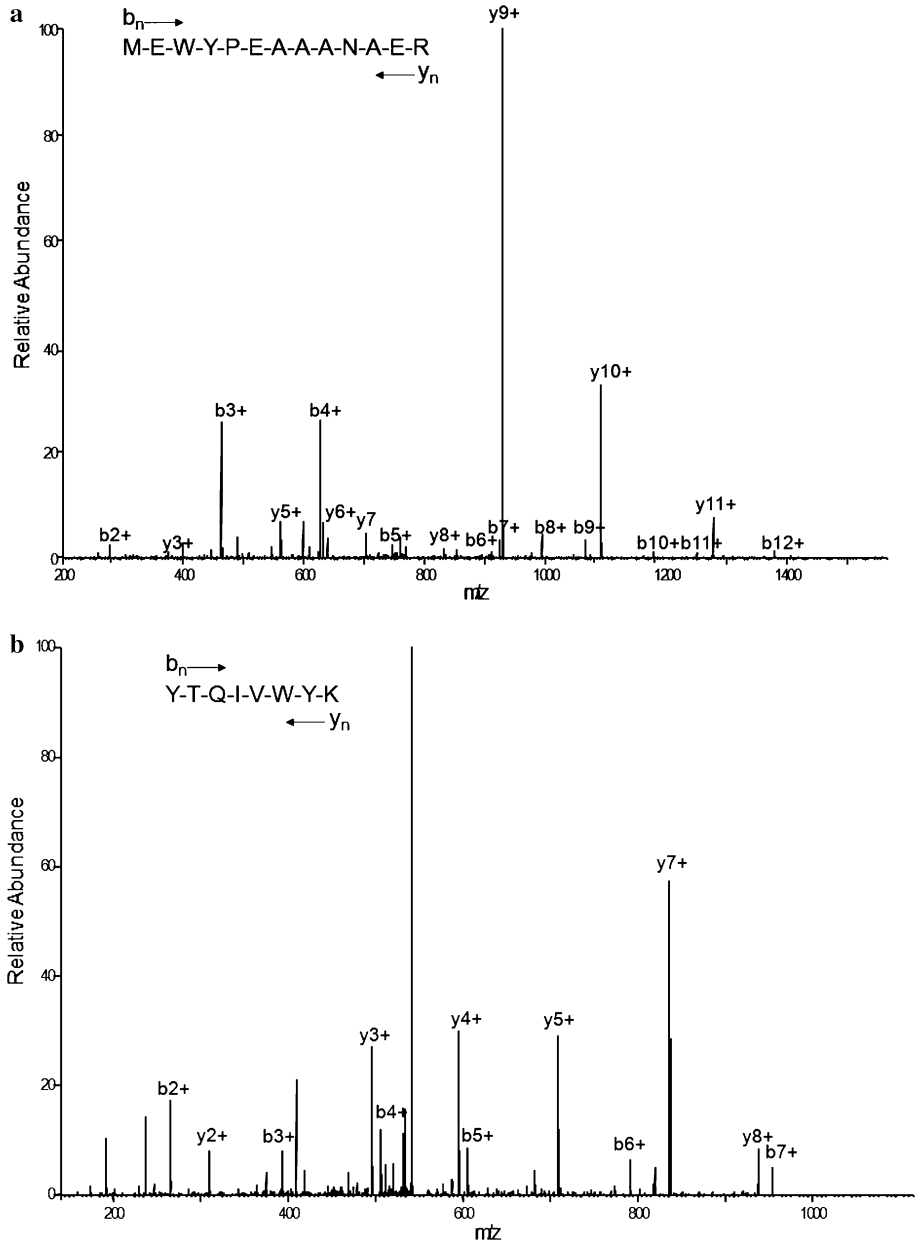

Fig. 3 CID spectra of helicopsin. **a** CID spectrum of peptide MEWYPEAAANAER (m/z 1537.660) and **b** CID spectrum of peptide YTQIVWYK (m/z 1100.270). The characteristic peptide bond fragment ions, type b and type y, are labeled

Discussion

The detection of neurotoxic peptides has significantly advanced throughout the precedent years due to the introduction of refined and efficient technical methods (Calvete et al. 2009). Numerous research corporations and groups have a wide interest in these small molecules for drug discovery and design (Heading 2002). Snake venom cysteine-rich secretory proteins are a group of vertebrate proteins that have potential in drug discovery since they are capable of disrupting homeostasis through various mechanisms, including inhibiting or activating factors in blood coagulation and blocking ion channels (Morrissette et al. 1995; Yamazaki et al. 2002; Yamazaki and Morita 2007; Gibbs and O'Bryan 2007).

This is the first report of a cysteine-rich secretory protein displaying neurotoxic activity, helicopsin, found in *H. angulatus* venom. The protein was isolated by three chromatographic procedures (Fig. 2a–c), which was subjected to LC-MS/MS resulting in two unique peptides (MEWYPEAAANAER, M(ox)EWYPEAAANAER, and YTQIVWYK) totaling 21 amino acids (Fig. 3a, b). The unsequenced snake genome of *H. angulatus* likely limited additional sequence identification from purified helicopsin, which is a known restriction for snake venom research and has been reported by other groups (Galán et al. 2008). The first peptide resides near the N-terminal part of the protein, while the second peptide is located on the C-terminal end (Fig. 4). In comparison with 36 CRISPs isolated from

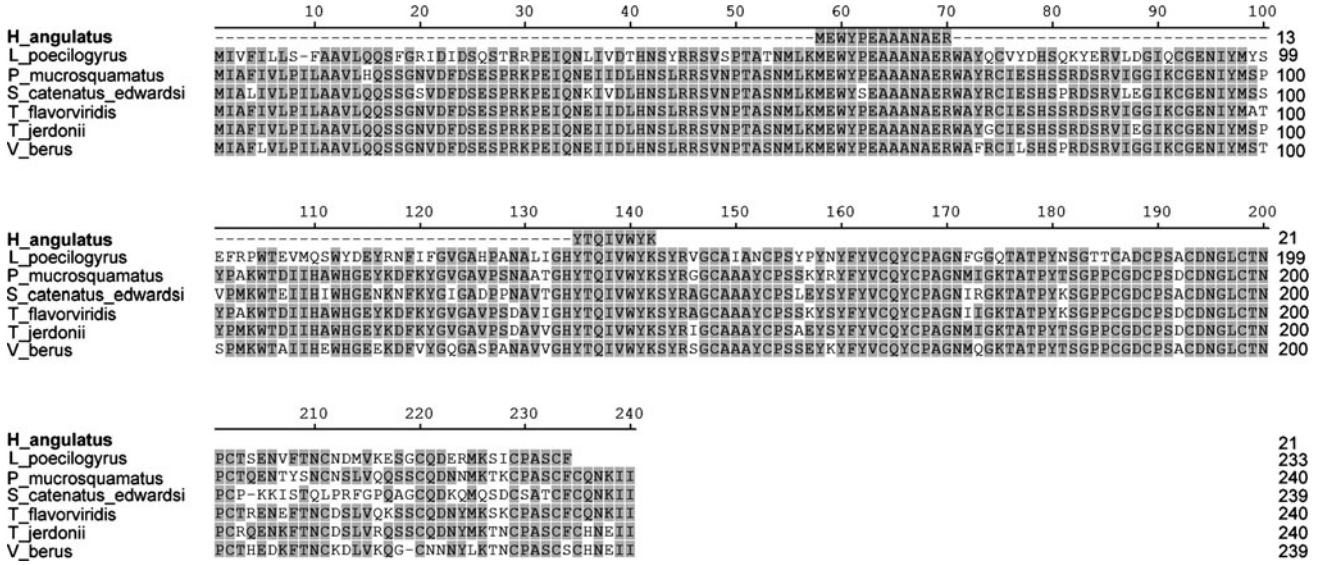

Fig. 4 Amino acid comparison of snake venom cysteine-rich secretory proteins with *H. angulatus* SE fractions. Conserved amino acids are shaded in *gray*. H_angulatus in *bold* was the CRISP, helicopsin, isolated in this work, and the others were selected from previously published papers. L_poecilogyrus (Q2XXQ0) from *Liophis poecilogyrus*, P_mucrosquamatus (P79845) from *Protobothrops mucrosquamatus*, S_catenatus_edwardsi (B0VXV6) from *Sistrurus catenatus edwardsii*, T_flavoviridis (Q8JI39) from *Trimeresurus flavoviridis*, T_jerdonii (Q7ZZN9) from *Trimeresurus jerdonii*, and V_berus (CAP74089) from *Vipera berus*. The comparison was limited to the CRISPs found in the NCBI database that share identical amino acids identified in the venom of *H. angulatus*

snake venoms (Heyborne and Mackessy 2009), for which primary structure information is available on NCBI, helicopsin showed a total amino acid sequence homology with cysteine-rich venom protein precursors from the venoms of *Liophis poecilogyrus, Protobothrops mucrosquamatus*, *Sistrurus catenatus edwardsi*, *Trimeresurus flavoviridis*, *Trimeresurus jerdonii,* and *Vipera berus* (Fig. 4). Of these six CRISPs, only one was isolated from a colubrid (*L. poecilogyrus*), which is also a water snake as is *H. angulatus*. Only two other CRISPs have been identified from another water snake, *Enhydris polylepis* (Fry and Wüster 2004); however, they contained amino acid differences of aspartic acid (Q2XXQ2) and serine (Q2XXQ3) in the alanine position 9 of the helicopsin amino acids (Fig. 5). Helicopsin is a CRISP of ~20 kDa (Fig. 2d), which is in the molecular weight range (20–30 kDa) of other CRISPs (Yamazaki and Morita 2004). These proteins are highly conserved with all 16 of their cysteines strictly conserved, and 10 of these cysteines are bundled in the C-terminal end of the proteins. The biological activities of some of these CRISPs have been identified; however, the functions of many remain highly unknown. The biological activities of the CRISP isolated from the water snakes *E. polylepis* and *L. poecilogyrus* have not been tested (Fry and Wüster 2004; Fry et al. 2006). Those that have been tested inhibit smooth muscle contraction and cyclic nucleotide-gated ion channels (Mochca-Morales et al. 1990; Morrissette et al. 1995; Nobile et al. 1994).

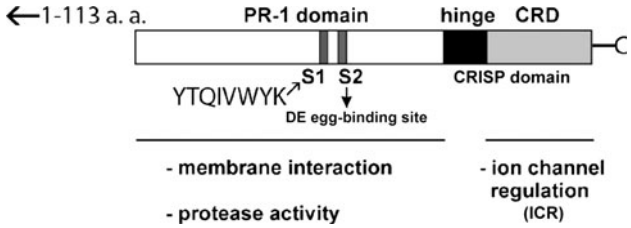

Fig. 5 Schematic illustration of CRISP showing the functional domains. The pathogenesis-related (PR-1) domain contains the Signature 1 and 2 (S1 and S2) regions involved in membrane binding and enzymatic activities, while the cysteine-rich domain (CRD) is responsible for ion channel regulation. This region begins approximately at the 114 amino acid and ends with the final amino acid of the entire CRISP. The YTQIVWYK peptide fragment identified in helicopsin is located in the S1 region. Although the whole protein is designated as a CRISP protein, the CRD region is also referred to as the CRISP domain. The illustration was adopted from that of Ellerman et al. 2006

Although these CRISPs have a highly conserved primary, secondary, and tertiary structure and can be easily identified on their primary structure alone (Heyborne and Mackessy 2009), a section at the C-terminal domain appears to determine their functionality (Fig. 5). The CRISP family contains a plant pathogenesis-related (PR-1) domain and a cysteine-rich domain (CRD), connected by a short hinge (Ellerman et al. 2006). Within the PR-1 domain, two evolutionarily conserved regions named Signature 1 (S1) and Signature 2 (S2) reside. Based on the sperm–egg penetration results reported by Ellerman et al.

(2006), the S2 region of the CRISP-1 (isolated from rat epididymis; Cameo and Blaquier 1976) is involved in the binding of the egg. Thus, the likely role for CRISP members include an ion channel regulatory activity in the CRD and both a protease activity and a membrane-interacting activity in the PR-1 domain (Fig. 5).

The lethality of helicopsin evaluated by the AOAC mouse bioassay method was significantly potent relative to that described from other snake venom neurotoxins (Rossetto and Montecucco 2008). The mouse bioassay has been shown to be a specific method for detecting, identifying, and typing neurotoxins (Franciosa et al. 1994). Helicopsin caused severe symptoms when injected into mice. The injected mice were initially shocked and then showed signs of tremors, which increased with time and lasted up to 6 min. In addition, the hind legs were contracted and the mice began to arch their backs, which resulted in a hunchback or slouching posture with recurrent whole body wrench. Impaired respiration, lethargy, and locomotor distress became evident in 3–5 min, and within 8 min, exophthalmia, tachycardia, sphincter relaxation, paralysis of hind limbs, and death followed. Control animals injected with saline rapidly recovered within 3 min. Given that helicopsin corresponds to a minority component of the SE, it could be suggested that it operates in synergy with other SE components comprising other neurotoxins to generate its biological actions. In summary, the current study gives information on the biological activity and partial amino acid composition of a novel neurotoxin isolated from *H. angulatus* SE.

The biological function of toxins in the SE of *H. angulatus* is unclear, as is the situation with Colubrid snakes (Mackessy 2002; Fry et al. 2008). In recent studies, Gibbs and O'Bryan (2007) demonstrated that the cysteine-rich secretory protein (CRISP-2) domain had a structure analogous to ion channel toxins ShK and BgK from the sea anemone (Tudor et al. 1996; Dauplais et al. 1997). CRISP-2 was able to regulate Ca^{2+} flux through ryanodine receptors (RyRs) such as those in helothermine, a cysteine-rich protein from the venom of the Mexican beaded lizard (*Heloderma horridum horridum*), which caused the reversible concentration-dependent blockage of voltage-gated Ca^{2+} and K^{+} channels and RyRS (Mochca-Morales et al. 1990). Yamazaki et al. (2002) isolated a 25-kDa novel snake venom protein, ablomin, from the venom of the Japanese Mamushi snake (*Agkistrodon blomhoffi*), deduced sequence of which showed high similarity to helothermine. Recently, the crystal structure of Stecrisp, a CRISP protein from the venom of the snake *Trimerusurus stejnegeri*, was proposed (Guo et al. 2005). These authors established that the CAP domain and the cysteine-rich CRISP domain existed as two discrete domains. The CRISP domain is further subdivided into two sub-regions, which is the hinge region and ion channel regulator (ICR) (Fig. 5). It is important to establish that other CAP proteins contain hinge-like sequences (Gibbs et al. 2008), but only the CRISP contains the ICR region, suggesting that the CAP and the ICR domains have separate biological activities (Gibbs and O'Bryan 2007). It is also important to note that the PR-1 domain in Fig. 5 is also part of the CAP domain.

In the in vivo neurological assay, it was noticed that helicopsin produced several neurotoxic symptoms in the inoculated mice consistent with a channel toxin and/or excitatory neurotoxins. To the best of our knowledge, this is the first report of in vivo neurological signs and symptoms produced by *H. angulatus* SE and its isolated CRISP, helicopsin. These results confirm the wide circulation of neurotoxins in the highly developed Colubroidea superfamily of snakes that could have potential in drug discovery.

Acknowledgments This research was supported by a grant from the FONACIT (G-2005000400), Venezuela. We would like to thank the anonymous referee for the important comments that improved the manuscript.

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