

Venom of the Brown Treesnake, *Boiga irregularis*: Ontogenetic shifts and taxa-specific toxicity

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Abstract

The Brown Treesnake (*Boiga irregularis*), a rear-fanged member of the polyphyletic family Colubridae, is an introduced predator on Guam which has been responsible for numerous human envenomations. Because little is known about this species' venom, we characterized venom proteins from *B. irregularis* using enzyme assays, one and 2D electrophoresis, Western blot analysis, mass spectrometry, HPLC and toxicity assays. Venom yields and protein content varied significantly with snake size, and large adult specimens averaged over 500 μ l venom (19.2 mg, protein content \sim 90%). Only two enzymes, azocaseinolytic metalloprotease and acetylcholinesterase, were detected in venoms, and both activities increased with snake size/age. Western blot analysis demonstrated a 25 kDa CRiSP homolog in venoms from both neonate and adult snakes. 2D electrophoresis showed variation between venoms from neonate and adult snakes, especially with respect to metalloprotease and acetylcholinesterase. Analysis by MALDI-TOF mass spectrometry revealed the presence of numerous proteins with molecular masses of \sim 8.5–11 kDa. Adult *B. irregularis* venom was quite toxic to domestic chickens (*Gallus domesticus*; 1.75 μ g/g) and lizards (*Hemidactylus* geckos: 2.5 μ g/g and *Carlia* skinks: 4.5 μ g/g), and intoxication was characterized by rapid paralysis of all species and neck droop in chickens. Toxicity of venom from neonates toward geckos was 1.1 μ g/g, consistent with the presence of a greater diversity of 8–11 kDa proteins (suspected neurotoxins) in these venoms. All of these values were notably lower than murine LD₅₀ values (neonate: 18 μ g/g; adult: 31 μ g/g). Like venoms of several front-fanged species, *B. irregularis* venom showed an ontogenetic shift in enzyme activities and toxicity, and neonate snakes produced more toxic venoms with lower protease and acetylcholinesterase activities. High toxicity toward non-mammalian prey demonstrated the presence of taxa-specific effects (and thus toxins) in *B. irregularis* venom, likely a characteristic of many colubrid snake venoms. We hypothesize that the lack of significant envenomation effects in humans following most colubrid bites results from this taxa-specific action of colubrid venom components, not from a lack of toxins.

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1. Introduction

The Brown Treesnake (*Boiga irregularis*) is an arboreal, nocturnal rear-fanged 'colubrid' snake indigenous to northern and eastern Australia, New Guinea and surrounding areas, and the Solomon Islands. *B. irregularis* was inadvertently introduced to the island of Guam sometime during the 1940s

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or early 1950s and has since been implicated in the decline and extirpation of indigenous bird, bat and lizard populations on the island (Savidge, 1987, 1991; Wiles, 1987; Fritts, 1988; Chiszar et al., 1988; Greene, 1989). Population densities of *B. irregularis* on Guam are very high and may have reached 50–100 snakes per hectare in some forest habitats, with suspected total numbers exceeding one million (Fritts, 1988; Rodda et al., 1999; Rodda, pers. comm.). Brown Treesnakes have a high potential to become established on other Pacific islands and they may already be established on Saipan; eight snakes have also been found on Hawaii (BTS Control Committee, 1996; Kraus and Cravalho, 2001). The diet of *B. irregularis* consists primarily of lizards, birds, mammals and bird eggs (Fritts, 1988; Greene, 1989). However, Savidge (1988) noted that juvenile and smaller Brown Treesnakes preyed almost exclusively upon ectothermic vertebrates such as lizards, while adult snakes also incorporated birds and small mammals into their diets, and a very recent observation on Guam confirms that ranid frogs will also be consumed (Brown Treesnake listserv, USGS, 2005). Such ontogenetic shifts from ectotherms to endotherms have been documented in many species of snakes (e.g. Mackessy, 1988; Mackessy et al., 2003) and these dietary changes are often accompanied by a shift in venom composition.

Although bites from some members of the genus *Boiga* are known to cause medically important symptoms in humans, these snakes generally pose much less risk to humans relative to sympatric elapid and viperid species. However, average adult *B. irregularis* are 1.4–1.5 m in length and occasionally reach 2.3 m (females) and 3.1 m (males) (Rodda et al., 1992), and because venom volume produced increases exponentially with snake length/mass (e.g. Mackessy, 1988, 2002; Mackessy et al., 2003), large adult snakes could produce quantities sufficient to cause serious human envenomation. *Boiga irregularis* is the only venomous snake on Guam, and there has been a rare opportunity to study the medical importance of this species. Bites from *B. irregularis* are becoming more common, and approximately 1 in 1200 emergency room visitors in Guam sought treatment from Brown Treesnake bites (Fritts et al., 1990). Clinical observations suggesting neurotoxicity, such as ptosis, poor muscle tone, lack of coordination, depressed heart rate and respiratory distress, and prominent swelling and discoloration beyond the bite wound, indicate that this venom can have systemic effects in humans (Fritts and McCoid, 1999; Fritts et al., 1994). The existence of neurotoxins in *B. irregularis* venom has been hypothesized (Weinstein et al., 1991; Weinstein et al., 1993; Mackessy, 2002; Fry et al., 2003; Lumsden et al., 2004a,b) but not proven conclusively. However, the unremarkable toxicity of venom from this species toward inbred mice ($LD_{50} = 10\text{--}80 \mu\text{g/g}$; Vest et al., 1991; Weinstein et al., 1991, 1993) has suggested otherwise.

The pharmacology, toxinology and biochemistry of venoms from many front-fanged snakes have been well documented (e.g. Menez, 2002), but much less is known about the venoms of rear-fanged ‘colubrids’ (see

Mackessy, 2002 for a review), which comprise over 700 species worldwide. Recent studies have begun to characterize the venom from several *Boiga* species, particularly venom of the Mangrove Snake, *Boiga dendrophila* (Broaders and Ryan, 1997; Broaders et al., 1999; Lumsden et al., 2004a,b; Lumsden et al., 2005). Venoms from several species of *Boiga*, including *B. irregularis*, showed inhibition of postsynaptic neuromuscular activity in chick biventer cervix muscle preparations, strongly indicating the presence of postsynaptic neurotoxins in the venoms (Lumsden et al., 2004). Venoms from other members of the genus *Boiga* have been shown to contain α -neurotoxin-like components (Levinson et al., 1976; Broaders et al., 1999). In the current study, we present analyses of Brown Treesnake venom and demonstrate that considerable component complexity exists. Age-related changes in venom composition and toxicity occur, reiterating a theme observed among several species of front-fanged snakes. Additionally, we show that pronounced tax-specific toxicity occurs in venom of this species, and avian and saurian species are 10–30 times more susceptible to *B. irregularis* venom than are inbred mice.

2. Materials and methods

2.1. Reagents

Buffers, enzyme substrates and other biochemicals (analytical grade or better) were purchased from Sigma Chemical Corp. (St Louis, MO, USA), and precast electrophoretic gels and Mark 12 molecular weight standards were obtained from Invitrogen, Inc. (San Diego, CA, USA). Protein concentration reagents were purchased from BioRad, Inc. (San Diego, CA, USA).

2.2. *B. irregularis* venom

Venom was extracted from Brown Treesnakes (*B. irregularis*) obtained from Guam (US Fish and Wildlife Service permit No. MA022452-0) and housed in the UNC Animal Facility in accordance with UNC-IACUC protocol No. 9204.1 and ASIH/SSAR guidelines. Additional samples were also extracted from large adult snakes from Guam in the care of Dr D. Chiszar (University of Colorado, Boulder, CO, USA) and from a snake originating from Indonesia (donated by T. Moisi). Extraction of snakes utilized a previously published method (Hill and Mackessy, 1997; Rosenberg et al., 1992). Briefly, snakes were anesthetized using a 20 mg/kg dose of the dissociative anesthetic ketamine-HCl. After a period of approximately 15 min, the snakes were injected with a 6 mg/kg dose of pilocarpine, and venom was collected into micropipettes. Snakes were extracted no more frequently than once every two months. Size classes were defined as neonates (<600 mm snout-vent length), juveniles (SVL = 650–1000 mm), adults (SVL = 1200–1500 mm)

and large adults (SVL > 1500 mm). Venoms from adult *B. dendrophila* (Sulawesi) and *B. cyanea* (locality unknown) were obtained using the same method.

2.3. Protein concentration determination

Protein concentration was assayed in triplicate according to Bradford (1976) as modified by BioRad Inc., using bovine gamma globulin as a standard. Enzyme specific activities and all other analyses were based on these protein concentrations.

2.4. Enzyme assays

Endoprotease activity was determined with azocasein (Aird and da Silva, 1991), and activity was expressed as $\Delta A_{342}/\text{min}$ per mg venom protein. Activity towards 4-nitroaniline-derived (pNA) synthetic substrates for thrombin (BzPheValArg-pNA), kallikrein (BzProPheArg-pNA), plasmin (D-ValLeuLys-pNA), trypsin (N- α -Bz-DL-Arg-pNA), elastase (SuccAlaAlaAla-pNA), collagenase (CbzGlyProLeuGlyPro-pNA), leucine aminopeptidase (L-Leu-pNA), dipeptidyl aminopeptidase (GlyPro-pNA) and arginine esterase (CBZ-L-Arg-pNA) activities were assayed according to Mackessy (1993). Phosphodiesterase activity was assayed by the method of Björk (1963) as modified by Mackessy (1988, 1998). Phospholipase activity was assayed using 4-nitro-3-(octanoyloxy) benzoic acid as a substrate (Holzer and Mackessy, 1996; method I) or egg yolk phosphatidylcholine Type IV (Sigma) (Wells and Hanahan, 1969; method II). L-amino acid oxidase activity was assayed according to Weisbach et al. (1961). Acetylcholinesterase activity was assayed according to Ellman et al. (1961) and activity was expressed as micromole product formed/min per milligram venom protein; venoms of *Naja melanoleuca* (Africa; locality unknown) and *Crotalus atrox* (SE Arizona, USA) were used as positive and negative controls, respectively. Endoprotease and acetylcholinesterase assays were also used to localize these enzymes following SE-HPLC (Section 2.8).

2.5. 1D gel electrophoresis

SDS-PAGE on Novex 14% acrylamide Tris–glycine gels (Invitrogen, Inc., USA) was used to determine the number and relative molecular masses of venom components; all solutions and reagents were prepared according to Hames (1990). Venom samples were prepared at a final concentration of 2.0 $\mu\text{g}/\mu\text{l}$ in 1 \times sample buffer (35 $\mu\text{g}/\text{lane}$) and were run under non-reduced or reduced (with 2-mercaptoethanol) conditions; standards (Invitrogen Mark 12) were run at 10 $\mu\text{l}/\text{lane}$. Invitrogen zymogram gels containing gelatin were used to determine if the venom (non-reduced; 5 $\mu\text{g}/\text{lane}$) had gelatin-degrading proteolytic activity and/or

endoprotease activity (Munekiyo and Mackessy, 1998). Gels were photographed using an Alpha Imager system.

2.6. Western blot analysis

Venom samples from large adult and neonate Brown Treesnakes were subjected to SDS-PAGE as above, but after electrophoresis, proteins were blotted to nitrocellulose membrane using a Novex Xi blot apparatus as described by the manufacturer (Invitrogen, Inc.). After transfer at 200 V for 1.5 h, the membrane was blocked with 3% bovine serum albumin in Tris buffered saline (TBS: 25 mM Tris pH 8.0 with 140 mM NaCl) for 2 h. The membrane was then incubated overnight at room temperature (with gentle agitation) in TBS containing 3% BSA and 5.0 μg lyophilized rabbit serum containing polyclonal anti-tigrin antibodies (Yamazaki et al., 2002; gift from Dr T. Morita); tigrin is a CRISP isolated from *Rhabdophis tigrinus* venom. Following three rinses with buffer, the membrane was incubated at room temperature for 2 h with goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma #A3687). The membrane was again rinsed with buffer and then developed for approximately 10 min in alkaline phosphatase substrate BCIP/NBT (Sigma No. B5655). Membranes were then dried and photographed using an Alpha Imager system.

2.7. 2D gel electrophoresis

Isoelectric focusing was performed on an Amersham Pharmacia IPGphor system according to the manufacturer's instructions. Briefly, we used 7 cm immobilized pH gradient (IPG) strips with a pH gradient of 3–10 (Amersham-Pharmacia). Lyophilized crude venom (175 μg) was dissolved in rehydration buffer (175 μl 8 M urea with 2% CHAPS, 0.5% IPG buffer, and 0.002% bromphenol blue to which DTT (final concentration 18.15 mM) was added immediately prior to use) and pipetted into the IPG focusing tray. The dehydrated IPG strips were placed into the trays, covered with a thin layer of mineral oil, and rehydrated on the IPGphor system for 15 h at 18 °C. Strips were then focused for 3 h with a voltage gradient (maximum of 5000 V, 50 $\mu\text{A}/\text{strip}$). After focusing, strips were removed from trays and equilibrated for 10 min first in SDS/Tris sample buffer containing 65 mM DTT (10 mg/ml) and then for 10 min with buffer (no DTT) containing 135 mM IAA (25 mg/ml) in order to ensure that the proteins were fully reduced and alkylated. SDS-PAGE was then performed according to Hames (1990) using Invitrogen ZOOM precast gels and Mark 12 standards. Gels were stained with Coomassie Brilliant Blue, destained and imaged as above.

2.8. Size exclusion HPLC

Samples of venoms from adult, juvenile and neonate snakes were dissolved in 100 mM HEPES buffer containing

100 mM NaCl and 5.0 mM CaCl₂ (pH 6.8). Two hundred microliters (250 µg) of each venom was injected onto a TosoHaas TSK G2000 SWXL size exclusion column (10 × 300 mm) at a flow rate of 0.3 ml/min using the same buffer (Waters HPLC), and chromatograms were recorded using Empower software.

2.9. MALDI-TOF mass spectrometry

Approximately 0.5 µg crude *B. irregularis* venom (neonate and adult-Guam; adult-Indonesia) was spotted onto 0.5 µl sinapinic acid matrix and the samples were subjected to matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis using an ABI Voyager DE-Pro mass spectrometer in linear mode. Samples were analyzed using a window of 5–15 kDa.

2.10. Toxicity assays

Lethal toxicity of crude venom was evaluated using house geckos (*Hemidactylus frenatus*) obtained from Bushmaster Reptiles (Longmont, CO, USA), curious skinks (*Carlia aylanpalai*) collected on Guam, chicks (*Gallus domesticus*) obtained from a local breeder and NSA mice (*Mus musculus*) bred in the UNC Animal Facility. All doses were delivered intraperitoneally in sterile saline, and doses were adjusted to individual animal body masses. Three animals per dose were utilized, and all animals were monitored for 24 h. Lethality was expressed as microgram venom per gram body mass producing 50% mortality after 24 h (Reed and Muench, 1938). All procedures were evaluated and approved by the UNC IACUC (protocol No. 9401).

3. Results

3.1. Venom yields

As with other colubrid and viperid snakes (e.g. Huang and Mackessy, 2004; Mackessy et al., 2003), venom yields from *B. irregularis* showed an exponential relationship with snake length (Fig. 1A and B). Venom mass and snake mass also showed an exponential relationship ($r^2=0.83$; data not shown). Average large adult venom yield was 518 µl (Table 1), and the largest single yield was over 950 µl. As seen in other colubrid snake venoms, solids content of *B. irregularis* venom was low (35.7 mg/ml; Fig. 1C), and mean protein content (w/w) was 90% for venom from large adult snakes. Approximate solids content of several species of rattlesnakes is much higher (225–250 mg/ml), but mean protein content is similar (90–92%; Mackessy, unpubl. obs.). Protein content also varied ontogenetically, and venoms from neonates averaged only 48% protein (Table 1). A subset of adult snakes ($n=5$) were extracted first without pilocarpine

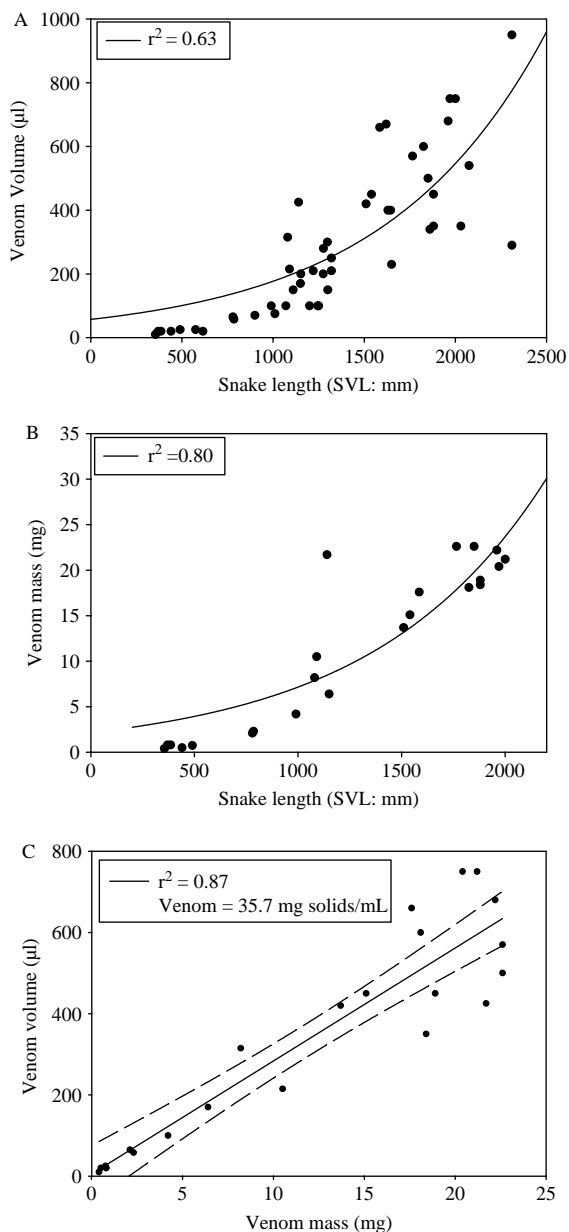


Fig. 1. Venom yields of Brown Treesnakes (*Boiga irregularis*). Both venom volume (A) and mass (B) show an exponential increase with snake length, and venom volume and mass are strongly correlated (C), indicating that the use of ketamine/pilocarpine to induce secretion produces a consistent product. Dashed lines in C are 95% confidence intervals.

(ketamine only) and then 30 min later after administration of pilocarpine as above (6 µg/g). Yields without pilocarpine were 14.8 ± 7.9 µl (range 5–27 µl); after pilocarpine, yields were 250 ± 136.3 µl (range 100–450), demonstrating the utility of pilocarpine stimulation.

Table 1

Venom yields for four size classes of *Boiga irregularis*: venom volume, mass and percent protein. All values are mean values \pm standard deviation

Size class (N)	Snout-Vent length (mm)	Snake mass (g)	Venom volume (μ l)	Venom mass (mg)	Percent protein
Neonate (9)	461.4 \pm 102.7	16.0 \pm .5	20.6 \pm 5.0	0.7 \pm 0.19	47.5 \pm 5.7
Juvenile (6)	909.0 \pm 130.6	102.7 \pm 45.4	92.2 \pm 47.7	2.2 \pm 0.14	74.9 \pm 3.2
Adult (17)	1200.9 \pm 97.4	284.6 \pm 67.4	204.4 \pm 89.7	10.2 \pm 6.8	80.5 \pm 8.1
Large adult (20)	1844.8 \pm 232.8	1166.7 \pm 319.2	517.5 \pm 184.1	19.2 \pm 3.0	90.2 \pm 15.4

3.2. Enzyme assays

Enzyme assays revealed only azocaseinolytic (metalloprotease) and acetylcholinesterase enzyme activities. For metalloprotease activity (Fig. 2), there was a trend toward increasing activity with increasing snake SVL (Fig. 2; $r^2 = 0.56$), and a significant difference between venoms from neonate/juvenile and adult snakes was observed ($p < 0.05$). Acetylcholinesterase activity followed the same trend, with venom from larger snakes showing approximately 2 \times higher activity (Fig. 3). Highest levels were approximately 1/4 that observed with the positive control (*N. melanoleuca* venom). For all size classes, no activity toward synthetic substrates for thrombin, kallikrein, trypsin, elastase, collagenase, leucine aminopeptidase, dipeptidyl aminopeptidase, PLA₂, phosphodiesterase or L-amino acid oxidase was detected.

3.3. 1D gel electrophoresis

Non-reducing SDS-PAGE of crude venoms revealed an average of 12 major and 6 minor bands, with molecular masses ranging from \sim 7–200 kDa (Fig. 4A and B). No major differences were seen between the venoms from neonate, juvenile and adult snakes, but bands at 50–60 kDa were less intense in venoms from neonates, and low molecular mass bands (8–10 kDa) were somewhat less intense in venoms from large adults

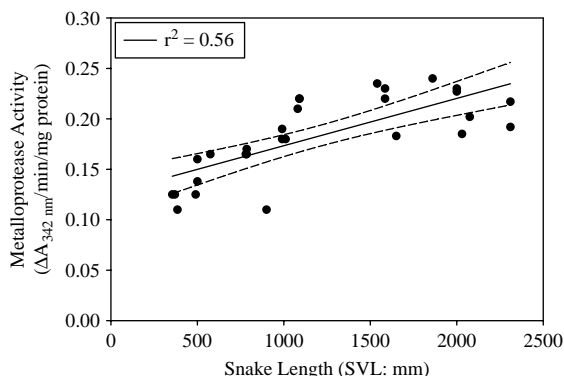


Fig. 2. Caseinolytic metalloprotease activity as a function of snake length. Activity tends to increase with length. Dashed lines are 95% confidence intervals.

(Fig. 4B). Several differences from *B. dendrophila* and *B. cyanea* were apparent: molecular masses of bands in the 50–53 and 17–20 kDa ranges were slightly higher for both of these species relative to *B. irregularis*, as was a band in the 25–28 kDa range for *B. dendrophila* (Fig. 4A). No protease activity was observed on zymogram gels (data not shown) for any of these *Boiga* venoms.

3.4. Western blot analysis

Probes of blotted venoms with anti-tigrin serum identified a single band in venoms from both neonate and adult *B. irregularis* (Fig. 5), indicating the presence of a CRISP homolog (Yamazaki and Morita, 2004). The approximate molecular mass of this protein was 25 kDa.

3.5. 2D gel electrophoresis

Approximately 40–45 protein spots were visualized following staining of 2D gels with Coomassie Brilliant Blue (Fig. 6A and B). The main differences between venoms from neonates and adults were seen in the higher molecular mass acidic region of the gels. Spots were more intense in the adult venom sample, and based on preliminary size exclusion HPLC experiments (see Section 3.7), they corresponded to acetylcholinesterases (approximately reduced molecular mass of 56 kDa) and metalloproteases (approximately reduced molecular mass of 49 kDa). Venoms from both neonates and adults showed numerous intense spots in the lower molecular mass region (8–14 kDa), with multiple \sim 8 kDa species which ranged from moderately acidic to highly basic.

3.6. MALDI-TOF mass spectrometry

Mass spectrometry confirmed the presence of numerous peptides in the 8–10 kDa range (Fig. 7). Peptides between samples were considered homologous if the mass differed by less than 0.3%. Comparison of venom samples from neonate and adult snakes from Guam and an adult snake from Indonesia, using an analysis window of 5–15 kDa, indicated at least 23 total peptides in this mass range, at least 6 of which appeared to be present in the Indonesian snake venom only (Table 2). A greater number of peptides (16)

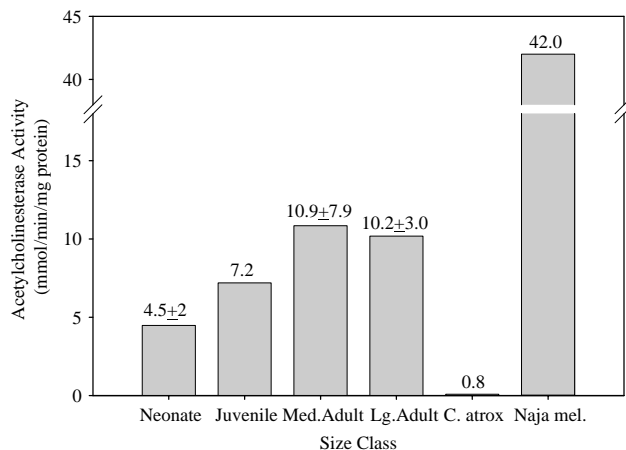


Fig. 3. Acetylcholinesterase activity as a function of snake size class. Activity is lowest in neonate snake venoms and highest in adult venoms. Venoms from *Crotalus atrox* and *Naja melanoleuca* were included as negative and positive controls, respectively. Mean values \pm SE are given above histograms.

were observed in the Indonesian sample, including two of approximate masses of 6.6 and 6.8 kDa, and only five peptides were present in all three samples. Some differences in peptide distribution in venoms from neonate and adult snakes were also apparent, with a total of 13 peptides in neonate venom and 9 in adult venom. A prominent peak at 25.4 kDa was also observed in all venoms (data not shown), likely corresponding to the CRiSP band observed following Western blot analysis.

3.7. Size exclusion HPLC

Size exclusion chromatography resolved 8–10 protein peaks, and detected enzyme activity (acetylcholinesterase,

metalloprotease) was limited to peak I (Fig. 8). Based on 1D SDS-PAGE, a 25 kDa CRiSP homolog and a 17 kDa protein were present in all chromatograms, and numerous 8–10 kDa proteins were present in peaks eluting between 35 and 52 min. Chromatograms from the three age classes were similar, but greater variation was seen in low molecular mass peaks in venom from neonates (Fig. 8C).

3.8. Lethal toxicity

In contrast to effects on inbred mice, crude venom was quite toxic to lizards and birds (Table 3). Chicks were most sensitive to venom, followed by *Hemidactylus* geckos and then *Carlia* skinks. It should be noted that on Guam, all of

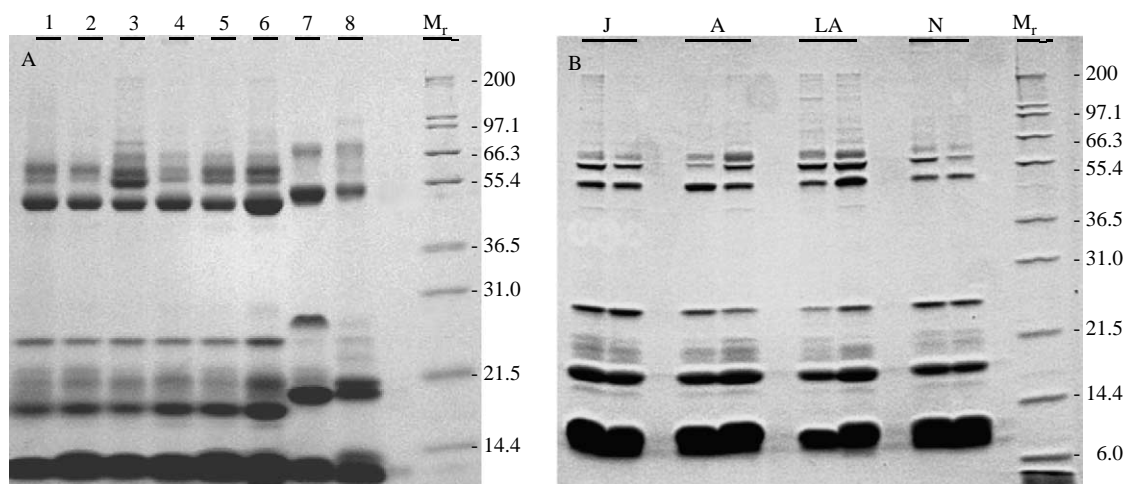


Fig. 4. One dimensional non-reducing SDS-PAGE analysis of venoms. (A) Comparison of six adult *B. irregularis* samples (lanes 1–6) with venoms from adult *B. dendrophila* (7) and *B. cyanea* (8). Note that patterns of individual *B. irregularis* venoms are quite similar. (B) Comparison of venoms of several size classes of *B. irregularis*. J, juvenile; A, adult; LA, large adult; N, neonate; M_r , molecular mass standards (in kiloDalton).

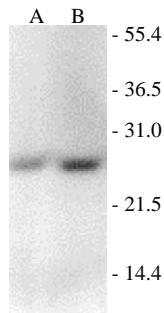


Fig. 5. Presence of a CRiSP homolog in *B. irregularis* venom. Anti-tigrin antibodies detected a single band of approximately 25 kDa in blotted crude venoms from neonate (A) and adult (B) snakes.

these species are typical prey species, but only the *Carlia* were actually from Guam. In lizards and chicks, rapid paralysis occurred following venom administration, and neck droop was observed in all chicks receiving lethal doses. LD₅₀ values in NSA mice were much higher (neonate:

18 µg/g; adult: 31 µg/g) and were similar to previously published values for Swiss-Webster mice (LD₅₀ = 10–80 µg/g; Vest et al., 1991; Weinstein et al., 1991). Venom from neonates was approximately twice as toxic to *Hemidactylus* geckos and to NSA mice as venom from adults, demonstrating that ontogenetic differences in venom toxicity are present in *B. irregularis*.

4. Discussion

As shown in previous studies, large venom yields from rear-fanged snakes such as *B. irregularis* can be obtained using ketamine and pilocarpine. Average yields of Brown Treesnake venom without ketamine/pilocarpine were approximately 80 µl (Weinstein et al., 1991) compared with 370 µl from treated Brown Treesnakes (Hill and Mackessy, 1997, 2000). In the present study, yields from the largest adult snakes averaged over 500 µl (Table 1), and as demonstrated by a subset of adult snakes extracted without and with pilocarpine, yields from pilocarpine-induced snakes were much higher. Although a larger percentage of water is seen in the venom collected from pilocarpine-treated snakes, a threefold increase in overall dry matter yield was also obtained (Chiszar et al., 1992). Large *B. irregularis* are therefore capable of delivering 20–25 mg venom during an extended bite.

The only venom enzymes detected in this study were metalloprotease and acetylcholinesterase activities. Metalloprotease activity tended to increase with age/size, but highest levels were only about half those seen in many rattlesnake venoms (e.g. Bjarnason and Fox, 1994; Munekiyo and Mackessy, 1998; Mackessy, unpubl. obs.). Vest et al. (1991) showed *B. irregularis* venom to contain very low kallikrein-like, L-amino acid oxidase, and phosphodiesterase activities, but these results were not supported by the current study. Broaders and Ryan (1997) demonstrated phospholipase A₂ activity in *B. blandingi* and *dendrophila*, but two different assays for PLA₂ failed to show activity in *B. irregularis* venom. Like the venoms of *B. blandingi* and *B. dendrophila* (Broaders and Ryan, 1997), *B. irregularis* venoms contain acetylcholinesterase, although maximum activity of *B. irregularis* venom (~12 µmol/min per mg) was considerably lower than that reported for *B. dendrophila* (122 µmol/min per mg). Additionally, Broaders and Ryan (1997) noted that acetylcholinesterase in crude venom was unstable in aqueous solution and lost approximately one-half its activity in 1 h. When water-solubilized *B. irregularis* crude venom was assayed one year after the initial investigation (stored frozen at –20 °C), there was virtually no difference in acetylcholinesterase activity levels. This stabilization of activity is similar to that seen in Blacktail Rattlesnake (*Crotalus m. molossus*) venom for other enzymes (Munekiyo and Mackessy, 1998) and suggests that endogenous inhibitors/stabilizers may also be present in *B. irregularis* venom.

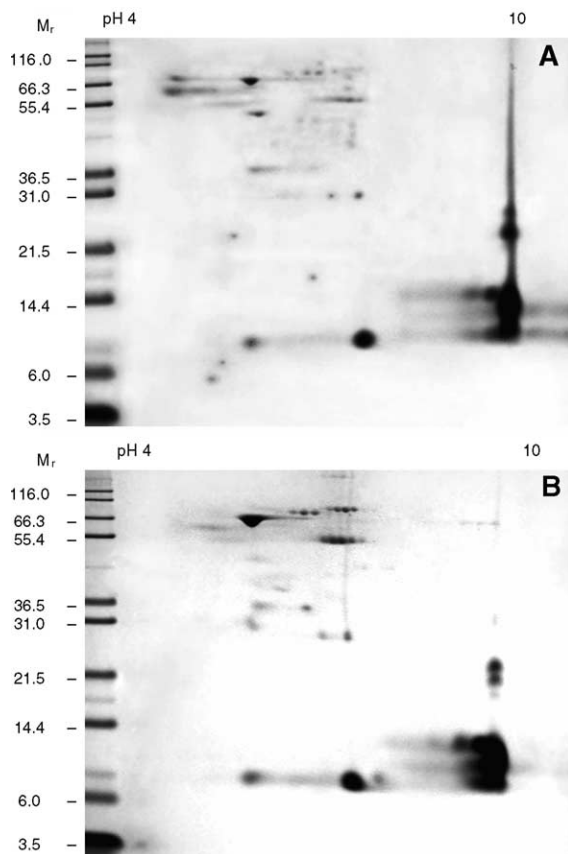


Fig. 6. 2D electrophoretic comparison of neonate (A) and adult (B) *B. irregularis* venoms; 175 µg venom was used for each gel. Many more individual proteins are visible (40–45) than in 1D SDS-PAGE, but note that overall patterns are similar in both. Approximate pH gradient is given at the top of each gel.

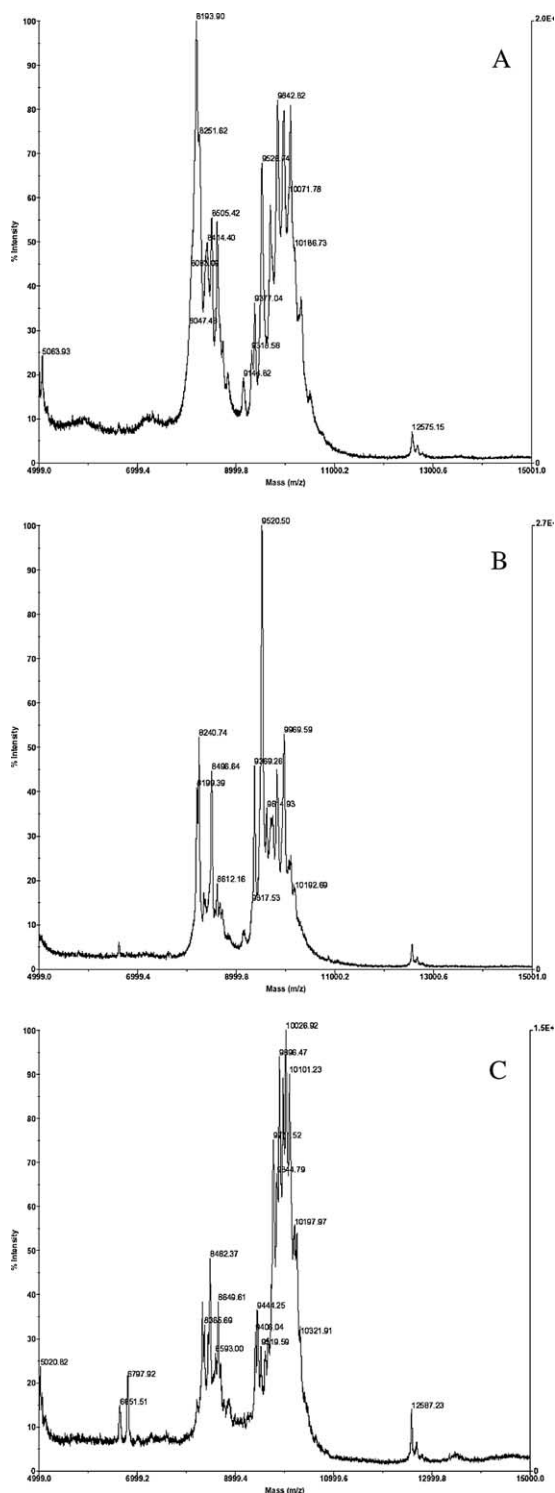


Fig. 7. MALDI-TOF mass spectra of neonate (A) and adult (B) venom samples of *B. irregularis* from Guam. An adult venom sample from an Indonesian *B. irregularis* (C) is shown for comparison. Note the abundance of peptides with masses of 8198–10,312.

Acetylcholinesterase enzymes from *Naja* and *Bungarus* typically are homodimers of 65–70 kDa subunits, while acetylcholinesterase monomer from the shore pit viper (*Trimeresurus purpureomaculatus*) was 58.6 kDa (Tan and Tan, 1987); purified AChE may show several isoforms (Raba et al., 1979; Grossman et al., 1979). In *B. irregularis*, partially purified acetylcholinesterase (data not shown) has a monomer mass of ~65 kDa and also appears to have several isoforms. We are currently characterizing this enzyme from *B. irregularis* venom.

2D electrophoresis revealed greater complexity in the venoms of *B. irregularis* than was previously observed, and a comparison of neonate and adult venoms revealed both qualitative and quantitative differences. At present, most components are not identified, but increased intensity of spots in the acidic 49–66 kDa range are consistent with higher levels of acetylcholinesterase and metalloprotease activities in adult venoms. Western blot analysis using polyclonal antibodies to the colubrid venom CRiSP tigrin demonstrated that a homolog was present in *B. irregularis* venoms. This class of proteins appears to be very broadly distributed among reptile venoms (Yamazaki and Morita, 2004), suggesting that they are functionally important components, but most venom CRiSPs have unknown activities. It is clear from this and other studies (e.g., Yamazaki et al., 2002; Huang and Mackessy, 2004; Fry et al., 2003; Lumsden et al., 2004a,b, 2005) that many (if not most) ‘colubrids’ possess venom protein components homologous with those in the venoms of front-fanged snakes.

Based on SDS-PAGE and MALDI-TOF-MS, there is an abundance of peptides in the 8–11 kD range in the venoms of *B. irregularis*. Venoms from other members of the genus *Boiga* have been shown to contain components with α -neurotoxin-like activity (Broaders and Ryan, 1997; Lumsden et al., 2004a,b, 2005), and several colubrid ‘three-finger’ neurotoxins have been isolated recently (Fry et al., 2003; Lumsden et al., 2005). These toxins have molecular masses of 8498 Da (colubritoxin; Fry et al., 2003) and 8769 Da (boigatoxin-A; Lumsden et al., 2005), generally larger than the well-characterized α -neurotoxins (such as α -bungarotoxin) from elapid snakes. *B. irregularis* venoms also contained four peptides with masses of 8.4–8.7 kDa (see Table 2). Because both human envenomation symptoms and animal toxicity data indicate neurotoxicity, we believe that these *B. irregularis* venom peptides are likely neurotoxin homologs of colubritoxin and boigatoxin-A. We are currently purifying and characterizing *B. irregularis* components in order to identify the precise nature of these abundant low molecular mass compounds.

Ontogenetic differences in venom composition are present in *B. irregularis*, and like some crotaline snakes (e.g. Mackessy, 1988), venoms from neonate snakes are more toxic to lizards and inbred mice than adult venoms. Higher toxicity of neonate venom is consistent with the presence of a greater diversity of 8.5–11 kDa proteins

Table 2
Masses of peptides (5–15 kDa) from *Boiga irregularis* venoms as determined by MALDI-TOF-MS

Peptide	Neonate-Guam	Adult-Guam	Adult-Indonesia
1	–	–	6651.5
2	–	–	6797.9
3	8047.5	–	–
4	8083.1	–	–
5	8193.9	8199.4	–
6	8251.6	8240.7	8365.7
7	8414.4	–	–
8	8505.4	8496.6	8482.4
9	–	8612.2	8593.0
10	–	–	8649.6
11	9144.8	–	–
12	9318.6	9317.5	–
13	9377.0	9369.3	9406.0
14	–	–	9444.3
15	9526.7	9520.5	9519.6
16	–	–	9771.5
17	9842.8	–	9844.8
18	–	–	9896.5
19	–	9969.6	–
20	–	–	10026.9
21	10071.8	–	10101.2
22	10186.7	10192.7	10197.9
23	–	–	10321.9

(suspected neurotoxins) in these venoms, as indicated by MALDI-TOF-MS and 2D gel analyses. Also like venoms of some crotalines, enzyme activities (metalloprotease and acetylcholinesterase) were higher in adult venoms. In several species of rattlesnakes, this shift in activity is associated with a decrease in venom toxicity and a reliance on larger prey by adults (Mackessy, 1988; Mackessy et al., 2003), and there may be a similar functional significance to these changes in *B. irregularis* venom. Neonate *B. irregularis* are dependent on lizard prey, particularly *Hemidactylus* geckos, but as they grow larger, they include birds and mammals as prey. Ontogenetic differences in venom composition, which produce greater effects on specific prey, therefore appear to be a general characteristic of many species of several families of snakes.

The large difference in susceptibility of lizards and birds to *B. irregularis* venom, relative to effects on mice, strongly indicates the presence of taxa-specific toxins. An analogous condition of toxins with specific potent effects toward one class of animal, with no or little effect toward a different class, is seen among some invertebrate venoms. For example, multiple high molecular mass neurotoxins (latrotoxins; ~120 kDa) have been well-characterized in black widow spider venoms (e.g. Grishin, 1999), and these show profound taxa-specific toxicity. Latroinsectotoxins are very potent against insects, latrocrustotoxins are toxic to crustaceans and α -latrotoxins affect primarily vertebrates. It appears likely that multiple variants of much smaller, similar-mass toxins (approximately 6–12 kD) in snake

venoms may also follow this pattern, and we predict that some of the low molecular mass proteins in *B. irregularis* venoms will show such differential effects. We hypothesize that the lack of significant envenomation effects in humans following most colubrid bites, including most bites by *B. irregularis*, results primarily from taxa-specific action of venom components toward non-mammalian prey, not from a lack of toxins.

Venom glands and a delivery apparatus are a synapomorphy among the Colubroidea (Vidal and Hedges, 2002, 2005), but for rear-fanged ‘colubrid’ snakes, the specific function/role of venoms and their components is less clearly defined. If *B. irregularis* are surgically deprived of venom, they will kill mouse prey by constriction just as rapidly as when the venom is available (Rochelle and Kardong, 1993), suggesting that venom is not necessary to overcome small mammalian prey. In our lab, we have observed that *B. irregularis* often constrict and hold mouse prey until it is quiescent (up to several minutes) before beginning the process of ingestion, while lizards are held in the jaws only until quiescent. In the wild, a combination of constriction and venom effects may help overcome fractious prey, and venom is differentially more effective toward specific prey types such as lizards and birds. Both *Hemidactylus* and *Carlia* were unintentionally introduced onto Guam, and with the demise of Guam’s avifauna, they are now important prey for *B. irregularis*, particularly for smaller snakes (Rodda, pers. comm.).

The present study demonstrates that the inbred mouse model is of marginal utility for understanding the biological roles of venoms from snakes that feed largely on non-mammalian prey. Differences are present even among the more susceptible taxa, and LD₅₀ values for adult venoms in *Carlia* skinks were nearly twice as high as for *Hemidactylus* geckos. Further, taxa-specific high toxicity of venoms strongly suggests *against* a neutral selection model for venom evolution in general, as has been suggested by several authors (Mebs, 2001; Sasa, 1999). Instead, it appears likely that the evolution of resistance mechanisms by prey is countered by a venom evolutionary response of the snakes, and this coevolutionary arms race (Heatwole et al., 2000; Mackessy et al., 2003) may be an important contributor to the observed high levels of venom composition complexity (e.g. Fox and Serrano, 2005; Serrano et al., 2005). Because rear-fanged colubrid snakes encompass a broader range of prey taxa in their diets than do front-fanged snakes (Greene, 1997), understanding the diversity of colubrid venom components and their differential effects toward specific prey will greatly facilitate an understanding of the selective mechanisms driving snake venom evolution. Although risks to humans following bites by most venomous ‘colubrid’ snakes appear to be minimal, this is not true for at least some native prey species (likely most). Colubrid snake venoms represent a distinct

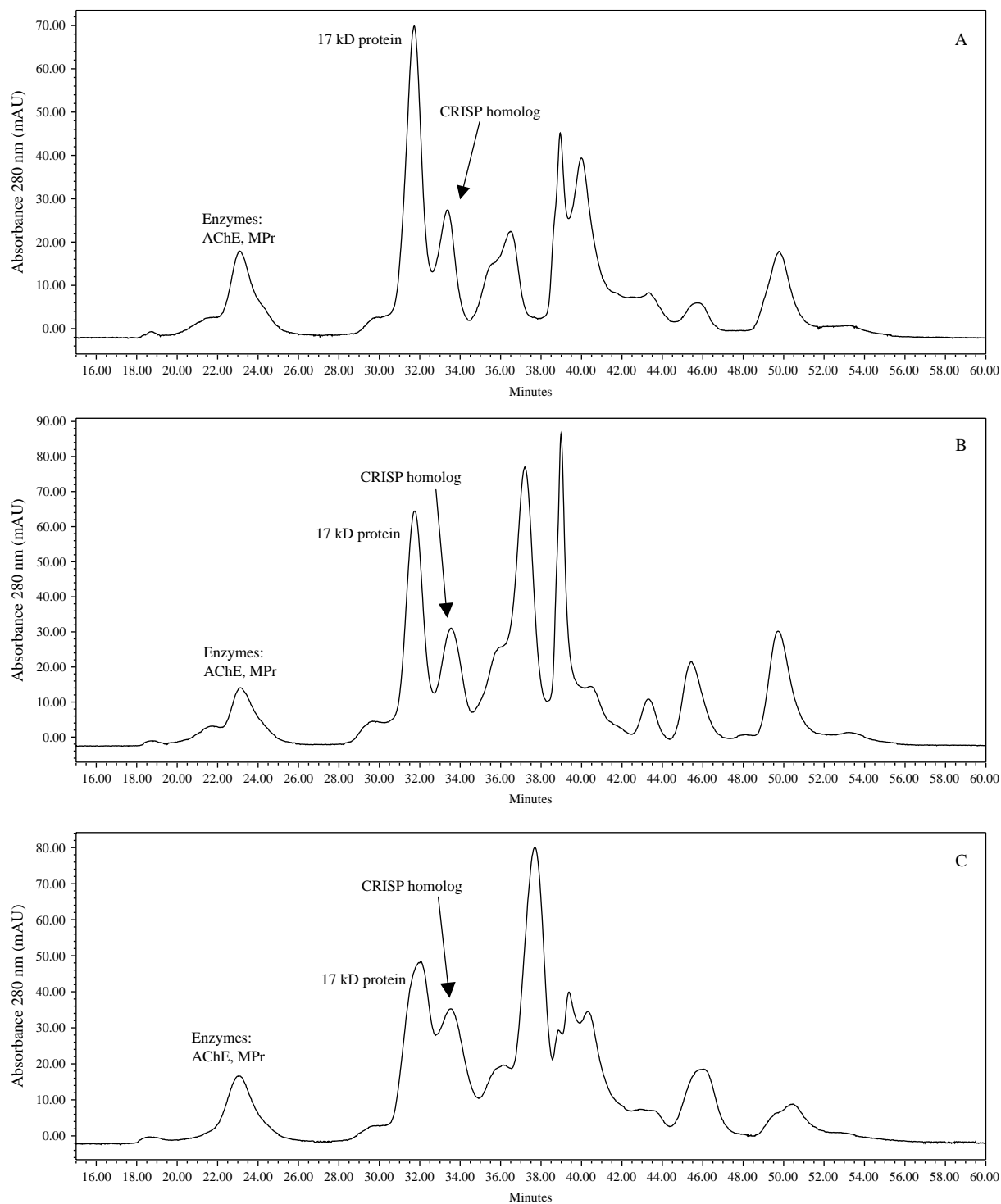


Fig. 8. Size exclusion HPLC separation of crude *B. irregularis* venom. Samples of 250 μg of each venom were fractionated, and components of several peaks were identified via enzyme assays and SDS-PAGE analysis of fractions. All proteins eluting between 35 and 52 min showed apparent molecular masses of 8–10 kDa; note that the greatest variation in chromatograms occurred in this region. A, adult; B, juvenile; C, neonate venom.

Table 3
Lethal toxicity (24 h LD₅₀) of neonate and adult Brown Treesnake (*B. irregularis*) venom toward several species of vertebrates

Animal	Intraperitoneal venom LD ₅₀ (µg/g body mass)	
	Neonate	Adult
Chicken (<i>Gallus domesticus</i>)	–	1.75
House Gecko (<i>Hemidactylus frenatus</i>)	1.1	2.5
Curious skink (<i>Carlia ailanpalai</i>)	–	4.75
NSA mouse (<i>Mus musculus</i>)	18.0	31.0

trajectory in the evolution of venom systems, and there is a vast pharmacological potential in these venoms.

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