PERGAMON

Toxicon 38 (2000) 1663–1687

TOXICON

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Characterization of venom (Duvernoy's secretion) from twelve species of colubrid snakes and partial sequence of four venom proteins

Robert E. Hill, Stephen P. Mackessy*

Department of Biological Sciences, 501 20th St., University of Northern Colorado, Greeley, CO 80639-0017, USA

Received 25 June 1999; accepted 22 December 1999

Abstract

R.E. Hill and S.P. Mackessy. Characterization of venom (Duvernoy's secretion) from twelve species of colubrid snakes and partial sequence of four venom proteins. Toxicon **XX**, xx–yy, 2000. — Venomous colubrids, which include more than 700 snake species worldwide, represent a vast potential source of novel biological compounds. The present study characterized venom (Duvernoy's gland secretion) collected from twelve species of opisthoglyphous (rear-fanged) colubrid snakes, an extremely diverse assemblage of non-venomous to highly venomous snakes. Most venoms displayed proteolytic activity (casein), though activity levels varied considerably. Low phosphodiesterase activity was detected in several venoms (*Amphiesma stolata, Diadophis punctatus, Heterodon nasicus kennerlyi, H. n. nasicus* and *Thamnophis elegans vagrans*), and acetylcholinesterase was found in *Boiga irregularis* saliva and venom, but no venoms displayed hyaluronidase, thrombin-like or kallikrein-like activities. High phospholipase A_2 (PLA_2) activity was found in *Trimorphodon biscutatus lambda* venom, and moderate levels were detected in *Boiga dendrophila* and *D. p. regalis* venoms as well as *B. dendrophila* and *H. n. nasicus* salivas. Non-reducing SDS–PAGE revealed 7–20 protein bands (3.5 to over 200 kD, depending on species) for all venoms analyzed, and electrophoretic profiles of venoms were typically quite distinct from saliva profiles. Components from *A. stolata, Hydrodynastes gigas, Tantilla nigriceps* and *T. e. vagrans* venoms showed protease activity when run on gelatin zymogram gels. N-terminal

* Corresponding author. Tel.: +1-970-351-2429; fax: +1-970-351-2335.
E-mail address: spmacke@bentley.unco.edu (S.P. Mackessy).

PII: S0041-0101(00)00091-X

protein sequences for three 26 kD venom components of three species (*H. gigas, H. torquata, T. biscutatus*) and one 3.5 kD component (*T. nigriceps*) were also obtained, and the 3.5 kD peptide showed apparent sequence homology with human vascular endothelial growth factor; these data represent the first sequences of colubrid venom components. Protease, phosphodiesterase and PLA_2 activities are also common to elapid and viperid snake venoms, but it is apparent that numerous other (as yet undescribed) components make up the majority of colubrid venom proteins. The complex nature of venoms produced by most species surveyed, and the high levels of protease or phospholipase A_2 activity of some venoms, suggest that many colubrids could become an important source of human health concern as encounters with these snakes increase.

1. Introduction

The composition of toxic oral secretions (venoms) from snakes of the family Colubridae are largely unknown, even though this exceptionally diverse family contains well over half of the described extant species of snakes, and perhaps half of these produce toxic secretions from the Duvernoy's gland (Gans, 1978; Underwood, 1979; Minton, 1990, 1996). A vast body of literature, including several recent reviews (see Kini, 1997; Bailey, 1998), exists on venoms from proteroglyphous (family Elapidae) and solenoglyphous (family Viperidae) snakes (here collectively termed front-fanged snakes). In contrast, the venoms of opisthoglyphous (rear-fanged) colubrids are rarely investigated, and no protein sequence data have been reported. A major impediment to the study of colubrid venoms has been the (typically) low venom yields of most species and the time-intensive collection techniques required to obtain venom.

The heterogeneous assemblage of advanced snakes currently referred to the family Colubridae are generally considered non-venomous, but there have been several cases of fatal human envenomations by *Dispholidus typus* (boomslang; Pope, 1958), *Thelotornis capensis* (formerly *T. kirtlandii*; twig or bird snake; FitzSimons and Smith, 1958) and *Rhabdophis tigrinus* (yamakagashi; Mittleman and Goris, 1976; Ogawa and Sawai, 1986; Nomura et al., 1989); the South American colubrid *Philodryas olfersii* has also been implicated in cases of severe and fatal envenomations (Salomão and di-Bernardo, 1995; de Araujo and dos Santos, 1997). The high potency and complex nature of these venoms suggest that colubrid venoms may contain compounds with some similarities to front-fanged snakes' venoms, but because the elapids and viperids are distantly related to the family Colubridae, similar activities in colubrid venoms are likely to show novel structural and/or specificity motifs.

Other species of rear-fanged colubrids have not caused human deaths but produce venoms with some characteristics similar to front-fanged snakes; examples include *Hydrodynastes gigas* (i.p. LD_{50} of 2.0 mg/kg; Glenn et al., 1992) and *Boiga irregularis* (i. v. LD_{50} of 10–80 mg/kg; Vest et al., 1991;

Weinstein et al., 1991, 1993). The former species is of concern due to common occurrence in the pet trade and a recent severe envenomation (Manning et al., 1999), and human envenomations by the latter are increasing due to increasing abundance of this snake on Guam; over half of 94 envenomation cases during a 2-year period on Guam involved infants (Fritts et al., 1994). Many other colubrids have also been implicated in human envenomations with less severe results (Cowles, 1941; Taub, 1967; McKinstry, 1978; Fuller, 1981; Vest, 1981a; Morris, 1985; Vest, 1988; Minton, 1990, 1996; Ribeiro et al., 1999).

Colubrid venoms that have been studied to date appear to lack a number of enzymatic properties that are characteristic of most front-fanged snake venoms (Weinstein and Kardong, 1994). Phospholipase A_2, hyaluronidase and L-amino acid oxidase have been found in most elapid and viperid venoms analyzed (for reviews see Tu, 1977; Lee, 1979; Rosenberg, 1990; Kini, 1997; Bailey, 1998); however, most colubrid venoms apparently lack these activities (but see Mebs, 1968; Durkin et al., 1981; Vest et al., 1991; Broaders and Ryan, 1997). Two properties common to colubrid and front-fanged snake venoms are hemorrhagic and caseinolytic protease activities, and these activities are widely distributed among colubrids (Grasset and Schaafsma, 1940; Robertson and Delpierre, 1969; Kornalik et al., 1978; Hiestand and Hiestand, 1979; Vest, 1981b; Sakai et al., 1983; Vest, 1988; Assakura et al., 1992, 1994; Glenn et al., 1992; Weinstein and Smith, 1993; Weinstein and Kardong, 1994). Kallikrein-like protease activity has been reported only in *B. irregularis* venom (Vest et al., 1991). Differences between colubrid and front-fanged snake venoms are due in part to divergent evolutionary and life histories.

Snakes contain numerous salivary and other glands lining the buccal cavity which condition prey and assist in swallowing, and the Duvernoy's and venom glands are the main cephalic glands producing protein-rich serous secretions which facilitate prey capture by inducing prey quiescence/death and/or initiating digestion. There has been some discussion of the biological role of colubrid venoms relative to the venoms of the front-fanged snakes (Kardong, 1980, 1982; Rodríguez-Robles and Thomas, 1992; Rodríguez-Robles, 1994; Kardong, 1996), but a lack of sufficient information on colubrid venoms has made it difficult to address this question.

The objectives of this study were to examine the composition of venoms from a diversity of species of opisthoglyphous colubrids and to compare colubrid venoms with the much better characterized venoms of elapid and viperid (front-fanged) snakes. Venoms from 12 colubrid species were analyzed using enzyme assays, electrophoretic protease assays and molecular fingerprinting via gel electrophoresis (SDS–PAGE), and four colubrid venom components were partially N-terminally sequenced. It was predicted that due to similar roles of venoms for snakes, colubrid venoms would have some components in common with front-fanged snake venoms, but because of differences in prey bases and in phylogeny, novel components would also be present.

2. Materials and methods

2.1. Reagents

Reagents (analytical grade or better) were purchased from Sigma Biochemical Corp., USA, and precast electrophoretic gels and molecular weight standards (Mark 12) were acquired from Novex Inc., USA. PVDF membrane (Immobilon P) for protein blots was obtained from Waters Corporation, USA. Protein concentration reagents were purchased from BioRad Inc, USA. Bovine casein yellow (Lot ♯603193) was obtained from CalBioChem Inc., USA.

2.2. Snakes

Twelve species of opisthoglyphous colubrid snakes were used in this study (Table 1); an aglyphous colubrid, *Pituophis melanoleucus sayi* (bullsnake), was used as a non-venomous saliva control. Colubrid snakes native to the United States were collected in Arizona (permit ♯MCKSY000221 to S.P.M.) and Colorado (permit ♯95-0456 to S.P.M.). Two specimens of *H. gigas* were on loan from Dr. Samuel S. Sweet and David Martin, and one specimen of *T. b. lambda* was on loan from Dr. Wade Sherbrooke. Permission to extract venom from two *B. irregularis* was granted by Dr. David Chiszar, and other snake species were obtained from commercial dealers.

2.3. Extraction of venom from Duvernoy's glands

Extraction for all snakes was based on the methodology reported by Rosenberg (1992) as modified by Hill and Mackessy (1997). Due to extremely low venom yields of some species and limited numbers of specimens, it was not possible to run all assays on all venoms. It should be noted that it is difficult to obtain venom samples *completely* free of saliva (and vice versa).

2.4. 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. A total volume of 1.0 ml was used for all assays. Enzyme specific activities were based on protein concentrations obtained from these assays.

2.5. Enzyme assays

Caseinolytic activity was assayed according to methods detailed by Mackessy (1993a), and specific activity was expressed as $\Delta A_{285\ nm}$/min/mg venom protein. Due to problems with later batches of casein yellow, protease activity was also determined with azocasein (Aird and da Silva, 1991), and activity was expressed as ΔA_{342}/min/mg venom protein. A venom sample from *Sistrurus catenatus*

Table 1
Species and habits of colubrid snakes used in this study

Species	Common name	Subfamily	General range	General prey base
Amphiesma stolata	Keeled water snake	Natricinae	India, China to Indochina	Fish, frogs, invertebrates
Boiga cyanea	Green treesnake	Colubrinae	North India, Indochina, China, Thailand	Primarily snakes
Boiga dendrophila	Mangrove snake	Colubrinae	Thailand, Malaysia, Philippines	Primarily birds
Boiga irregularis	Brown treesnake	Colubrinae	New Guinea, N Australia, Guam	All small vertebrates
Diadophis punctatus regalis	Regal ringneck snake	Natricinae	USA: Arizona, New Mexico, northcentral Mexico	Snakes and lizards
Heterodon nasicus kennerlyi	Mexican hognosed snake	Xenodontinae	USA: SE Arizona, SW New Mexico, S Texas; NE Mexico	Primarily toads and frogs
Heterodon nasicus nasicus	Plains hognosed snake	Xenodontinae	USA; Great Plains	Primarily toads and frogs
Hydrodynastes gigas	False water cobra	Xenodontinae	South America, Amazon basin	Primarily frogs
Hypsiglena torquata texana	Texas night snake	Xenodontinae	USA: Colorado, New Mexico, Texas; NE Mexico	Lizards, small snakes, frogs
Salvadora grahamiae grahamiae	Mountain patchnose snake	Colubrinae	USA: Arizona, New Mexico, Texas; northcentral Mexico	Lizards, small rodents
Tantilla nigriceps	Plains blackheaded snake	Xenodontinae	USA: Central/southern Great Plains; northcentral Mexico	Centipedes, spiders
Thamnophis elegans vagrans	Wandering garter snake	Natricinae	Western USA; SW Canada	Vertebrates and invertebrates
Trimorphodon biscutatus lambda	Sonoran lyre snake	Colubrinae	Southwestern USA; W Mexico	Lizards, small mammals
Pituophis melanoleucus sayi	Bullsnake	Colubrinae	USA: Great Plains; NE Mexico	Small mammals, birds, lizards

edwardsi (desert massasauga rattlesnake; collected in Colorado) was also run for comparison in both caseinolytic assays. Thrombin-like, kallikrein-like and plasmin-like proteolytic activities and arginine peptidase activity (using BAPNA) were assayed according to Mackessy (1993b). Phosphodiesterase activity was assayed by the method of Laskowski (1980) as modified by Mackessy (1988) with activity expressed as ΔA_{400}/min/mg venom protein. Phospholipase A_2 activity was assayed using 4-nitro-3-(octanoyloxy) benzoic acid as substrate (Holzer and Mackessy, 1996), with activity expressed as nmol chromophore released/min/mg venom protein (PLA_2I). Phospholipase A_2 activity was also assayed with egg yolk phosphatidylcholine Type IV (Sigma) following the procedure of Wells and Hanahan (1969), and specific activity was expressed as μmol product formed/min/mg venom protein (PLA_2II). L-amino acid oxidase activity was assayed according to Weissbach et al. (1961). Acetylcholinesterase activity was assayed as described by Ellman et al. (1961), using forest cobra (*Naja melanoleuca*) and red diamond rattlesnake (*Crotalus ruber*) venoms as positive and negative controls, respectively; activity was expressed as μmol of product formed/min/mg venom protein. Hyaluronidase activity (di Ferrante, 1956) was expressed as μg hyaluronic acid hydrolyzed/min/mg venom protein.

2.6. Electrophoresis: SDS Tris–glycine gels

SDS–PAGE was used to determine the number and relative molecular weight of proteins found in the colubrid venoms; all solutions and reagents (except gels) were prepared according to Hames (1990). Venom samples were prepared at a final concentration of 2.0 mg/ml crude venom in 1 × SDS sample buffer (62.5 mM Tris–HCl, 10% glycerol, 1% SDS and 0.001% bromphenol blue; no β-mercaptoethanol) and were centrifuged at 6–7000 rpm for 5 min to pellet any particulates. Novex 14% acrylamide Tris–glycine gels were rinsed with fresh reservoir buffer, and typically 15–20 μl samples were loaded in each well; gels were run at 100 V for 2–3 h. The gel was stained overnight in 0.1% Coomassie blue R-250 (in 30% methanol, 10% acetic acid) and then destained. After destaining, the gel was placed in 7.5% acetic acid for preservation and imaged with a CCD camera attached to a PowerMac 7200 (with Adobe Photoshop and PageMaker software).

2.7. Zymogram gels

Novex zymogram gels were used to determine if the venoms had gelatin-degrading proteolytic activity (general endoprotease activity); this method also provided information on the number and relative size of components with endoprotease activity (Heussen and Dowdle, 1980; Munekiyo and Mackessy, 1998). Amounts of venom loaded varied between samples (typically 10–40 μg/lane). Samples were treated with SDS (but *not* β-mercaptoethanol) in sample buffer and during electrophoresis; this potential inhibitor is removed during a subsequent wash in 2.5% Triton X-100 (Heussen and Dowdle, 1980).

2.8. Electroblot transfer

Several proteins from the venoms of *H. gigas, H. t. texana, T. b. lambda* and *T. nigriceps* were isolated via reducing SDS–PAGE (5% β-mercaptoethanol in sample buffer) followed by electroblot transfer with a Novex X-Cell (Trans-Blot Electrophoretic Transfer Cell) as described by Wilson and Yuan (1989). The PVDF membrane was stained for 10 min with 0.1% Coomassie R-250 stain and then destained in 50% methanol. The protein bands of interest were excised from the membrane and stored frozen until sequenced. The 26 kD proteins were chosen because of high concentration in the venoms and prevalence of this band in venoms of several species (therefore a common venom protein component). A 3.5 kD protein of venom from *Tantilla* was chosen for analysis because specific toxins from snake venoms are often low molecular weight peptides/proteins.

2.9. Protein sequencing

Partial N-terminal sequence was obtained from electroblots of 26 kD components from *H. gigas* venom, *H. t. texana* venom and *T. b. lambda* venom, and the 3.5 kD *T. nigriceps* venom peptide. Sequencing was accomplished via automated Edman degradation on an ABI 473a protein sequencer (MacroMolecular Resources, Colorado State University, Fort Collins, CO, USA).

2.10. Protein sequence homologies

Sequences obtained were evaluated for sequence homology with previously described proteins via the National Center for Biotechnology Information's NR Protein Database (FASTA: Pearson and Lipman, 1988), with post-processing provided by the Human Genome Center, Baylor College of Medicine (BEAUTY: Worley et al., 1995). All searches were conducted via the Internet (http://dot.imgen.bcm.tmc.edu:9331/seq-search/protein-search.html).

2.11. Observations of colubrid envenomations

The effects of envenomation on other snakes were observed for three species of opisthoglyphous colubrids (*D. punctatus, H. t. texana* and *T. e. vagrans*). In addition, the summary of a field report of human envenomation by *H. gigas* is reported (N. Scott, personal communication, 1997).

3. Results

3.1. Protein concentrations

Protein concentrations of venoms (Table 2) were generally much higher than salivas and were similar to those found in a previous study (Hill and Mackessy,

Table 2
Yields, protein concentrations and percent protein of venoms and salivas from 12 species of opisthoglyphous colubrids

Species	*N*	Total length $\bar{X}$ (range; mm)	Body weight $\bar{X}$ (range; g)	µl Venom yield $\bar{X}$ (range)	mg Venom dry yield $\bar{X}$ (range)	% Protein in venom $\bar{X}$ (range)	% Protein in saliva, $\bar{X}$ (range)
A. stolata	3	634 (526–665)	37.5 (20.9–55.7)	12.7 (6–22)	0.07 (0.05–0.10)	23.1 (6.8–39.3)	NC
B. cyanea	1	1920	819.7	240	13.16	55.9	48.9
B. dendrophila	2	1565 (1504–1625)	357.0 (286.3–427.6)	260 (250–270)	10.4 (8.2–12.5)	46.2 (41.6–50.8)	43.8 (39.4–48.1)
B. irregularis	2	approx. 2310	1499 (1487–1510)	370 (290–450)[b]	7.7 (2.4–13.0)[b]	79.4 (58.8–100.0)[b]	27.3[a]
D. p. regalis	4	510[a]	17.4 (9.6–26.9)	10 (5–20)[b]	2.88[b,c]	100[c]	NC
H. n. kennerlyi	2	320	22.4 (20.7–24.1)	15 (10–20)[b]	ND[d]	55.8[b,c]	21.9[b]
H. n. nasicus	2	480 (424–535)	58.7 (41.8–75.5)	24 (20–28)[b]	ND[d]	73.2 (64.3–84.0)[b]	NC
H. gigas	11	1977 (1760–2146)	2050 (1639–2709)	423 (110–840)[b]	7.31 (0.3–15.2)[b]	67.1 (31.8–97.8)[b]	30.9 (13.5–48)[b]
H. t. texana	8	384 (310–428)	16.2 (7.8–37.1)	12 (5–30)[b]	0.53 (0.28–1.05)[b]	49.8 (21.7–100)[b]	NC
S. grahamiae	1	648	33.1	15	0.6	22.8	NC
T. nigriceps	2	317 (281–351)	7.3 (4.5–10.0)	13 (10–15)[b]	0.08 (0.05–0.10)[b]	95.6[b,c]	NC
T. e. vagrans	10	775 (656–895)	91.3 (44.7–167.9)	23 (10–45)[b]	0.39 (0.10–0.46)[b]	51.6 (32.7–84.6)[b]	8.7 (3.3–12.6)[b]
T. b. lambda	3	916 (910–921)	85.4 (75.1–101.4)	130 (125–135)[b]	6.34 (4.98–7.70)[b]	98.2 (96.4–100)[b]	NC
P. melanoleucus	1	1245	424	N/A	N/A	N/A	8.2[a]

[a] Data from one specimen.
[b] Data from Hill and Mackessy (1997).
[c] Only one sample determined.
[d] Samples were too small to weigh accurately. ND, Not determined. NC, Saliva not collected. N/A, Not applicable.

1997), indicating that secretions collected were primarily Duvernoy's gland secretions. Total carbohydrate concentrations were determined (Dubois et al., 1956) for *Boiga irregularis, H. n. kennerlyi* and *H. gigas* venoms (0.1%, 1.0% and 4.7%, respectively), and *B. irregularis* saliva had a carbohydrate concentration of 3.7%.

3.2. Enzyme assays

Protease activity towards casein yellow substrate was detected in most colubrid venoms (Table 3), and *H. gigas* venom showed the highest levels of activity (average of 1.98 units; Fig. 1). All venoms tested that displayed activity toward casein yellow also showed activity towards azocasein (Table 3), though values were generally less than one-half of the casein yellow assay values. A venom sample from the desert massasauga rattlesnake showed high azocaseinolytic activity (0.719 units), typical of many rattlesnake venoms. Salivas analyzed showed very low caseinase activity (Table 4), and all venoms and salivas lacked arginine peptidase, thrombin-like and kallikrein-like activities. *Hydrodynastes gigas* venom also showed no activity toward the plasmin-like pNA substrate.

Phosphodiesterase activity was present in the venoms of some species, such as *D. punctatus, H. n. nasicus* and *T. e. vagrans*, and activity was also detected in *B. dendrophila* and *P. melanoleucus* salivas (Tables 3 and 4). The venom of *T. b. lambda* showed considerable phospholipase A_2 activity towards the 4-nitro-3-

Table 3
Enzymatic activities of venoms from 12 species of opisthoglyphous colubrids[a]

Species	Cas	Azo	Thr	Kal	AP	PDE	PLA_2	PLA_2II	LAAO	Acet	Hyal
A. stolata	ND	0.244	0	0	ND	0.647	ND	ND	0	ND	ND
B. cyanea	ND	0.224	0	0	0	0	0	0	0	ND	0
B. dendrophila	ND	0.084	0	0	0	0	0	90.0	0	ND	0
B. irregularis	0.582	0.255	0	0	0	0	0	ND	0	0.059	0
D. p. regalis	ND	0.003	0	0	0	2.550	0	102.5	0	ND	0
H. n. kennerlyi	0.187	0.167	0	0	0	0.882	0	ND	0	0	0
H. n. nasicus	ND	0.378	0	0	0	1.706	0	ND	ND	ND	0
H. gigas	1.982	0.586	0	0	0	0	0	0	0	0	0
H. t. texana	0.820	0.492	0	0	0	0	0	ND	0	0	0
S. grahamiae[b]	ND	0.342	0	0	ND	1.059	ND	ND	0	ND	ND
T. nigriceps	0.043	0.042	0	0	ND	0	ND	ND	0	ND	0
T. e. vagrans	0.357	0.205	0	0	0	1.882	0	0	0	0	0
T. b. lambda	ND	0.398	0	0	0	0	63.73	327.5	0	ND	0

[a] 0 indicates no activity detected; ND = not determined; all figures shown are $\bar{X}$ values (2 samples/species, run in triplicate). Cas = caseinase, units = ΔA_{285nm}/min/mg; Azo = azocaseinase, units = ΔA_{342nm}/min/mg; Thr = thrombin-like; Kal = kallikrein-like; AP = arginine peptidase; PDE = phosphodiesterase, units = ΔA_{400nm}/min/mg; PLA_2 = phospholipase A_2, units = nmol product formed/min/mg; PLA_2II = -phospholipase A_2, units = μmol product formed/min/mg; LAAO = L-amino acid oxidase; Acet = acetylcholinesterase, units = μmol product formed/min/mg; Hyal = hyaluronidase.

[b] = One sample analyzed.

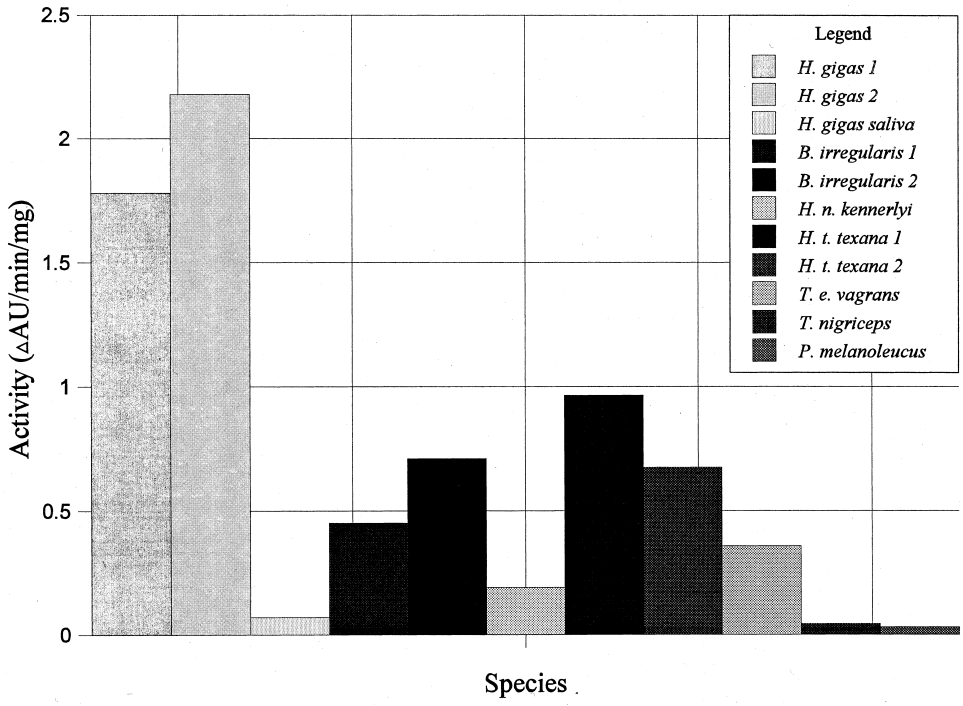

Fig. 1. Proteolytic activity of colubrid venoms and salivas toward casein yellow substrate. Activity is expressed as ΔA_{285} nm/min/mg protein.

Table 4
Enzymatic activities of saliva from eight species of colubrids[a]

Species	Cas	Azo	Thr	Kal	PDE	PLA_2	PLA_2II	LAAO	Acet	Hyal	AP
B. cyanea	ND	0.107	0	0	0	0	ND	0	ND	0	0
B. dendrophila	ND	0.005	0	0	0.941	0	77.5	0	ND	0	0
B. irregularis	0.050	0.116	0	0	0	0	ND	0	0.064	0	0
D. p. regalis	ND	0.076	0	0	0	0	ND	0	ND	0	0
H. n. nasicus	ND	0.426	0	0	0	50.0	115.0	ND	ND	0	0
H. gigas	0.065	0.319	0	0	0	0	0	0	0	0	0
T. e. vagrans	ND	0	0	0	0	0	ND	0	ND	0	0
P. melanoleucus	0.048	0	0	0	4.412	0	ND	0	0	0	0

[a] 0 indicates no activity detected; ND = not determined; all figures shown are $\bar{X}$ values (one sample/species, run in triplicate). Cas = caseinase, units = ΔA_{285nm}/min/mg; Azo = azocaseinase, units = ΔA_{342nm}/min/mg; Thr = thrombin-like; Kal = kallikrein-like; AP = arginine peptidase; PDE = phosphodiesterase, units = ΔA_{400nm}/min/mg; PLA_2 = phospholipase A_2, units = nmol product formed/min/mg; PLA_2II = -phospholipase A_2, units = μmol product formed/min/mg; LAAO = L-amino acid oxidase; Acet = acetylcholinesterase, units = μmol product formed/min/mg; Hyal = hyaluronidase.

(octanoyloxy) benzoic acid substrate (63.7 nmol/min/mg; PLA_2I assay, Tables 3 and 4), as did *Heterodon n. nasicus* saliva (8.69 nmol/min/mg), but other venoms and salivas showed no apparent activity. To verify these results, a more sensitive assay using egg yolk phosphatidylcholine was conducted on several samples (PLA_2II, Tables 3 and 4). With the second assay, *T. b. lambda* venom, *Boiga dendrophila* venom, *D. punctatus* venom, *B. dendrophila* saliva and *H. n. nasicus* saliva showed moderate activity levels. Saliva and venom from *B. irregularis* displayed low levels of activity when assayed for acetylcholinesterase (0.059 and 0.062 μmol/min/mg, respectively), though it was not nearly as active as the venom from the forest cobra (positive control: 3.68 μmol/min/mg). Venom from the red diamond rattlesnake (negative control) displayed no acetylcholinesterase activity. No other venoms or salivas assayed showed acetylcholinesterase activity, and none of the venoms or salivas analyzed showed L-amino acid oxidase or hyaluronidase activities.

3.3. Gel electrophoresis

All venom samples had multiple protein bands (Figs. 2–4), and molecular weights ranged from <6 to >200 kD (Table 5). The venom from some species, such as *T. b. lambda* and *H. gigas*, showed considerable complexity (Fig. 2a);

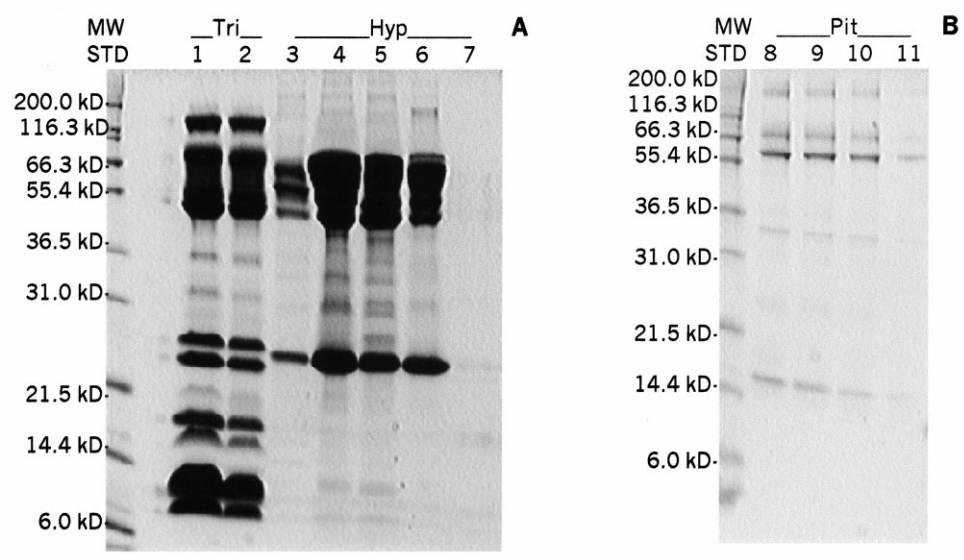

Fig. 2. (a) SDS–PAGE (14% acrylamide gel) of *T. b. lambda* (Tri) venoms (1, 2) and *H. t. texana* (Hyp) saliva (7), venoms (4–6) and venom–saliva mixture (3). Forty micrograms of sample were loaded in each lane. (b) Protein bands of *P. melanoleucus* (Pit) saliva (8–11). Lanes 8, 9, 10 and 11 contained 48, 36, 20 and 10 μg of sample, respectively. Note that saliva lanes, even at the highest concentrations, show very low intensity bands. MW STD = Novex Mark 12 molecular weight standards (in kDa).

saliva from the aglyphous bullsnake (*P. melanoleucus*), which lacks a Duvernoy's gland, showed very few bands and very low protein concentration (Fig. 2b). The venoms of *H. gigas, T. e. vagrans* and *B. irregularis* showed significantly more protein components than the salivas of those same species (Figs. 3 and 4; Table 5). β-mercaptoethanol treatment of *H. gigas* venom showed that several of the venom components are multiple subunit proteins (data not shown). Generally, protein profiles of the salivas contained fewer bands and differed considerably from the venoms of the same colubrids (Table 5).

Several of the venoms analyzed showed proteolytic activity towards the gelatin substrate of zymogram gels (Figs. 5 and 6), and a summary of the number and relative molecular weights of these proteases is given in Table 5. Crude *H. gigas* venom diluted to a concentration of 0.05 ng/lane had two venom components that showed detectable enzymatic activity (data not shown). Three very faint high molecular weight bands were observed in *T. nigriceps* venom, and one venom component from *T. e. vagrans* also showed low activity (data not shown). None of the other venom samples run on the zymogram gels displayed activity, including *T. b. lambda* venom, which showed proteolytic activity towards azocasein (data for *Heterodon* and *Hypsiglena* not shown). There was no detectable proteolytic activity in *P. melanoleucus* saliva (Fig. 5).

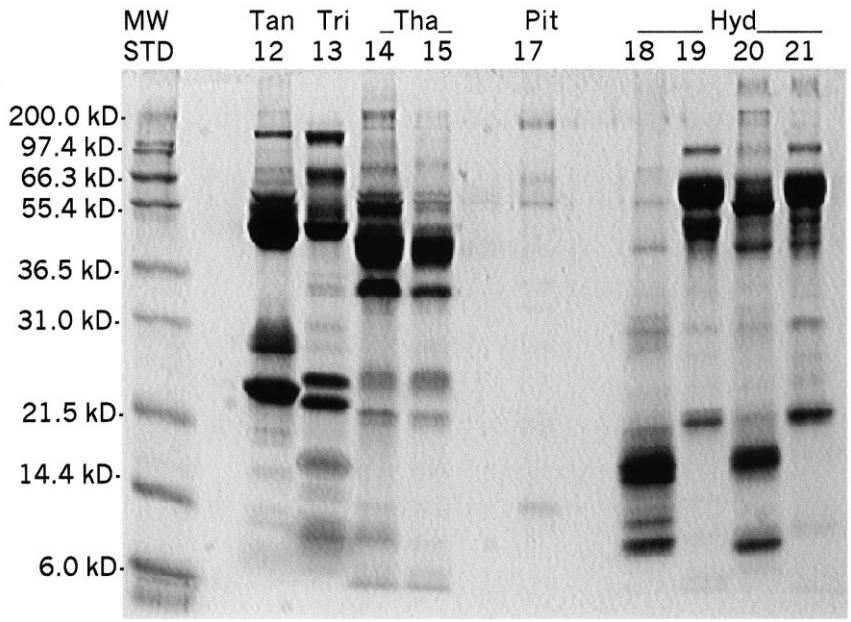

Fig. 3. SDS–PAGE (14% acrylamide gel) of *T. nigriceps* (Tan) venom (12), *T. b. lambda* (Tri) venom (13), *T. e. vagrans* (Tha) venom (14–15), *P. melanoleucus* (Pit) saliva (17), and *H. gigas* (Hyd) saliva (18), venom (19, 21) and venom–saliva mixture (20). Thirty micrograms of sample were loaded in each lane. MW STD = Novex Mark 12 molecular weight standards (in kDa).

3.4. Protein sequencing

N-terminal sequences (5–21 residues) were obtained for four colubrid venom proteins (Table 6). The 26 kD components from *H. gigas, H. t. texana* and *T. b. lambda* venoms showed considerable sequence identity, and the 3.5 kD peptide from *T. nigriceps* venom showed no homology with the 26 kD proteins.

3.5. Protein sequence homology

High sequence identity was observed among the 26 kD components (Table 6), but there was no apparent sequence homology discovered between these components and previously reported protein sequence data (National Center for Biotechnology Information's NR Protein Database). The 3.5 kD peptide showed limited sequence homology (< 30%) with internal sequences of laminin and laminin-type endothelial growth factor-like domain regions; this peptide also showed moderate sequence identity (43%) with the N-terminal region of vascular endothelial growth factor (Table 6).

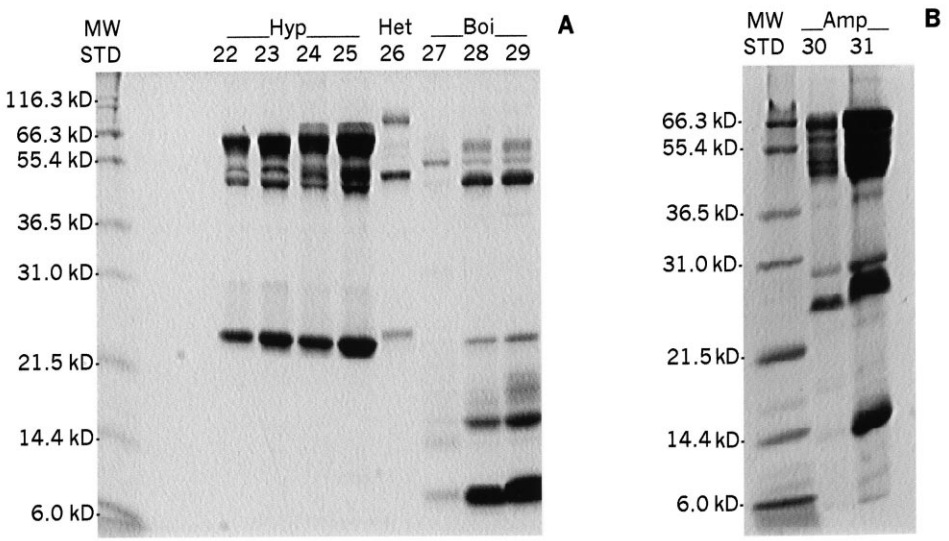

Fig. 4. (a) SDS–PAGE (14% acrylamide gel) of *H. t. texana* (Hyp) venom (23, 25) and venom-saliva mixture (22, 24), *H. n. kennerlyi* (Het) venom-saliva mixture (26) and *B. irregularis* (Boi) saliva (27) and venom (28, 29). Thirty micrograms of sample were used in each lane. (b) *Amphiesma stolata* (Amp) venom–saliva mixture (30) and venom (31) run on a 14% Tris–glycine polyacrylamide gel. Approximately 30 μg of sample were used in each lane. MW STD = Novex Mark 12 molecular weight standards (in kDa).

Table 5
Summary of electrophoretic patterns of colubrid venom and salivas: size distribution and proteolytic activity

Species	Number of bands	Size of protein bands (in Daltons)	Size of protein(s) with endoproteolytic activity
Venoms			
A. stolata	7–8 major; 7–9 minor	21,500- > 200,000	At least 6 bands from 31,000- > 200,000
B. cyanea	9 major; 5–6 minor	6000- > 116,300	–[a]
B. dendrophila	9 major; 5–7 minor	6000- > 200,000	–[a]
B. irregularis	8 major; 3–4 minor	6000–66,300	None detected
H. n. kennerlyi	7 bands	21,500–97,400	None detected
H. gigas	11 bands with at least 1 low MW band	< 6000- > 200,000	1 band at >200,000; 2 bands at 41,000 and 46,000
H. t. texana	9 major; at least 4–9 minor	21,500–97,400	None detected
S. grahamiae	at least 3 bands	6000–56,000	–[a]
T. nigriceps	at least 10 major; at least 6 minor	< 4000- > 200,000	3 minor bands between 66,300–97,400[b]
T. e. vagrans	7 major, at least 6 minor	6000- > 200,000	1 band approx. 75,000[b]
T. b. lambda	10 major; at least 9 minor	6000–200,000	None detected
Salivas			
B. cyanea	9 major; 5–6 minor	6000- > 116,300	–[a]
B. dendrophila	9–10 major; 5–7 minor	6000- > 200,000	–[a]
B. irregularis	4–6 major	6000–53,000	None detected
H. gigas	10 minor	8000–63,000	None detected
T. e. vagrans	4 major; 5–6 minor	14,400- > 200,000	None detected
P. melanoleucus	5–6 minor	14,400–200,000	None detected

[a] This sample was not subjected to zymogram analysis.
[b] Not visible in gel photograph.

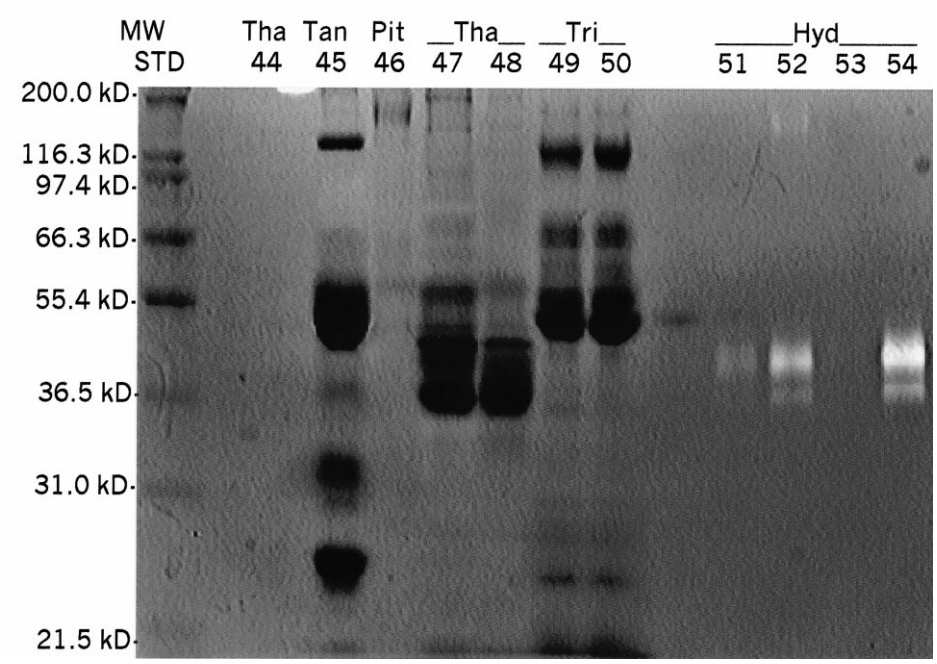

Fig. 5. Endoproteases in colubrid venoms: protease activity appears as a clear band on a 10% acrylamide–gelatin zymogram gel. Venom–saliva mixture (44) and venom (47, 48) of *T. e. vagrans* (Tha), venom–saliva mixture (45) of *T. nigriceps* (Tan), saliva (46) of *P. melanoleucus* (Pit), venom–saliva mixture (49) and venom (50) of *T. b. lambda* (Tri), and saliva (51, 53) and venom (52, 54) of *H. gigas* (Hyd). Samples 44–50 were loaded at 40 μg per lane, and 51–54 were loaded at 0.1 μg per lane. MW STD = Novex Mark 12 molecular weight standards (in kDa).

Table 6
N-terminal sequences of *H. gigas, H. t. texana, T. b. lambda* and *T. nigriceps* venom proteins[a]

	1 **5** **10** **15** **20**
HG 26[b]	Q-**D**-**F**-**N**-**S**-E-P-**P**-**R**-K-**P**-**E**-**I**-**Q**-**R**-V-S-V-D-T-N-
HT 26	Y-**V**-**D**-**F**-**N**-**S**-Q-S-**P**-**R**-R-**P**-**E**-**I**-**Q**-**R**-S-I-A-N-
TB 26	N-**V**-**D**-**F**-**N**
TN 3.5	L-M-F-Q-C-D-**Q**-**H**-K-**K**-**C**-**E**-**C**-T
HVEGF	G-Q-H-I-G-E-M-S-F-L-**Q**-**H**-N-**K**-**C**-**E**-**C**-R-P-K-
	1 **5** **10** **15** **20**

[a] Amino acid abbreviations: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

[b] HG 26 = 26 kD protein from *H. gigas* venom; HT 26 = 26 kD protein from *H. t. texana* venom, TB 26 = 26 kD protein from *T. b. lambda* venom; TN 3.5 = 3.5 kD protein from *T. nigriceps* venom. HVEGF = human vascular endothelial growth factor (96 aa). Numbering for lower panel follows HVEGF sequence.

Bold = Sequence identity.

3.6. Observations of colubrid envenomations

An adult *T. e. vagrans* (approx. 500 mm) bit a large adult (>700 mm) *Thamnophis sirtalis parietalis* (red-sided garter snake) approximately 5 cm behind the head. Severe hemorrhage and tissue necrosis was evident at the site of the bite within 2 h, and the snake died in approximately 36 h. Bites by *D. p. regalis* (regal ringneck snake) proved fatal to neonate specimens of *Elaphe guttatus* (corn snake), *T. e. vagrans* and *Salvadora hexalepis* (western patch-nosed snake) within 4–6 min; no local reactions were observed. A juvenile specimen (approximately 180 mm) of *Lichanura roseofusca* (rosy boa), which was bitten mid-body by an adult *H. torquata*, showed severe local edema, hemorrhage and necrosis within 1 h, and the snake died within 4 h.

3.7. Human envenomation by Hydrodynastes gigas

An adult male Caucasian (N. Scott, personal communication, 1997) provided the following description of a bite by *H. gigas* in Paraguay. The victim was bitten on the left inner thigh by a 1.8 m specimen that maintained its bite for an

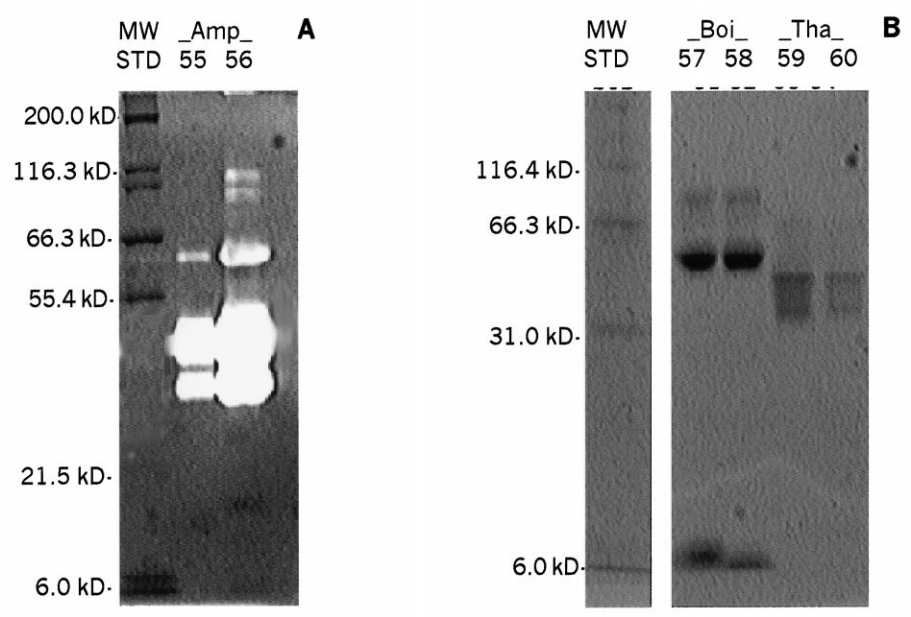

Fig. 6. Endoproteases in colubrid venoms: protease activity appears as a clear band on a 10% acrylamide–gelatin zymogram gel. (a) Protease activity of *A. stolata* (Amp) venom–saliva mixture (71) and venom (72). Approximately 10 μg of sample were loaded per lane. (b) Profiles of *B. irregularis* (Boi) venom (57, 58) and *T. e. vagrans* (Tha) venom (59, 60). Forty micrograms of sample were loaded in lanes 57 and 58, 20 μg in lane 59 and 10 μg in lane 60. MW STD = Novex Mark 12 molecular weight standards (in kDa).

undefined period. Three deep puncture wounds resulted, and the wounds bled profusely. After 6 h the wound was painful and slightly swollen, but there was no discoloration. The thigh became very painful after 24 h, and there was edema and red discoloration at the site of envenomation. The immediate area was hard and swollen after 48 h with a slight yellow discoloration, and a burning sensation occurred when the envenomated area was touched. The yellow discoloration and soreness remained for 4 days after the envenomation, and after 7 days there was no pain, swelling or discoloration. The fang puncture marks remained red, and there was never any sign of infection.

4. Discussion

Using ketamine hydrochloride in conjunction with pilocarpine, venoms were obtained from not only the large colubrids such as *B. irregularis* and *H. gigas*, but also from small species such as *H. t. texana* and *T. nigriceps*. In addition, manipulation of the snakes was greatly facilitated. Although collection of venom from colubrids is much more labor-intensive than venom extraction from front-fanged snakes, venom sufficient for numerous analyses can be obtained from even the smallest species (such as *T. nigriceps*; ~5 g body weight).

Because the Duvernoy's gland is a serous gland, secretions were expected to be high in protein concentration; the generally high protein content of venoms indicated that primarily Duvernoy's gland secretions (venom) had been collected. Salivary glands produce primarily mucopolysaccharides, and the protein content of salivas was typically low. Some venom components are undoubtedly glycosylated, but the results of limited carbohydrate assays in the present study suggested that carbohydrates were minor components of *B. irregularis* (0.1%) and *H. n. kennerlyi* (1%) venoms; the carbohydrate concentration in *H. gigas* venom (4.7%) was similar to that of several rattlesnake venoms (S.P. Mackessy, unpublished data). Undefined carbohydrate–protein complexes have been reported in the Duvernoy's glands of *H. gigas* (Glenn et al., 1992), but carbohydrates were not found in the cytoplasmic granules of secretory cells from *B. irregularis* Duvernoy's glands (Zalisko and Kardong, 1992).

Endoproteolytic activity was common in venoms analyzed in this study, and the highest proteolytic activities were observed in *H. gigas* and *A. stolata* venoms. Significant proteolytic activity was also observed in *B. irregularis, H. nasicus, H. t. texana* and *T. e. vagrans* venoms. Based on observations of effects on tissues of other snakes, some of these venoms may also contain hemorrhagic toxins, and (based on the action of viperid venom hemorrhagic proteases) these may be metalloproteases (e.g., Takeya et al., 1990; Fox and Long, 1998; Takeya and Iwanaga, 1998).

Several colubrid venoms contained components that very effectively digested the gelatin substrate in zymogram gels (notably *H. gigas* and *A. stolata*), and activity from *H. gigas* crude venom components (2 bands) could readily be detected even at concentrations as low as 0.05 ng crude venom per lane. In comparison,

rattlesnake venoms, which often have very high activity and 4+ components, usually require 0.5–1.0 μg/lane to visualize proteases (e.g., Munekiyo and Mackessy, 1998). The large number of proteases in *A. stolata* venom (≃6) and their high activity toward the zymogram substrate were somewhat unusual for a colubrid venom and are the subject of further study. Venoms from other species, such as *H. t. texana* and *T. b. lambda*, showed proteolytic activity when assayed with casein substrates but showed no apparent activity on zymogram gels; these venoms may lack gelatin-degrading proteases, or the proteases may be irreversibly denatured following short-term exposure to SDS. Most caseinolytic and gelatin-hydrolyzing proteases from snake venoms are metalloproteases (Fox and Long, 1998; Munekiyo and Mackessy, 1998; S. P. Mackessy, unpublished data, 1998), and these colubrid proteases may also be metalloenzymes.

In rattlesnake venoms, high proteolytic activity has a prominent role in predigestion of prey tissues, facilitating digestion and allowing for broader activity and distribution patterns (e.g. Thomas and Pough, 1979; Mackessy, 1988, 1993b). Enzymatic activities of colubrid venoms, particularly proteases which promote tissue damage, are likely also correlated with prey type(s) and/or activity patterns. The wandering garter snake (*Thamnophis elegans vagrans*) is very broadly distributed in western North America, occurring at high latitude and at high elevation, and venom which is proteolytic and hemorrhagic (casein assays, effects on other snakes) may have provided this species with the ability to occupy these thermally variable environments and still capitalize on bulky mammalian prey (e.g. Finley et al., 1994); a similar biological role for these components is seen in viperid venoms. Conversely, species of colubrids that feed on smaller prey items with high surface-to-volume ratios, such as *D. punctatus regalis* feeding on other snakes and small lizards and *T. nigriceps* feeding on arachnids, would not likely possess a venom with a prominent predigestive role (see Mackessy, 1988 for details), and protease activity in these species' venoms was quite low.

High levels of phospholipase A_2 were detected in venom from *B. dendrophila, D. p. regalis* and *T. b. lambda*; this enzyme is characteristically present in venoms of most front-fanged snakes (Rosenberg, 1990; Kini, 1997) but appeared to be lacking in most rear-fanged colubrid venoms (Weinstein and Kardong, 1994; but see Broaders and Ryan, 1997). The apparent lack of this common venom component in colubrid venoms is more likely due to inadequate sampling or insensitive assays, and we predict that phospholipases will be more common among colubrid venoms than was previously assumed. Phospholipase A_2 enzymes from several colubrid venoms are currently being isolated for further characterization and protein sequencing.

Phosphodiesterase assays showed that approximately half of the colubrid venoms in the present study contained detectable levels of activity, and this component was also reported for several other colubrid venoms (Kornalik et al., 1978; Rosenberg et al., 1985; Vest et al., 1991). Phosphodiesterase activity is common to most front-fanged snake venoms, and though the exact role of phosphodiesterases in those venoms is not known, it may involve disruption of cAMP- and ADP-mediated events (Mackessy, 1998). It is possible that this

enzyme has an important functional role in colubrid venoms as well. Because venom enzymes showed *much* higher activity toward some substrates (phospholipids and thymidine 5-nitrophenyl phosphate) than toward synthetic chromogenic substrates (4-nitro-3-(octanoyloxy) benzoic acid and Ca-bis-nitrophenylphosphate), it is recommended that several different substrates be used when assaying colubrid venoms for a specific enzymatic activity before concluding that an activity is lacking.

The low but detectable level of acetylcholinesterase activity found in *B. irregularis* saliva (and to a lesser degree the venom) is consistent with a recent report of this activity in the venom of two other species of *Boiga* (Broaders and Ryan, 1997). However, the activity in the venom sample may have been due to contamination with saliva, since one *B. irregularis* venom sample assayed showed no activity.

Electrophoretic analysis demonstrated that venoms from most colubrids contained numerous protein bands and that venoms were typically quite distinct from salivas. At least 10–20 components, ranging in size from $\leqslant 4$ to > 200 kD, were present in most venoms, and profile comparisons demonstrated species-specific components as well as several shared components. Congeneric species, such as *Boiga cyanea* and *B. dendrophila*, showed very similar protein banding patterns, while non-related species patterns were quite distinct. When the crude venoms were treated with reducing agents (β-mercaptoethanol), higher molecular weight components dissociated into smaller subunits, demonstrating higher order organization among some venom components, as is seen in most viperid and many elapid venoms.

The colubrid venoms assayed in the present study lacked many of the enzymatic properties (such as L-AAO, hyaluronidase, several serine proteases, etc.) typically found in front-fanged snake venom (see also review by Weinstein and Kardong, 1994). However, other components important to the biological roles of venoms, including endoproteases, phospholipases and phosphodiesterases, are present in many colubrid *and* front-fanged snake venoms. Many components appear to be novel proteins or proteins unique to colubrid venoms; these proteins likely reflect the divergent evolutionary histories of colubrids and convergence on an orally-delivered venom system among snakes. Non-ophidian venoms also contain numerous enzymatic activities (e.g. Hoffman, 1996), and assays based on non-ophidian venom components may help identify protein components in colubrid venoms with (at present) unknown activities.

There are considerable structural and sequence data available for viperid and elapid venom components (e.g. Shannon et al., 1989; Rosenberg, 1990; Takeya et al., 1990; Sanchez et al., 1991; Hite et al., 1992; Tu, 1991; Kini, 1997; Bailey, 1998), but there are no published data for colubrid venom components. A recent study of a myotoxic protein from the venom of *Philodryas olfersii* (green snake, a xenodontine colubrid) indicated that the protein was N-terminally blocked (Prado-Franceschi et al., 1998). N-terminal sequencing of the first 5–21 amino acid residues for four colubrid venom proteins represents the first protein sequence obtained for any colubrid venom components. However, in spite of the large

amount of sequence data available for front-fanged snake venom components, as well as for non-venom proteins, no protein homologies have been discovered for the 26 kD colubrid venom components. Regions of low homology with internal sequences of a DNA J-like protein and a glial fibrillary acidic protein likely represented spurious and chance sequence homology over a short stretch of these proteins. The level of sequence identity among the 26 kD venom components of *H. gigas, H. t. texana* and *T. b. lambda* indicates a similar structure (and likely function) in these proteins, which are prevalent venom components for the three species. At present, the 26 kD venom proteins remain unidentified.

A low molecular weight peptide (~3.5 kD) from *T. nigriceps* venom has no sequence homology with any published low molecular weight toxin, such as myotoxin a (Fox et al., 1979), suggesting that it may be a novel peptide. As with the 26 kD proteins, this peptide showed moderate homology with internal sequence of several larger proteins, such as laminin and the endothelial growth factor domain. However, the 3.5 kD peptide also showed significant sequence identity (6/14 residues) with the N-terminus of a 96 amino acid vascular endothelial growth factor (see Table 6). If this apparent homology is real, the peptide from *Tantilla nigriceps* venom may represent another example of convergence of a venom peptide on the sequence of a native regulatory peptide. A common "evolutionary strategy" adopted by venomous snakes is to target homeostatic mechanisms of prey (e.g. Stocker and Meier, 1989) and produce specific toxins which disrupt these mechanisms, and it is probable that colubrid venoms show a similar trend. An example are the sarafotoxins, 21-residue peptides (Kochva et al., 1982; Takasaki et al., 1988) isolated from the venoms of the mole snake *Atractaspis engaddensis* (formerly included in the Colubridae), which show high sequence and receptor homology with a group of native vasoconstrictive peptides, the endothelins (Ambar et al., 1988; Kloog et al., 1988, 1989; Galron et al., 1991). Sarafotoxins are potent venom toxins which produce cardiotoxic and vasoconstrictive effects, resulting in rapid prey death (Kochva et al., 1982; Takasaki et al., 1988). We predict that the 3.5 kD peptide from *T. nigriceps* venom will be a specific toxin, perhaps targeting a homeostatic mechanism of the arthropod prey (centipedes, spiders) of this species. It should be noted, however, that only 14 residues were available for homology searches.

Fatal envenomations of humans by colubrids are presently limited to four species: *Dispholidus typus, Philodryas olfersii, Rhabdophis tigrinus* and *Thelotornis capensis*; however, serious envenomations may occur from the bites of many species. Because rear-fanged snakes lack an efficient hollow-fanged injection system (Kardong and Young, 1991; Weinstein and Kardong, 1994), specific factors of a bite, such as length of contact time by the snake, can greatly influence the severity of envenomation by colubrids. Severe systemic effects following envenomation by *H. gigas* (Manning et al., 1999) likely resulted from the long contact time (1.5 min) of the bite. Extreme care should be exercised when handling *H. gigas* and *A. stolata* (because of the high level of endoproteolytic activity of their venoms) or any other rear-fanged colubrid whose venom has not been completely characterized. Lack of

an appropriate regard for the potential hazard from a bite of such snakes has resulted in human fatalities even among herpetologists (Pope, 1958; FitzSimons and Smith, 1958).

In conclusion, based on the complexity of most venoms obtained, the presence of activities which are common to front-fanged snakes and the extensive differences of these venoms from other oral secretions (saliva), the Duvernoy's gland secretions of many colubrid snakes should be considered as venoms homologous with (but not the same as) venoms of the front-fanged snakes. Previous studies have also shown that the colubrid Duvernoy's gland is morphologically and embryonically homologous with the venom glands of front-fanged snakes (Kochva, 1965; Ovadia, 1984). Colubrid venoms, like the venoms of front-fanged snakes, are trophic adaptations which facilitate feeding (Kardong, 1986; Mackessy, 1988, 1993a; Mackessy and Tu, 1993; Kardong, 1996). Diet and venom composition are intricately interwoven for many species of front-fanged snakes (Mackessy, 1988, 1993a; Daltry et al., 1996), and colubrid venoms likely have been shaped by specific aspects of prey. Taxa-specific toxins are known from the venom of the black widow spider (*Latrodectus mactans*; Grishin, 1998), and many of the small specialized colubrids may produce analogous venom toxins. Opisthoglyphous colubrid snakes represent a vast source of unknown venoms which deserve further investigation. The isolation, identification and characterization of colubrid venom components will provide insight into their function and biological roles and likely will produce unique molecular probes for use in other biological systems.

Acknowledgements

Support for this work was provided by grant GM52665-01 from the National Institutes of Health, National Institute of General Medical Sciences to S. P. Mackessy. The following individuals provided snakes or allowed extraction of animals under their care, and their assistance is appreciated: D. Chiszar, J. Hobert, D. Martin, C. Montgomery, W. Sherbrooke, S. S. Sweet and B. Tomberlin. Additional support was provided by a research incentives grant from the University of Northern Colorado Research Corporation. David Chiszar and Jennifer Clarke provided numerous comments on an earlier version of this manuscript, and the helpful suggestions of an anonymous reviewer are also acknowledged. Permission to collect several species used in this study was granted by the Colorado Division of Wildlife and the Arizona Game and Fish Department. Thanks go to Norm Scott for his account of a human envenomation by *H. gigas* and to Sean Munekiyo for his assistance with some of the enzyme assays.

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