Toxicon 55 (2010) 558–569

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Biological and proteomic analysis of venom from the Puerto Rican Racer (*Alsophis portoricensis*: Dipsadidae)

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ARTICLE INFO

Article history:
Received 12 August 2009
Received in revised form
27 September 2009
Accepted 2 October 2009
Available online 14 October 2009

Keywords:
Biological roles
Colubrid
CRISP
Enzymes
α-Fibrinogenase
Gland histology
Hemorrhage
Mass spectrometry
Metalloproteinase
Toxin

ABSTRACT

The Puerto Rican Racer *Alsophis portoricensis* is known to use venom to subdue lizard prey, and extensive damage to specific lizard body tissues has been well documented. The toxicity and biochemistry of the venom, however, has not been explored extensively. We employed biological assays and proteomic techniques to characterize venom from *A. portoricensis anegadae* collected from Guana Island, British Virgin Islands. High metalloproteinase and gelatinase, as well as low acetylcholinesterase and phosphodiesterase activities were detected, and the venom hydrolyzed the α-subunit of human fibrinogen very rapidly. SDS-PAGE analysis of venoms revealed up to 22 protein bands, with masses of ~5–160 kDa; very little variation among individual snakes or within one snake between venom extractions was observed. Most bands were approximately 25–62 kD, but MALDI-TOF analysis of crude venom indicated considerable complexity in the 1.5–13 kD mass range, including low intensity peaks in the 6.2–8.8 kD mass range (potential three-finger toxins). MALDI-TOF/TOF MS analysis of tryptic peptides confirmed that a 25 kDa band was a venom cysteine-rich secretory protein (CRiSP) with sequence homology with tigrin, a CRiSP from the natricine colubrid *Rhabdophis tigrinus*. The venom was quite toxic to NSA mice (*Mus musculus*: $LD_{50} = 2.1$ μg/g), as well as to *Anolis* lizards (*A. carolinensis*: 3.8 μg/g). Histology of the venom gland showed distinctive differences from the supralabial salivary glands (serous vs. mucosecretory), and like the Brown Treesnake (*Boiga irregularis*), another rear-fanged snake, serous secretory cells are arranged in densely packed secretory tubules, with little venom present in tubule lumina. These results clearly demonstrate that venom from *A. portoricensis* shares components with venoms of front-fanged snakes as well as with other rear-fanged species. Venom from *A. portoricensis*, in particular the prominent metalloproteinase activity, likely serves an important trophic function by facilitating prey handling and predigestion of prey.

1. Introduction

A prominent evolutionary trend seen among the advanced snakes is toward the production of toxic oral secretions (venoms) and away from constriction as the dominant method of subduing prey (Kochva, 1987). Venoms vary in complexity and composition, in part as a function of phylogeny and diet, as well as the influence of several other factors (Mackessy, 2009). An important biological role of venom in snakes is the immobilization of potentially dangerous, struggling prey, and by injecting venom which slows, paralyzes or kills the prey, the snake secures a meal and avoids injury. Venom of many species, particularly viperids, also produces extensive tissue necrosis and likely acts to increase rates of digestion of large prey items by "predigesting" the prey (Thomas and Pough, 1979; Mackessy, 1988). Because many venomous snakes are capable of consuming prey items of much larger

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doi:10.1016/j.toxicon.2009.10.010

size in proportion to their bodies than non-venomous snakes (Greene, 1997), the increased surface area available for gastric digestion caused by the venom breaking down the prey internally as the snake digests the prey's external surface is selectively advantageous (Mackessy, 1988).

The paraphyletic family "Colubridae" is the largest family of modern snakes, containing at least 700 venomous species world-wide (Cadle, 1994; Vidal, 2002). This clade of advanced snakes has been the subject of numerous morphological and molecular analyses, and the phylogenetic hypotheses of recent work (Vidal et al., 2007; Quijada-Mascareñas and Wüster, 2009) will be followed here; however, the term "colubrid" will be used generically and for convenience. These snakes vary greatly in size, distribution and diet, and although various species are not closely related, many possess enlarged rear teeth and a Duvernoy's gland (Weinstein and Kardong, 1994), a homolog of the venom gland of front-fanged snakes. Colubrid snakes are generally considered harmless, but there have been documented human fatalities from envenomation by the Boomslang (*Dispholidus typus*) (Pope, 1958) and species from three other genera of colubrids (*Thelotornis, Rhabdophis* and *Philodryas*) (FitzSimons and Smith, 1958; Mittleman and Goris, 1976; Kornalik and Taborska, 1978; Ogawa and Sawai, 1986; Prado-Franceschi et al., 1996). It is probable that the "non-venomous" nature of most colubrids is not due to a lack of enzymes or toxins in the venom, but instead is a result of evolution of venoms that differentially affect non-mammalian taxa (Mackessy et al., 2006; Pawlak et al., 2009). With the vast majority of colubrid venoms still unstudied, there is opportunity to discover novel biological compounds with possible medical implications; however, one obstacle to the characterization of colubrid venoms is the exceedingly small quantities of raw material previously obtainable (Mackessy, 2002). Most colubrids are of a small size and, therefore, yield small amounts of venom. While this previously hindered study, efficient extraction is now possible (Hill and Mackessy, 1997, 2000).

Snake venoms are of considerable interest pharmacologically because they contain compounds useful as probes of ion channels and other physiological processes and as leads for drug development. Colubrid snakes typically have less complex venoms than those of front-fanged snakes (Viperidae, Elapidae), but they share many of the same components including metalloproteinases, serine proteases, phosphodiesterases, acetylcholinesterases, phospholipases A_2 (PLA_2) and three-finger toxins (3FTXs) (Mackessy, 1998, 2002, 2009; Hill and Mackessy, 2000; Fry et al., 2003; Pawlak et al., 2006). Several of these (metalloproteinase and acetylcholinesterase activities) have been previously reported in the venom of the Puerto Rican Racer, *Alsophis portoricensis* (Hegeman, 1961).

Metalloproteinases are the most abundant and diverse enzymatic proteins found in most viperid venoms (Fox and Serrano, 2005; Pahari et al., 2007). Hemorrhagic metalloproteinases are responsible for the severe local inflammation and tissue necrosis seen in human envenomations (Rucavado et al., 1995, 1999), and are biologically very important to the predigestion effects of these venoms on prey (Mackessy, 1988, 1993b). Colubrid envenomations are commonly characterized by minor to significant bleeding (Kuch and Mebs, 2002) due in large part to the presence of hemorrhagic toxins (Takeya and Iwanaga, 1998), which can be serine proteinases or metalloproteinases, although hemorrhage is not necessarily due only to the proteinase components. Metalloproteinases may also show muscle-damaging activity, and myotoxic snake venom metalloproteinases have been isolated from the venoms of several colubrid snakes (e.g., *Philodryas olfersii*: Prado-Franceschi et al., 1998; *Rhabdophis tigrinus*: Komori et al., 2006; *P. patagoniensis*: Peichoto et al., 2007).

The Puerto Rican racer (*Alsophis portoricensis*), now considered a member of the family Dipsadidae (Vidal et al., 2007), is a rear-fanged colubrid snake found on numerous islands in the Caribbean. *Alsophis portoricensis* are ground-dwelling, diurnal snakes that have been observed envenomating prey before consumption (Rodriguez-Robles and Thomas, 1992). The diet of *A. portoricensis* primarily includes lizards (*Anolis* sp.) and *Eleutherodactylus* frogs, but they have also been observed preying on dead fish, bird eggs, iguanas, other snakes, and rats (Rodriguez-Robles and Leal, 1993a, b; Norton, 1993). Although typically hesitant to bite when confronted, *A. portoricensis* has been documented to cause cases of human envenomation resulting in edema, paraesthesia and ecchymosis at the bite site (Heatwole and Banuchi, 1966; R. Platenberg and K. Lindsay, pers. comm.). Because venom composition appears to be related to prey type and form (Mackessy, 1988; Daltry et al., 1996; Gibbs and Mackessy, 2009), the types of toxins utilized by particular snake species to facilitate prey handling may differ depending on the type of prey animal utilized. For example, venom from the Brown Treesnake (*Boiga irregularis*) has been shown to possess taxon-specific toxins that affect birds and reptiles to a much greater extent than mammals, and this snakes feeds primarily on non-mammalian prey (Mackessy et al., 2006; Pawlak et al., 2009). *Alsophis portoricensis* venom may also be more toxic to reptiles than to mammals because reptiles make up the majority of the diet of this snake. Hegeman (1961) commented on the hemolytic and proteolytic activities of *A. portoricensis* venom, but was unable to characterize it further due to a lack of both crude material and technical sophistication. The predatory use of venom by *A. portoricensis* has been documented, and envenomated *Anolis* lizards appeared to suffer respiratory distress resulting from hemorrhage in the lungs (Prieto-Hernandez, 1985), perhaps due to the action of SVMPs. In the current study, biological assays and proteomic techniques were employed to characterize more fully the venom of *A. portoricensis* and to provide a biochemical explanation for the effects of envenomation previously described. We also hypothesized that like *B. irregularis* venom, *A. portoricensis* venom would have taxon-specific effects, with greater potency toward lizard prey.

2. Materials and methods

2.1. Reagents

Protein concentration reagents were purchased from BioRad Inc. (San Diego, CA). Novex Mark 12 unstained molecular mass standards, MES running buffer, LDS sample

buffer and precast electrophoretic gels (Zoom, NuPAGE Novex Bis-Tris, Novex Zymogram) were obtained from Invitrogen, Inc. (San Diego, CA). Phospholipase A_2 assay kit was purchased from Cayman Chemical Co. (Ann Arbor, MI). Immobiline DryStrip immobilized pH gradient (IPG) strips, buffers and an Ettan IPGphor isoelectric focusing unit were obtained from GE/Amersham Biosciences, Inc. (Piscataway, NJ). Acetyl-L-Lys-L-Leu-L-Asn-L-Lys-L-Arg-paranitroaniline was synthesized by EZ Biolab (Carmel, IN). A Trypsin Profile IGD kit and all other buffers and reagents (analytical grade) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO).

2.2. Alsophis portoricensis venom

Venom was extracted from *A. portoricensis* collected on Guana Island, British Virgin Islands and housed at the UNC Animal Facility in accordance with UNC-IACUC protocol No. 9204.1. Snakes were extracted no more frequently than once every 6 weeks using the method of Hill and Mackessy (1997) with ketamine-HCl (32 mg/kg) followed by pilocarpine HCl (6 mg/kg), and venom was collected from the base of the enlarged rear maxillary teeth into microcapillary tubes. The venom was lyophilized and stored frozen at −20 °C until needed. Protein content of the venom was determined using bovine gamma globulin as a standard and the method of Bradford (1976) as modified by BioRad Inc. These calculated protein concentrations, performed in triplicate for each crude venom sample, were the basis of all other analyses and enzymatic activity calculations.

2.3. Enzyme assays

Metalloproteinase activity of the crude venom was evaluated using azocasein as a substrate (Aird and da Silva, 1991). The activity was expressed as $\Delta A_{342\ nm}$/min per mg venom protein. Phosphodiesterase activity was assayed using the methods of Bjork (1963) and Laskowski Sr., 1980 as modified by Mackessy (1998). Thymidine 5′-monophospho-*p*-nitrophenyl ester substrate was incubated with crude venom and specific activity was expressed as $\Delta A_{400\ nm}$/min per mg protein. Acetylcholinesterase activity was determined using 10 μg crude venom incubated with acetylthiocholine iodide substrate (Ellman et al., 1961) in a jacketed cuvette at 37 °C. The absorbance at 412 nm was recorded every minute for 15 min and the linear portion of the graph was used to calculate activity, reported as μmol product formed per minute per mg venom protein. Phospholipase A_2 activity (Huang and Mackessy, 2004) was evaluated using a commercially available kit (Cayman Chemical Co.) as described by the manufacturer using 1.0 μg crude *A. portoricensis* venom (200 μL final total volume). Absorbance was measured at 414 nm every minute for 15 min (37 °C) and activity was reported as μmol product/min/mg protein. Activity toward paranitroaniline-derived (pNA) synthetic substrates for thrombin (BzPhe-ValArg-pNA), trypsin (BzArg-pNA), thrombin/trypsin (TosylGlyProArg-pNA), plasmin (ValLeuLys-pNA), arginine esterase (CBZ-L-Arg-pNA), kallikrein (ProPheArg-pNA), elastase (SuccAlaAlaAla-pNA), leucine aminopeptidase (Leu-pNA), and cone snail CRiSP (AcLysLeuAsnLysArg-pNA) was assayed and evaluated at 405 nm (Mackessy, 1993a). Ten μg of crude venom was used and specific activities were reported as nmol product/min/mg protein. All enzyme assays were performed on venom from three individual snakes, all done in triplicate. Negative controls were also performed in triplicate. Positive controls included numerous rattlesnake venoms assayed previously (Mackessy, 2008). The metalloproteinase and phosphodiesterase assays described above were also used to determine activities in fractions generated from SE-HPLC.

Some colubrid snake venoms, including those of *P. olfersii* and *P. patagoniensis*, have been shown to degrade fibrinogen, interfering with blood coagulation via hypofibrinogenemia (Assakura et al., 1994; Peichoto et al., 2007). Fibrinogenase activity was determined using 20 μg of crude venom incubated with human fibrinogen (final concentration 0.5 mg/mL) in a total volume of 200 μL for periods of 0, 1, 5, 10, 30, and 60 min (Ouyang and Huang, 1979). Twenty μL of this reaction mixture was removed at each time point and mixed with an equal volume of 4% SDS and 5% 2-mercaptoethanol. Five μL aliquots of the resulting solutions were combined with 2× LDS buffer, electrophoresed on a 12% NuPAGE gel, stained with Coomassie Brilliant Blue, and destained. All gels were imaged using an Alpha Imager system.

2.4. One-dimensional gel electrophoresis

Crude venom from two extractions of three different *A. portoricensis* was used to assess the number and relative molecular masses of venom components as well as to determine whether variation in venom composition exists among individuals and/or between extractions. NuPage Bis-Tris gels (Invitrogen, Inc.) and MES running buffer were utilized for SDS-PAGE analysis, with samples and buffers prepared according to the manufacturer as described previously (Mackessy et al., 2006). Thirty μg of crude *A. portoricensis* venom was added to each lane. Other crude colubrid venoms (30 μg each) as well as 2.5 μg purified denmotoxin (a 3FTx from *Boiga dendrophila* venom; Pawlak et al., 2006) were run for comparison, and 5 μL Mark 12 standards (Invitrogen, Inc.) was used to estimate masses. The gel was then stained with Coomassie Brilliant Blue, destained and imaged with an AlphaImager.

The number and relative size of components with endoproteinase (gelatinase) activity were determined using varying amounts of crude venom (0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, and 5.0 μg per lane) run on a 10% gelatin Zymogram gel (Munekiyo and Mackessy, 1998). The gel was placed sequentially in renaturing and developing buffers (1 h each) and incubated at 37 °C overnight with gentle agitation. The gel was then stained with 0.1% Coomassie Brilliant Blue, destained, and imaged. Areas of gelatinase activity appeared clear on a blue field.

2.5. Two-dimensional gel electrophoresis

Seven cm IPG strips (GE/Amersham Biosciences, Inc.) of pH 3–11 were rehydrated with crude venom solutions from either *A. portoricensis* or *Crotalus ruber* (Red Diamondback Rattlesnake) and subjected to isoelectric focusing using an

Ettan IPGphor isoelectric focusing unit (GE Amersham Biosciences, Inc.) as per manufacturer instructions. Briefly, 160 μg of lyophilized venom from each snake was dissolved in 125 μL of rehydration buffer (8 M urea, 2% CHAPS, 2.0% IPG buffer, trace bromphenol blue, and freshly added DTT (18.2 mM)). These solutions were added to each strip in IPG strip holders, covered with mineral oil, and rehydrated for 12 h at 22 °C and 30 V on the IPGphor system. The strips were then focused for 4.5 h at 22 °C with a maximum of 5000 V (50 μA/strip). After focusing, the strips were reduced (4 mL of Tris/SDS equilibration buffer with 65 mM DTT) for 15 min and then alkylated (4 mL of Tris/SDS equilibration buffer with 135 mM iodoacetamide) for 15 min. Next, the strips were equilibrated with 4 mL NuPAGE LDS sample buffer (1×) for 10 min, placed in the IPG wells of ZOOM gels, overlain with 0.5% agarose, and run according to the manufacturer's instructions. After staining with Coomassie Brilliant Blue and destaining, the spots on the two gels were visually compared with each other as well as with the molecular mass standards.

2.6. MALDI-TOF mass spectrometry and trypsin digestion

A sample of lyophilized crude venom was analyzed using a Bruker Ultraflex MALDI-TOF/TOF mass spectrometer (Proteomics and Metabolomics Facility, CSU, Fort Collins, CO). Approximately 1 μg of crude venom was spotted onto sinapinic acid matrix. For identification of venom proteins, bands of interest (50, 25 and 16 kDa) were excised from a 1D SDS-NuPAGE gel of crude *A. portoricensis* venom and proteins were digested using a trypsin in-gel digest kit (Sigma-Aldrich, Inc.; product #PP0100). Digest samples were C_{18} ZipTip cleaned and then subjected to MALDI-TOF-MS/MS analysis on an ABI 4800 mass spectrometer. Singly-charged ions of selected peptides from the MALDI-TOF mass fingerprint spectra were sequenced by collision-induced dissociation tandem mass spectrometry. The spectra generated were then analyzed using ABI ProteinPilot software, and sequence data were compared against the BLASTP database (http://blast.ncbi.nlm.nih.gov/Blast.cgi, 15 January 2009) in order to identify venom proteins.

2.7. Size exclusion (SE) HPLC

Five hundred μg of crude venom was dissolved in 200 μL of 25 mM HEPES buffer, pH 6.8, containing 100 mM NaCl and 5.0 mM $CaCl_2$, injected onto a TosoHaas TSK G2000 SWxl size exclusion column (7.8 mm ID × 300 mm, 5.0 μm) equilibrated with the same buffer and run at a flow rate of 0.3 mL/min using a Waters HPLC system (Mackessy et al., 2006). The fractions were collected at 1 min intervals and absorbance was measured at 280 nm (Empower 2 software). Fractions from each peak were run on a 1D gel and compared with results for crude *A. portoricensis* venom. Fractions were also assayed for acetylcholinesterase and metalloproteinase activity as above.

2.8. Toxicity tests

Because lizards make up a substantial part of *Alsophis* diet, taxon-specificity and lethal toxicity of crude *A. portoricensis* venom were evaluated using both lizards (*Anolis carolinensis*) and non-Swiss albino (NSA) mice (*Mus musculus*). Mice were bred in the UNC Animal Facility and lizards were obtained from Animal Attractions (Greeley, CO); all procedures were reviewed and approved by the UNC IACUC (protocol No. 9401). Venom was introduced intraperitoneally (IP) in sterile saline, with doses adjusted for the body mass of each animal. Three animals were used at each dosage in an effort to minimize animal use numbers and were monitored for 24 h post injection. The 24 h LD_{50} (Reed and Muench, 1938) was expressed in μg venom/g animal body mass. Necropsies were performed on animals that succumbed to the venom to analyze further the extent of tissue damage.

2.9. Hemorrhagic assay

The hemorrhagic activity of the crude venom was evaluated using a method based on Bjarnason and Fox (1983). Lyophilized crude venom was dissolved in 25 μL of 0.9% sterile saline and injected intradermally (ID) into 4 week old female NSA mice. After a 2 h incubation period, the mice were euthanized using CO_2. The skins were then removed, pinned fur side down, photographed and hemorrhagic spots measured to the nearest mm. Dosages were given in duplicate at 0 (control), 1.0 and 5.0 μg. The mice used in this assay (six total) were bred in the UNC Animal Facility and all procedures were reviewed and approved by the UNC IACUC (protocol No. 9401).

2.10. Histology of the venom (Duvernoy's) gland

One adult *A. portoricensis* was euthanized and the glands were rapidly removed and preserved in buffered formalin as described previously (Mackessy and Baxter, 2006). Preserved tissue was then serially dehydrated, embedded in paraffin and 5 μm sagittal sections were obtained and stained with either hematoxylin/eosin or Periodic Acid-Schiff (Humason, 1978; sectioning and staining performed by Colorado Histo-Prep, Ft. Collins, CO). Light micrographs were then recorded digitally using an Olympus CX41 microscope with a CCD camera, and images were captured using SPOT Digital software (Diagnostics Instruments, Inc.) version 4.0.5. Images were optimized using Adobe Photoshop Creative Suites 2.0.

3. Results

3.1. Snake sizes, venom yields and protein content

Average snake length was 582 mm (SVL), and mass was 68.9 g ($N = 33$). Based on 30 extractions, venom yields for each of six snakes averaged 160 μL (SD = ±70 μL). Dry mass of venom averaged 5.9 mg (±2.0 mg), and protein content of venoms averaged 89%.

3.2. Enzyme assays

There was substantial metalloproteinase activity toward azocasein, with activities of the venoms of the three specimens averaging 1.25 ($\Delta A_{342\ nm}$/min/mg venom protein).

Table 1
Specific activities of *A. portoricensis* venom toward various substrates. All values are mean values for three individual snakes, assayed in triplicate, ±standard deviation.

Assay	Substrate	Activity	St. Dev.	Units
Metalloproteinase	Azocasein	1.254	0.086	$\Delta A_{342\ nm}$/min/mg
Phosphodiesterase	Thymidine 5′-monophospho-*p*-nitrophenyl ester	0.046	0.023	$\Delta A_{400\ nm}$/min/mg
Acetylcholinesterase	Acetylthiocholine iodide	0.450	0.010	μmol product/min/mg
Phospholipase A_2	1, 2-Dithiodiheptanoyl phosphatidylcholine	0.000	0.000	μmol product/min/mg
Thrombin	BzPheValArg-pNA	1.187	0.002	nmol/min/mg
Plasmin	ValLeuLys-pNA	0.251	0.0003	nmol/min/mg
Elastase	SuccAlaAlaAla-pNA	0.084	0.0002	nmol/min/mg
Thrombin/trypsin	TosylGlyProArg-pNA	37.85	0.011	nmol/min/mg
Trypsin	BzArg-pNA	0.028	0.0001	nmol/min/mg
Kallikrein	ProPheArg-pNA	0.527	0.001	nmol/min/mg
Leucine aminopeptidase	Leu-pNA	3.590	0.002	nmol/min/mg
Cone snail CRiSP	AcLysLeuAsnLysArg-pNA	1.940	0.001	nmol/min/mg
Arginine esterase	CBZ-L-Arg-pNA	0.419	0.0004	nmol/min/mg

Phosphodiesterase activity of *A. portoricensis* venom was 0.069 ($\Delta A_{400\ nm}$/min/mg venom protein), relatively low compared with several other colubrids (Hill and Mackessy, 2000). The acetylcholinesterase assays revealed low activity levels of 0.45 μmol product formed/min/mg venom protein. Low activity toward the thrombin/trypsin-like pNA substrate (TosylGlyProArg-pNA) was observed (37.8 nmol product/min/mg protein). Assays for activity toward all other pNA substrates showed very low or no activity (Table 1), and there was no detectable PLA_2 activity. The zymogram gel showed prominent gelatinase activity, as evidenced by the large cleared area in the gel at approximately 45 kDa (Fig. 1). The optimal amount of venom was 0.05 μg/lane, but activity was detectable at 0.005 μg/lane. The fibrinogen digest revealed an almost immediate hydrolysis of the α-subunit of fibrinogen (20 μg venom), and after 60 min there appeared to be some degradation of the β-subunit as well (Fig. 2).

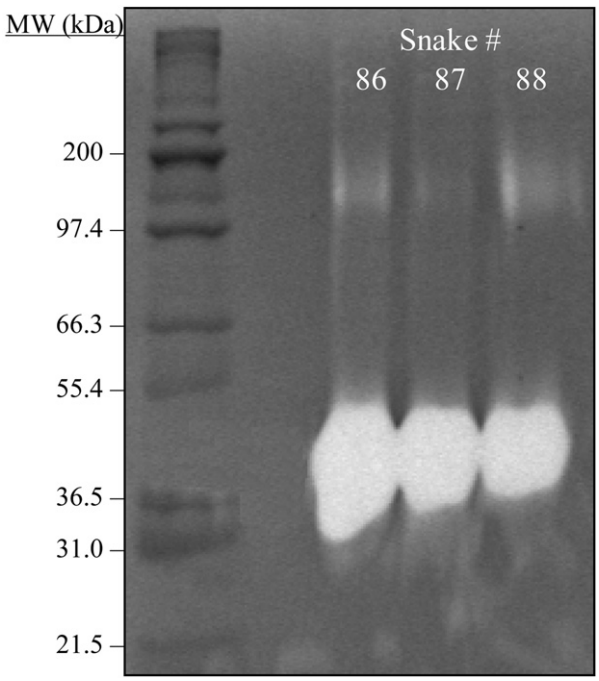

Fig. 1. Ten percent acrylamide zymogram gel image showing gelatinase activity of *A. portoricensis* venom. Activity of crude venom (0.05 μg/lane) from each of three *A. portoricensis* (#86, #87, #88) is seen as clear regions of the gel. Molecular mass standards appear in the left lane.

3.3. One-dimensional gel electrophoresis

Approximately 20 protein bands were observed between 10 and 80 kDa, with minimal variation in relative band intensity between venom extractions and among three different *A. portoricensis* individuals (Fig. 3, lanes 11–16). Prominent bands were found between 45 and 62 kDa, and one at 25 kDa, and three very faint, high molecular weight bands (~90–160 kDa) were also observed. There were several very faint, lower molecular mass bands (~5–9 kDa) also present (not visible in Fig. 3). Many of the other colubrid venoms compared also had prominent bands at 25 kDa and at 50–60 kDa (lanes 3–9), but *A. portoricensis* venom appeared to have greater

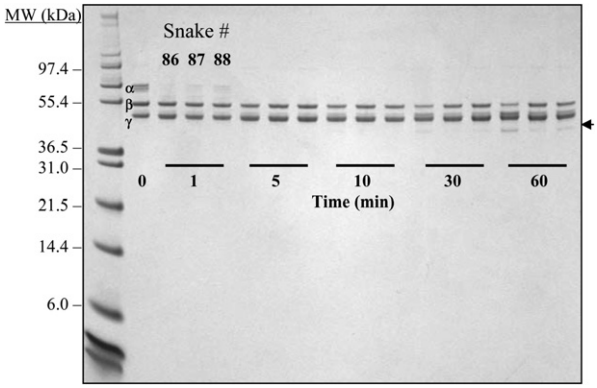

Fig. 2. Fibrinogen digest assay gel showing degradation of subunits after incubation with *A. portoricensis* venom for specific time intervals. Molecular mass standards appear in the far left lane. First lane after standards shows the three fibrinogen subunits (α, β, γ) intact at time 0. The following groups of three lanes (venom from snake #86, #87 and #88) show rapid loss of α-subunit after incubation with venom (1, 5, 10, 30 and 60 min). Note the degradation products visible below the γ-subunit band indicating partial degradation of the β-subunit by 60 min (arrow at right).

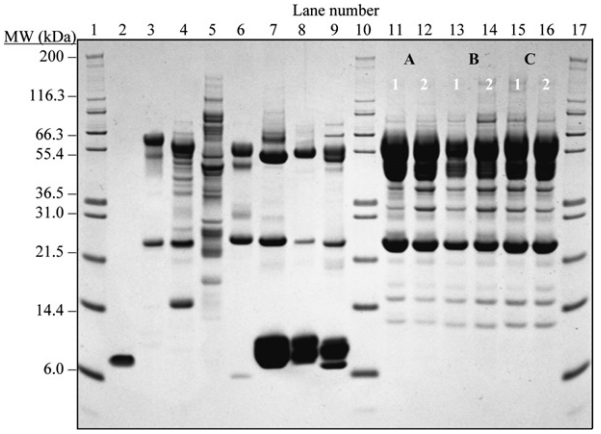

Fig. 3. 1-D SDS PAGE comparing venoms of *A. portoricensis* to other colubrids. Molecular mass standards appear in lanes 1, 10, and 17. Samples: 2.5 μg purified denmotoxin (lane 2); crude venom (30 μg) from other colubrids: *Hydrodynastes gigas* (lane 3), *Lioheterodon madagascariensis* (lane 4), *Leptodeira annulata* (lane 5), *Ahaetulla nasuta* (lane 6), *Boiga cyanea* (lane 7), *Boiga dendrophila* (lane 8), *Boiga irregularis* (lane 9). Lanes 11–16: *A. portoricensis*, three individual snakes (A–C), first and second extractions (1 & 2) for each. Note the lack of variation between repeat extractions and between individuals.

complexity in the 31–50 and 12–20 kDa range. *Alsophis* venoms appeared to lack detectable amounts of homologs of denmotoxin (lane 2), a taxon-specific, monomeric 3FTx, whereas homologs were present in all three *Boiga* venoms analyzed (lanes 7–9).

3.4. Two-dimensional gel electrophoresis

The 2D *A. portoricensis* venom gel had approximately 30 protein spots that spanned most of the 3–11 pH range, with the majority of the spots in the higher molecular mass, acidic region (Fig. 4A). Spots of the same molecular mass but of differing isoelectric points (likely isoforms of single proteins) were visible at ~45, 58, and 62 kDa, as evidenced by a series of repeating spots (Fig. 4A, arrows). Discrete protein spots were visible at ~35 and 50 kDa, but several were horizontally streaked, possibly due to incomplete focusing. Probable or confirmed identity of several protein spots is indicated for Fig. 4A. The *C. ruber* gel (Fig. 4B) showed many more distinct proteins than the *A. portoricensis* gel, demonstrating the comparatively lower complexity of the proteome of *A. portoricensis*, typical of many colubrid snakes (Mackessy et al., 2006).

3.5. Mass spectrometry

MALDI-TOF spectrograms of the crude venom were generated using 1–14 and 15–70 kDa windows. The 1–14 kDa window revealed several species of low molecular mass, including 3.56, 4.25, 11.62 and 12.59 kDa peaks as well as numerous lower intensity peaks (Fig. 5A). Four major peaks at 17.47, 23.13, 25.18, and 50.36 kDa were visible in the 15–70 kDa scan (Fig. 5B). Following band excision and trypsin digestion, MALDI-TOF-MS/MS spectrometry and analysis with ProteinPilot software revealed that a peptide derived from the 25 kDa protein band had a sequence of WAYQCAYDHSPRSSR (Fig. 6). A BLASTP search for this peptide revealed 100% sequence identity with residues 52–66 of tigrin (mature protein chain), a 25 kDa CRiSP isolated from *Rhabdophis tigrinus* venom (Yamazaki et al., 2002). The band at 16 kDa was identified as a hemoglobin blood contaminant; a peptide sequence derived from this protein matched a hemoglobin peptide only (data not shown). Attempts to obtain internal sequence of the putative metalloproteinase (~50 kDa) via MALDI-TOF-MS/MS were unsuccessful, perhaps due to the presence of numerous other proteins with similar masses.

3.6. Size exclusion HPLC

Five peaks were observed eluting between 19 and 47 min, and enzyme activities (metalloproteinase and

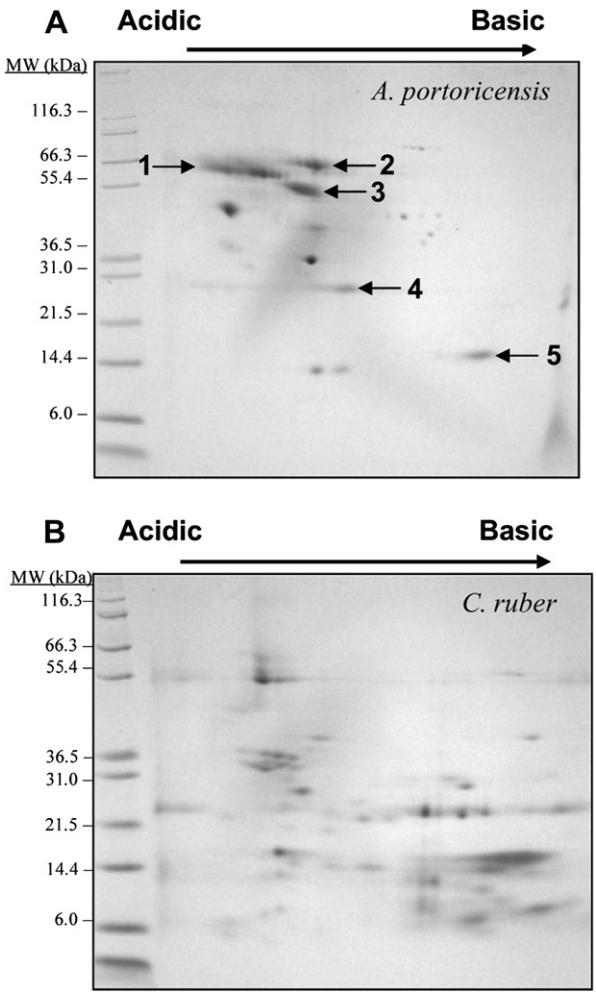

Fig. 4. Two-dimensional SDS PAGE gels comparing complexity of *A. portoricensis* venom (A) to that of *Crotalus ruber* (B); 160 μg venom each. Molecular mass standards appear along the left of both gels. Top three arrows in (A) indicate possible protein isoforms in the acidic region between 50 and 60 kDa (likely 1 – metalloproteinases, 2 – acetylcholinesterases and 3 – phosphodiesterases); identified proteins are indicated by arrows 4 (CRiSP) and 5 (hemoglobin contaminant). Note the general lack of proteins in the lower molecular mass region on the *A. portoricensis* gel compared with the same region on the *C. ruber* gel.

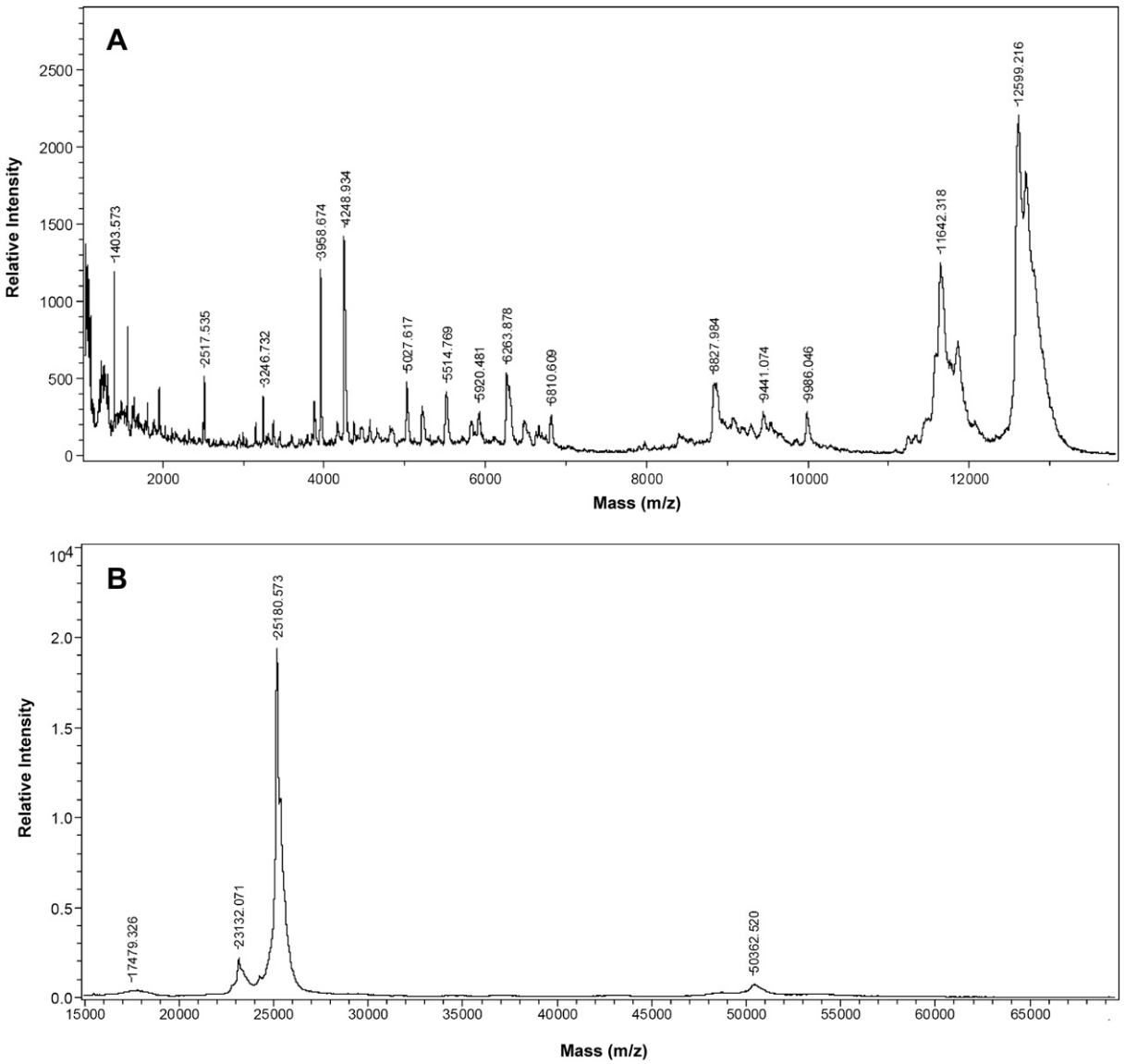

Fig. 5. MALDI-TOF spectrograms of crude *A. portoricensis* venom generated using a Bruker Ultraflex mass spectrometer. (A) 1–14 kDa and (B) 15–70 kDa window scans. Note the numerous small mass peptides (~1400–9900 Da) in A which appear to be of very low abundance (based on comparison with 1D & 2D SDS-PAGE).

phosphodiesterase) were detected in peak I fractions (19–26 min) only (Fig. 7). Aliquots taken from the fractions that corresponded to each of the five peaks were electrophoresed on a 1D gel. When compared to the 1D SDS-PAGE gel of crude venom, peak I encompassed the 45–58 kDa bands, peak II (28–32 min) corresponded to the 25 kDa band (CRiSP), and the three peaks that eluted between 34 and 47 min corresponded to the four lower molecular weight bands observed between 11 and 20 kDa.

3.7. Lethal toxicity

The venom was quite toxic to both mice and lizards, with an IP LD_{50} of 2.1 μg crude venom/gram body mass in mice and an IP LD_{50} for *Anolis carolinensis* of 3.8 μg/g. Necropsies for animals of both species which received lethal doses revealed substantial hemorrhage at the injection site and surrounding muscle tissue, as well as to the lungs.

3.8. Hemorrhagic assay

Hemorrhage was present in the subcutaneous tissue at all ID dosages of crude venom (Fig. 8). The largest hemorrhagic spot, present from the 5.0 μg venom assay, was 12 × 11 mm (Fig. 8C).

3.9. Gland histology

Sagittal sections of the venom gland (Fig. 9) showed an overall pattern of organization similar to that of the Brown

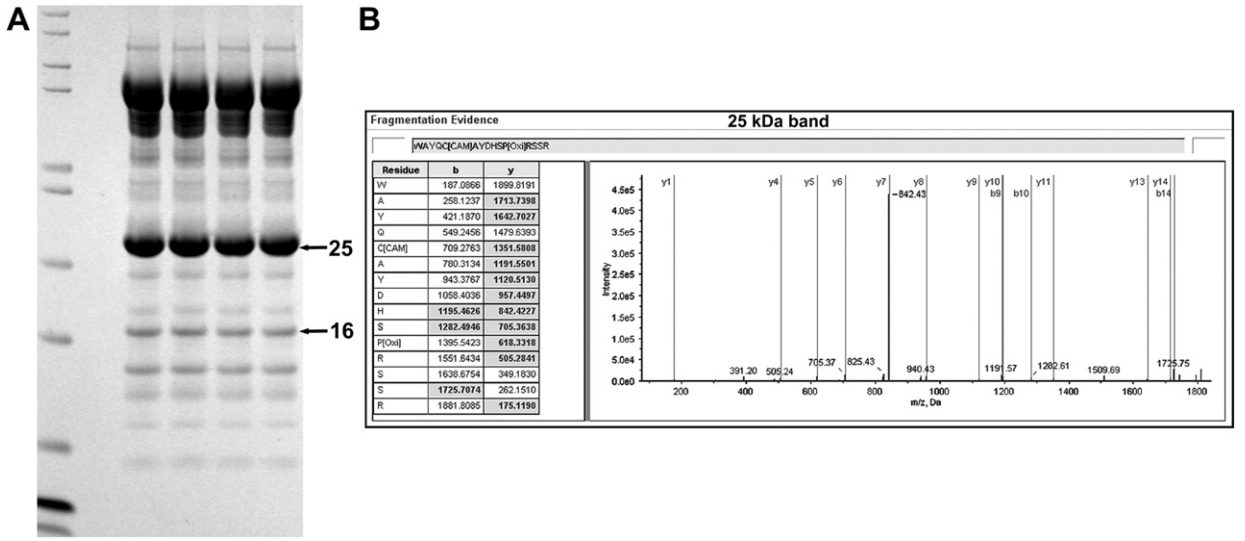

Fig. 6. MALDI-TOF MS/MS analysis of *A. portoricensis* crude venom. (A) Reducing SDS-PAGE image of *A. portoricensis* crude venom (50 μg/lane) from which the 25 and 16 kDa bands were excised and in-gel digested with trypsin. (B) Spectrogram and analysis using ProteinPilot software of a peptide derived from the 25 kDa protein from *A. portoricensis* venom.

Treesnake, *Boiga irregularis* (Zalisko and Kardong, 1992). The *Alsophis* gland is composed of lobular subunits arranged into numerous secretory tubules which are lined by serous secretory cells. The difference of these secretory cells from the (muco)polysaccharide-secreting cells of the supralabial salivary gland, which abuts the anterior end of the venom gland, was apparent by the differential staining of the glands (Figs. 9A,B,D). Mucosecretory cells of the salivary gland contained large diffuse cytoplasmic granules which were absent from the (serous) cells of the venom gland. Most secretory tubules were empty of venom. A large (often basal) storage lumen, common among front-fanged snakes, was absent in *Alsophis*, as is typical of most colubrid venom glands.

4. Discussion

Many enzymes and toxins appear to be present in both front-fanged snakes and colubrids, and the biological roles of venom in prey handling and digestion appear to be universal (Mackessy, 1988, 1993a; Mackessy et al., 2006; Pawlak et al., 2006, 2009; Gibbs and Mackessy, 2009). Despite the diminutive size of most venomous colubrid snakes, they are still very capable predators, and many exclusively use a chemical means of dispatching prey. The relatively small size of the rear teeth is likely compensated for by extreme maxillary rotation that allows for engagement of these teeth into prey tissues. By "chewing" their

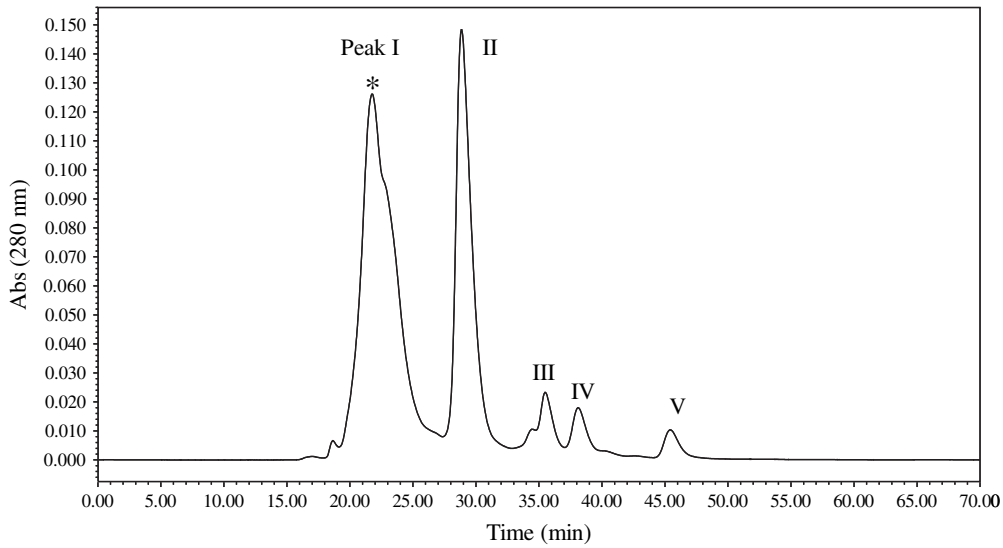

Fig. 7. HPLC size exclusion chromatogram of 500 μg crude venom. The first peak in the chromatogram represents the metalloprotease and phosphodiesterase-containing peak (*), the second peak is the CRiSP peak, and the small peaks further downstream are the lower molecular mass toxins (~10–16 kDa). Flow rate was 0.3 mL/min, and absorbance was monitored at 280 nm.

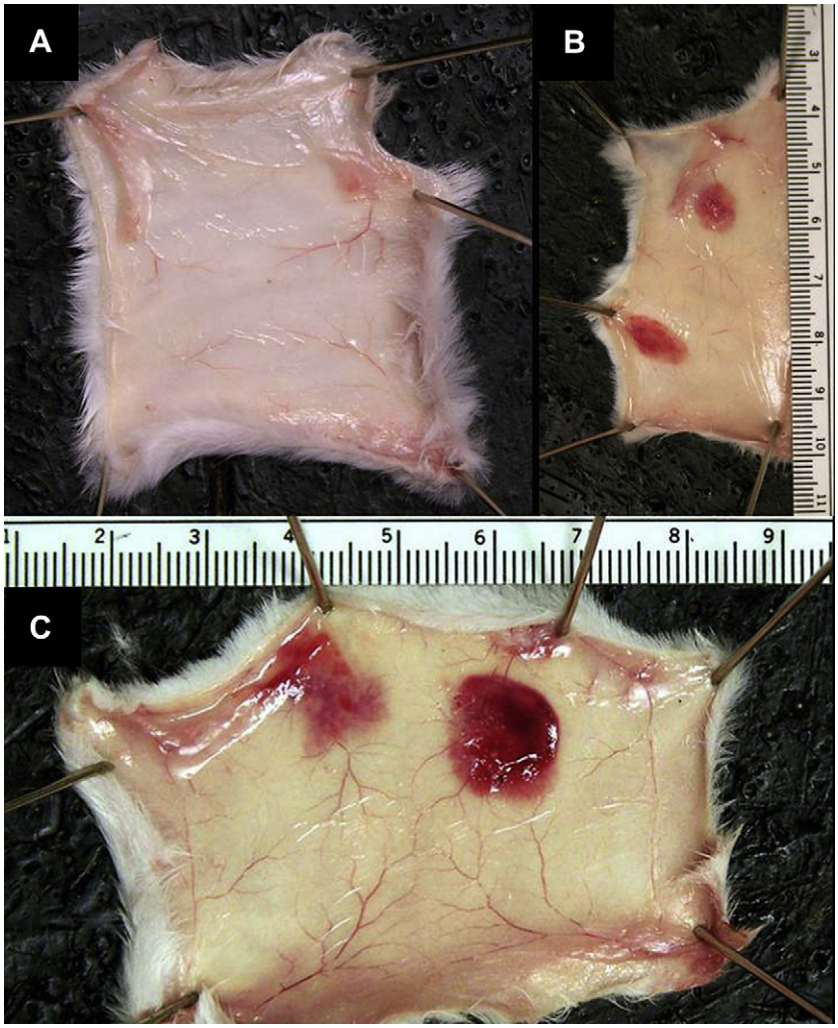

Fig. 8. Hemorrhagic effects of *A. portoricensis* venom on NSA mice. Inner surface of mouse skin 2 h after intradermal injection with (A) 0.9% saline control, (B) 1 μg venom and (C) 5 μg venom dosages. Rule has 1 mm increments.

prey as opposed to striking and releasing, colubrid snakes are able to introduce venom into prey tissues at multiple sites, allowing the venom to produce diffused toxic effects. Relatively long contact time appears to be necessary for significant effects in humans (e.g., Kuch and Mebs, 2002), due in part to the intracellular storage of venom, and bites from most colubrid snakes are not of clinical concern. However, *A. portoricensis* occasionally are responsible for more serious human envenomations, and symptoms of a recent extended bite included edema, generalized arthralgia, paraesthesia in the bitten digit and minor ecchymosis in the bitten limb (R. Platenberg and K. Lindsay, pers. comm.). Given the toxicity of *A. portoricensis* venom toward both mice and lizards and anecdotal observations of human cases, an extended bite by a very large adult snake could easily result in clinically significant symptoms of envenomation.

Rattlesnakes use venom as a means of promoting digestion of large food items through "predigestion" of the prey (e.g. Thomas and Pough, 1979; Mackessy, 1988), and it is likely that *A. portoricensis* venom, with high levels of metalloproteinase activity, has a similar role. The levels of metalloproteinase activity detected were very high for a colubrid snake, and were comparable to those measured for venoms from some species of rattlesnakes (Bjarnason and Fox, 1994; Hill and Mackessy, 2000; Mackessy, 2008). Because metalloproteinases cause both localized and systemic hemorrhage and necrosis (Bjarnason and Fox, 1995; Gutiérrez and Rucavado, 2000), one or more metalloproteinases are likely responsible for at least some of the observed damage in the mouse and lizard tissues. The severity of an envenomation depends on the nature of the venom components and, as demonstrated in this and other studies, colubrid snakes possess some venom proteins in common with the venoms of front-fanged snakes. Crude venom from *A. portoricensis* caused severe localized ecchymosis, hemorrhage and necrosis in both mice and lizards, a finding consistent with the feeding behavior observations of Thomas 1985); hemorrhage was particularly pronounced in the lungs.

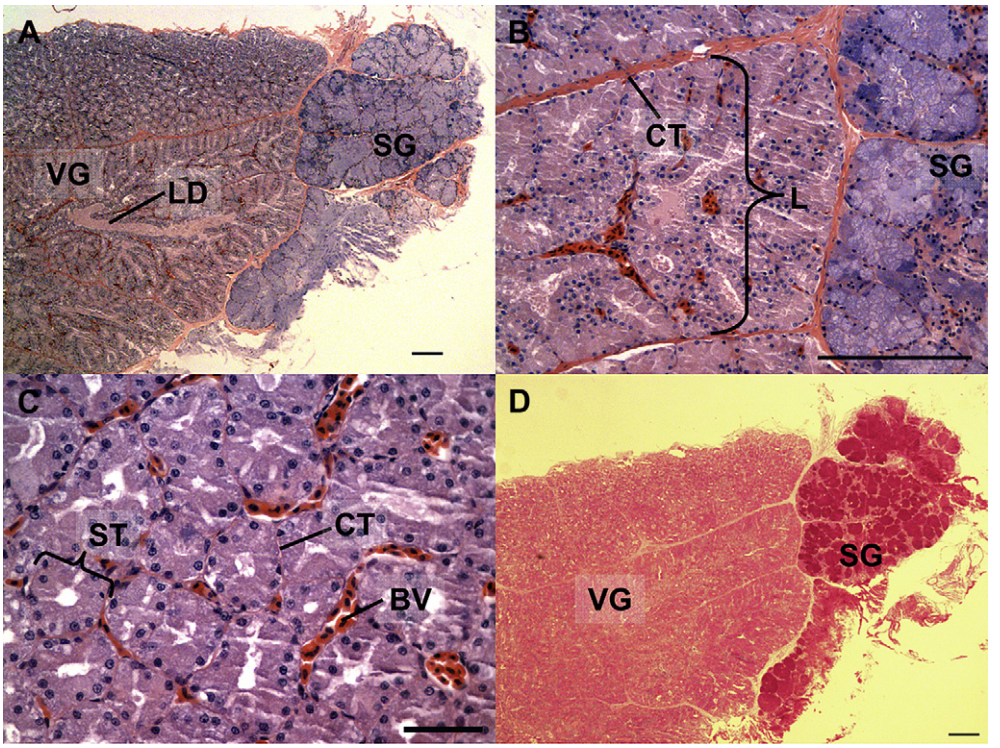

Fig. 9. Structure of the venom (Duvernoy's) gland of *A. portoricensis*. Overall organization is similar to that of *Boiga irregularis*, with the secretory epithelial tissue organized into lobules (L) containing secretory tubules (ST) and surrounded by connective tissue (CT), often also supporting blood vessels (BV). Secretory tubules join to form a larger lobular duct (LD), apparently filled with venom. Note differential staining of supralabial salivary gland (SG) compared to the venom gland (VG) in A, B & D; large (muco)polysaccharide-rich cytoplasmic granules are apparent in the SG, whereas the cells of the VG are primarily serous. A, B and C – hematoxylin/eosin stain; D – PASH stain. Scale bars = 200 μm (A, B, D) and 50 μm (C).

However, the venom did not appear to show taxon-specific toxicity and was somewhat less toxic to Green Anoles (*Anolis carolinensis*) than it was to inbred mice. Perhaps because envenomation is only one aspect of the feeding sequence, the snake may rely more heavily on mechanically subduing prey via constriction rather than using chemical means (venom). It is also possible that species of *Anolis* which are native to Guana Island (including *A. cristatellus* and *A. stratulus*; Rodda et al., 2001) may be more susceptible to venom than *A. carolinensis*, and two different species of lizards have been shown to display different sensitivities to *Boiga irregularis* (also a colubrid snake) venom (Mackessy et al., 2006). *Alsophis* venom possessed very high gelatinase activity, with quantities as low as 0.005 μg producing discernable activity on zymogram gels. Gelatinase and collagenase activities of metalloproteinases, which degrade structural proteins in the basement membrane, contributed to the hemorrhage and necrosis observed in envenomated prey tissues. The venom also contained α-fibrinogenase activity, with levels similar to that seen in venoms from snakes in the genus *Philodryas* (Peichoto et al., 2007) and from many rattlesnakes (Mackessy, 1993a). By disrupting the blood clot cascade and inducing hypofibrinogenemia, fibrinogenases are likely responsible for much of the bleeding associated with colubrid snake bites.

Although some species of colubrid snakes have high levels of acetylcholinesterase in their venoms (e.g., Broaders and Ryan, 1997; Anderson and Dufton, 1998), *A. portoricensis* apparently does not. The low acetylcholinesterase activity was consistent with the low cholinesterase levels reported by Hegeman (1961). The combination of potent hemorrhagic, metalloproteinase, gelatinase and fibrinogenase activities (with low phosphodiesterase and acetylcholinesterase activities) could certainly explain the pain and inflammation associated with reported bites from *A. portoricensis*. By damaging structural proteins and interfering with the blood clot cascade, *Alsophis* venom is most likely used both to immobilize and to digest prey. The snake's relatively small size and hesitation to bite are the primary reasons it is not considered more of a hazard to humans.

One dimensional electrophoresis verified that a consistent venom product was obtained from *A. portoricensis* specimens and that little variation exists in the venom composition among individual snakes. This lack of variation has been noted among venoms of captive Brown Treesnakes (Mackessy et al., 2006) and among venoms sampled at 6 month intervals from wild Prairie Rattlesnakes (*Crotalus viridis viridis*) in Colorado (Mackessy, unpubl. data). When compared with several other colubrid species on 1D SDS-PAGE, *A. portoricensis* venom also appeared to have greater complexity in the higher molecular mass region. Only ~20 proteins were visible following 1D SDS-PAGE, but there were over 30 distinct proteins observed following 2D SDS-PAGE. As with most colubrid venoms (Mackessy, 2002), the venom proteome was less

complex than that of front-fanged viperids such as *C. ruber* (30 vs. ~100), and the region of greatest complexity appeared to be restricted to the 45–60 kDa acidic region of the gel. These proteins likely represent different isoforms of one or more phosphodiesterases, metalloproteinases and/or acetylcholinesterases in *A. portoricensis* venom, as indicated by SE-HPLC and SDS-PAGE of fractions.

The MALDI-TOF/TOF-MS-derived sequence of a tryptic peptide from the 25 kDa protein showed identity with an internal peptide of tigrin, a CRiSP isolated from the venom of a colubrid snake, *Rhabdophis tigrinus* (Yamazaki et al., 2002), confirming preliminary identification of this band. This family of proteins is a common component of most reptile venoms (Mackessy, 2002; Yamazaki and Morita, 2004; Peichoto et al., 2009). While the function of most CRiSPs is not fully understood and its biological role in venoms is unknown, its (near ubiquitous) presence in reptile venoms strongly suggests a common ancestry and a core importance to venom function. Three-finger toxins (often neurotoxins), commonly found in other colubrid venoms (Levinson et al., 1976; Broaders et al., 1999; Mackessy, 2002; Fry et al., 2003; Lumsden et al., 2005; Pawlak et al., 2006, 2009), were not observed in *A. portoricensis* venom following routine SDS-PAGE. However, very low intensity peaks in the 8–9 kDa mass range were seen following MALDI-TOF-MS, suggesting that if 3FTXs are present in *A. portoricensis* venoms, they are only minor constituents of the venom proteome.

While several venom components have close similarity with those from front-fanged snakes, the overall organization of the gland of rear-fanged snakes is much different, as has been noted previously (Taub, 1967; Zalisko and Kardong, 1992). An important functional difference is that the venom is not stored in a large basal lumen, as observed in viperids (Mackessy, 1991), with moderate amounts of venom in the lobule ducts and only small amounts of venom observed in secretory ductules. During a bite, or when the gland is stimulated with pilocarpine-HCl, venom proteins are exocytosed from these serous secretory cells and flow down the secretory tubules to a (presumed) central duct leading to the base of (typically 2) enlarged rear maxillary teeth. Venom is then introduced into the puncture wound(s). Envenomation therefore requires rapid exocytosis of intracellular venom followed by low-pressure delivery into prey tissues, resulting in the requirement of longer contact time for significant envenomation of larger animals such as humans.

Colubrid snake venoms are thus rich sources of potent biological compounds that, until recently, have been difficult to obtain in amounts sufficient for detailed analyses, particularly from small species. With the vast majority of colubrid venoms still unstudied, there is an opportunity to discover novel biological molecules with possible medical implications. Additionally, because of the often specialized diets (Greene, 1997) and complex phylogenetic history (Vidal et al., 2007; Zaher et al., 2009) of colubrids, understanding the composition and evolution of venoms from rear-fanged snakes is central to understanding the evolution of squamate venoms generally. We are currently isolating and characterizing the metalloproteinase and lower molecular mass peptide components of this venom.

Acknowledgements

Funding for this project was provided by a BioScience grant from the Colorado Office of Economic Development and International Trade. We thank Gad Perry and Robert Powell for providing the *Alsophis* specimens used for this research.

Conflict of interest

The authors declare that there are no conflicts of interest.

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