ORIGINAL PAPER

Enzymatic and toxinological activities of *Hypnale hypnale* (hump-nosed pit viper) venom and its fractionation by ion exchange high performance liquid chromatography

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Abstract: *Hypnale hypnale* (hump-nosed pit viper) has been recently identified as one of the medically important venomous snakes in Sri Lanka and on the southwestern coast of India. The characterization of its venom is essential for understanding the pathophysiology of envenomation and for optimizing its management. In the present study, the biological properties of *Hypnale hypnale* venom and venom fractions obtained using Resource Q ion exchange chromatography were determined. The venom exhibited toxic activities typical of pit viper venom, comparable to that of its sister taxon, the Malayan pit viper (*Calloselasma rhodostoma*). Particularly noteworthy were its high activities of thrombin-like enzyme, proteases, phospholipase A$_2$, L-amino acid oxidase and hyaluronidase. The thrombin-like enzyme was mainly acidic and distributed over several chromatography fractions, indicating its existence in multiple isoforms. The hemorrhagic and necrotic activities of the venom were likely associated with the proteolytic enzyme found mainly in the basic fraction. Phospholipase A$_2$ and phosphomonoesterase exist in both acidic and basic isoforms, while L-amino acid oxidase and hyaluronidase are highly acidic. The venom clotting activity on fibrinogens showed distinct species specificity in the following increasing order for clotting time: bovine < rabbit < goat < human < horse < < dog, and was comparable to that of *C. rhodostoma* venom. Its clot formation on human fibrinogen is gradual and prolonged, a phenomenon suggestive of consumptive coagulopathy as a complication observed clinically. At an intramuscular sublethal dose, the venom did not cause acute kidney injury in a rodent model, contrary to the positive control group treated with *Daboia russelii* venom. Nephrotoxicity may result from higher venom doses in the context of coagulopathy, as a complication provoked by venom hematoxicity.

Key words: *Hypnale hypnale*, venom, enzymes, toxins, fibrinogen, nephrotoxicity.

INTRODUCTION

Snakes from the Viperidae family, including both vipers (viperines) and pit vipers (crotalids), are known to produce venoms that are principally hematoxic (1, 2). These venoms generally contain well over one hundred protein constituents, of which toxins belong to only a few major protein families that include such enzymes as serine proteases, Zn^{2+}-metalloproteases, L-amino acid oxidase and group II PLA$_2$; as well as non-enzymatic proteins (constituting a smaller portion) including disintegrins, C-type lectins, myotoxins, cysteine-rich secretory protein (CRISP) toxins etc. (3, 4). This context reflects the emergence of toxins from a restricted set of physiological protein families recruited into the venom proteome at the base of the Colubroidea radiation, ideal for their primary role in predation and defense (5, 6). Nevertheless, envenomation of humans causes serious public health problems, for which it has been duly categorized as a "disease" according to the WHO International Classification of Disease (ICD-10),

and appropriately categorized as a Neglected Tropical Disease (7). This problem also affects most tropical and subtropical countries, many of which are still underdeveloped, hence its apt description as a disease of poverty (8). Despite this, the envenomation issue remains systemically neglected in many parts of the world (9).

In South Asia, annual mortality due to snakebites has been estimated to be 1,000 in Sri Lanka and 20,000 in India (10). *Hypnale hypnale* (hump-nosed pit viper) has been recently recognized as a medically important venomous snake in Sri Lanka and on the southwestern coast of India (11, 12). It is now listed as a category I snake of medical importance by the World Health Organization, owing to its wide distribution and high prevalence of bites which cause significant morbidity and mortality in the region (13). This snake is mainly nocturnal and both arboreal and terrestrial. It inhabits both wet and dry deciduous zones; can be found in settings ranging from secondary forests, hilly areas, various plantations to low land gardens and even latrines within home compounds. Being well camouflaged and tiny in size (body length rarely exceeds 0.5 m), it is easily missed and trodden upon even in the daytime, resulting in its defensive bite (11). In Sri Lanka, it is not uncommon to come across *H. hypnale* envenoming even in the capital city of Colombo, although more cases occur in the suburban and rural areas.

Although the local effects of its bites were well observed and documented, it remains controversial after more than a century as to whether *H. hypnale* bites could be fatal (14-18). Recent clinical studies indicated that envenomation by this snake resulted in local edema, necrosis, regional lymphadenopathy, as well as systemic effects characterized by hemostatic dysfunction (coagulopathy, fibrinolysis, thrombocytopenia, spontaneous systemic hemorrhage) and acute renal failure, with an overall fatality rate of 1.7% in authenticated cases (11, 12, 19-22). To date, apart from the *Calloselasma rhodostoma* antivenoms (produced by the Thai Red Cross Society) which were shown to confer paraspecific neutralization in rodent models, there is no specific antivenom that is clinically effective against the *H. hypnale* venom (11, 12, 23).

In order to address the envenoming issue holistically, the venom's toxinological profile should be studied to elucidate its pathogenesis. In

this paper, we report the biological properties of the venom and its ion exchange chromatography-fractionated components, using *in vitro* and/or *in vivo* methods. Its clotting activity on several mammalian fibrinogens was also investigated. A rodent model was also used to study the *in vivo* nephrotoxic effects of this venom.

MATERIALS

Venoms

Hypnale hypnale venom was a pooled sample obtained from the milking of adult snakes captured in Sri Lanka (Gamapha, Kelaniya, Avissawela, Colombo regions). The snakes were kept at the snake farm in the University of Colombo, Sri Lanka and were identified by Anslem de Silva, an expert herpetologist. Venoms of *Calloselasma rhodostoma*, *Cryptelytrops purpureomaculatus* and *Daboia russelii* (Myanmar) were purchased from Latoxan (Valence, France).

Reagents and Animals

All reagents and chemicals were purchased from Sigma Chemical Company (USA) and were of analytical grade. Albino mice (ICR strain) and Sprague Dawley rats were supplied by the Laboratory Animal Center, Faculty of Medicine, University of Malaya. The use of animals was approved by the institute and these animals were handled according to the guidelines of the Council for International Organizations of Medical Sciences (CIOMS) on animal experimentation (24).

METHODS

Fractionation of *H. hypnale* venom by Resource® Q Ion-Exchange Chromatography

Five milligrams of the venom dissolved in 200 μL of starting buffer (20 mM Tris-HCl, pH 8.5) was filtered through a 0.2 μm pore size syringe filter. The filtrate was then injected into Resource® Q ion-exchange column (GE Healthcare, USA) (6.4 mm x 30 mm, 1 mL gel volume, 15 μm particle size, 200-10,000 Å) pre-equilibrated with starting buffer, and subsequently eluted by a linear, 0.0 to 0.5 M sodium chloride gradient (0-30% from 5 to 30 minutes, followed by 30-100% from 30 to 55 minutes), at the flow rate of 1 mLmin^{-1}. The solvent delivery and gradient formation over 60 minutes was achieved by using the Shimadzu LC-20AD® high performance liquid chromatography

(HPLC) system (Japan). Protein peaks were monitored by measuring the absorbance at 280 nm. The protein content of the fractions was determined by the Bradford method (25).

Determination of Enzymatic Activities

This portion of the study was done on the venoms of *H. hypnale* and *Calloselasma rhodostoma* for comparison, as well as on the HPLC-isolated protein fractions of *H. hypnale* venom. Two independent experiments were carried out on each test for each sample, and the average results were reported.

Protease assay

Protease activity was measured by modification of the method described by Kunitz (26). One milliliter of 1% casein in 0.25 M sodium phosphate buffer, pH 7.75, and 50 μL of sample solution were incubated for 30 minutes at 37°C. The reaction was terminated by adding 1 mL of 5% trichloroacetic acid. After centrifugation at 10,000 x g for ten minutes, the absorbance of the supernatant was measured at 280 nm. One unit of protease activity was defined as an increase of one absorbance unit per hour at 280 nm.

Phospholipase A$_2$ assay

Phospholipase A$_2$ activity was determined by the acidimetric method (27). The egg yolk substrate suspension was prepared by mixing one part chicken egg yolk, one part 18 mM calcium (II) chloride, and one part 8.1 mM sodium deoxycholate. The pH of the substrate suspension was adjusted to 8.0 with 1 M sodium hydroxide. The suspension was stirred to ensure good mixing. One hundred microliters of venom solution was added to 15 mL of the substrate suspension and the rate of decrease in pH was recorded using a pH meter. A decrease of 1 pH unit of the egg yolk suspension corresponded to 133 μmol of fatty acids released.

Arginine ester hydrolase assay

Arginine ester hydrolase was assayed using α-benzoyl arginine ethyl ester as substrate (28). The assay mixture contained 0.95 mL of 0.8 mM substrate in 0.05 M Tris-HCl buffer, pH 7.8, and 50 μL of sample solution. The reaction was monitored by measuring the rate of increase in absorbance at 255 nm. The difference in the extinction coefficient at 255 nm is 815 cm^{-1}M^{-1}.

Phosphodiesterase assay

Phosphodiesterase activity was determined by a method modified from Lo *et al.* (29). One hundred microliters of sample was added to an assay mixture containing 0.5 mL of 2.5 mM calcium bis-p-nitrophenylphosphate, 0.3 mL of 0.01 M magnesium sulfate and 0.5 mL of 0.17 M veronal buffer, pH 9.0. The hydrolysis of the substrate was monitored by measuring the rate of increase of absorbance at 400 nm. The extinction coefficient is 8100 cm^{-1}M^{-1}.

Alkaline phosphomonoesterase assay

Alkaline phosphomonoesterase activity was determined by a method modified from Lo *et al.* (29). One hundred microliters of sample was added to an assay mixture containing 0.5 mL of 0.5 M glycine buffer (pH 8.5), 0.5 mL of 10 mM p-nitrophenylphosphate and 0.3 mL of 0.01 M magnesium sulfate. The mixture was incubated at 37°C for 30 minutes. At the end of the incubation period, 2 mL of 0.2 M sodium hydroxide was added and allowed to stand for 20 minutes at room temperature. The absorbance at 400 nm was then measured. The extinction coefficient is 18500 cm^{-1}M^{-1}.

5'-Nucleotidase assay

5'-Nucleotidase activity was determined using 5'-AMP as substrate (30). One hundred microliters of sample was added to an assay mixture containing 0.5 mL of 0.02 M 5'-AMP (preadjusted to pH 8.5), 0.5 mL of 0.2 M glycine buffer, pH 8.5, and 0.1 mL of 0.1 M magnesium sulfate. The mixture was incubated for ten minutes at 37°C, and the reaction was terminated by the addition of 1.5 mL of 10% trichloroacetic acid. The ascorbic acid method was used to determine the inorganic phosphate content (31). To the above mixture, 1 mL of ascorbic acid reagent – containing equal parts of 3 M sulfuric acid, 2.5% ammonium molybdate, 10% ascorbic acid and water – was added. The mixture was left at room temperature for 30 minutes, and the absorbance at 820 nm was then measured. A standard curve was constructed using known concentrations of inorganic phosphate.

Hyaluronidase assay

Hyaluronidase activity was determined turbidimetrically (32). The assay mixture contained 0.45 mL of 0.2 M acetate buffer, pH 5.0,

containing 0.15 M sodium chloride and 0.2 mg of human umbilical cord hyaluronic acid and 50 μL of sample solution. The mixture was incubated for 1 h at 37°C. The reaction was terminated by the addition of 1 mL of 2.5% cetyltrimethyl-ammonium bromide in 2% sodium hydroxide solution. After 30 minutes, the absorbance at 400 nm was measured. Enzymatic activity was expressed as NFU/mg (National Formulary Units). Appropriate dilutions of sample were performed to ensure that the difference in absorbance between the blank and sample was less than 0.4. Hyaluronidase (EC 3.2.1.35), originating from bovine testes, was purchased from Sigma Chemical Co. (USA) and used as a standard; its activity is 270 NFU per mg. Several dilutions of hyaluronidase representing a series of NFUs were tested on solution containing 0.2 mg of hyaluronic acid. The absorbance values were plotted against the NFUs for calibration.

L-amino acid oxidase

L-amino acid oxidase activity was determined as described by Tan *et al*. (33). Fifty microliters of horseradish peroxidase (100 purpurogalin unit/mg) was added to 0.9 mL of 0.2 M triethanolamine buffer, pH 7.6, containing 0.1% L-leucine and 0.0075% o-dianidisine and incubated for three minutes at room temperature. Sample solution (50 μL) was then added and the increase in absorbance at 436 nm was measured. The molar absorption coefficient is 8.31×10^3 cm^{-1}M^{-1}.

Determination of Lethality, Hemorrhagic, Procoagulant and Necrotic Activities

Median lethal dose (LD$_{50}$)

The LD$_{50}$ values of *H. hypnale* venom were determined by intraperitoneal injection of the venom into mice (18-22 g, n = 4 per dose) whose survival ratio was recorded after 48 hours. The intraperitoneal LD$_{50}$ (and the 95% confidence intervals, CI) of the venom was calculated using the Probit analysis method of Finney (34). The intramuscular LD$_{50}$ value of *H. hypnale* was based on a previous determination from the same laboratory as reported by Tan *et al*. (23), while that for *D. russelii* was determined via intramuscular injection of the venom into hind legs of mice (n = 4 per dose), and analyzed as previously mentioned.

Hemorrhagic activity

Hemorrhagic activity was determined according to Theakston and Reid (35) with modifications. The venom dissolved in phosphate-buffered saline (50 μg in 50 μL) or the venom fraction (50 μL) was injected intradermally into the shaved dorsal skin of lightly anesthetized mice. After 90 minutes, the animals were euthanized with an overdose of diethyl ether. The dorsal skin was removed carefully, and the mean diameter of the hemorrhagic lesion on the inner surface of the skin was measured.

Procoagulant activity

Procoagulant activity was determined by the method described by Bogarin *et al*. (36). The venom (50 μg in 0.1 mL) or venom fraction (0.1 mL) was added to 0.2 mL of bovine fibrinogen solution (2 g/L) at 37°C. The coagulation time (time required for fibrin strands to form) was recorded. A shorter coagulation time suggests higher procoagulant activity.

Necrotic activity

Necrotic activity was determined by a modified version of the method of Theakston and Reid (35). The venom, (50 μg in 50 μL) or venom fraction (50 μL) was injected intradermally into the shaved dorsal skin of lightly anesthetized mice. After 72 hours, the animals were euthanized with an overdose of diethyl ether, the dorsal skin was removed, and the mean diameter of the necrotic lesion was measured.

Fibrinogen clotting activity

The fibrinogen clotting activity (procoagulant effect) of *H. hypnale* venom was tested further on fibrinogens from several mammalian sources. Venoms from two other Asiatic pit vipers, *C. rhodostoma* and *C. purpureomaculatus*, well known for their procoagulant effects, were studied simultaneously for comparison (37, 38).

The venom (50 μg in 0.1 mL) was added to 0.2 mL of fibrinogen solution from various animal sources (2 g/L) at 37°C. The coagulation time (time elapsed for fibrin strands to form) was recorded. To study the time course of venomous procoagulant activities, each venom (50 μg in 0.1 mL) was added to 0.2 mL of fibrinogen solution (2 g/L) preincubated at 37°C. The absorbance of the mixture at 450 nm was read for ten minutes to monitor the formation and degradation of fibrin clots.

Nephrotoxicity study

Sprague dawley rats were divided into three groups of three animals each: normal saline (NS) group, *H. hypnale* venom (HV) group and *Daboia russelii* venom (DV) group. The NS group and DV groups represented, respectively, the negative and positive controls. Rats from each group received intramuscular injections of treatment as follows: 200 µL of normal saline, or 200 µL of venom solution corresponding to one third of the venom's intramuscular LD_{50}. The rats were kept for 24 hours in metabolic cages with access to feed and water *ad libitum*. Urine was collected throughout 24 hours, while blood was collected via cardiac puncture under anesthesia at the end of experiment. The urine and blood were sent for biochemical analysis, to an outsourced service at a pathology analytical laboratory. Major organs harvested from rats upon death following cardiac punctures were sent for histopathological studies. Specimens were fixed in 10% formalin, followed by paraffin wax-embedding, sectioning of tissue, dewaxing, rehydration, and staining with hematoxylin and eosin. The sections were cover-slipped and images were captured using a Nikon Research Trinocular Teaching Microscope Eclipse 80i® (Japan) equipped with a camera.

Statistical analysis

The variability of lethality assays was expressed within 95% confidence intervals (CI). The significance of the differences of the means was determined by two-tailed unpaired Student's *t*-test or one-way ANOVA. Statistical analyses were carried out using the statistical software SPSS.

RESULTS AND DISCUSSION

Milking of Snake Venom

In an investigation of venom yield, the milking of six adult *H. hypnale* snakes yielded 10.0 mg, 8.7 mg, 11.4 mg, 20.0 mg, 12.2 mg and 15.6 mg, respectively, of dried venom. The average amount of venom obtained per milking is therefore 13 mg (± 4.2 mg). Hence the maximum amount of venom injected per bite is expected to be less than 20 mg, much lower than the roughly 50 mg per yield reported in its sister taxon, *C. rhodostoma* (37). This is predictable as the body size *of H. hypnale* is small and rarely grows beyond 0.5 m.

Lethality of the Venom

H. hypnale venom has an intraperitoneal median lethal dose (LD_{50}) of 6.0 µg/g (95% CI 4.2-8.1 µg/g) of mouse body weight, which is intermediate between the intravenous LD_{50} (0.9 µg/g) and the intramuscular LD_{50} (13.7 µg/g) reported by our research group in an earlier study (23). This suggests that the potency of toxins causing lethality differs depending on the administration route. A much lower intravenous LD_{50} indicates that the venom is likely to be principally hematoxic in action, while the intraperitoneal LD_{50} is higher than the intravenous value possibly due to delayed an/or incomplete absorption (as in first pass phenomenon) of the toxins before reaching the systemic circulation (39, 40). The fact that the intraperitoneal LD_{50} value is lower than that of the intramuscular route is likely due to higher bioavailability given that more venom is quickly absorbed intraperitoneally (larger surface area and richer blood supplies) without having to bind and interact with muscle and connective tissues.

The median intramuscular lethal dose of *D. russelii* venom tested in mice was 0.70 µg/g (95% CI, 0.45-0.9 µg/g) of body weight, which was determined for use in the nephrotoxicity study.

Biological Properties of *H. hypnale* and *C. rhodostoma* Venoms

The *H. hypnale* venom used in this study showed higher activities of proteases, phospholipase A_2, L-amino acid oxidase and hyaluronidase than those found in a previous report (41) (Table 1). This may be due to ontogenic differences as the venom used in this study was a recently pooled sample. The procoagulant activity, however, did not differ markedly between the two reports.

H. hypnale venom exhibited enzymatic properties similar to *C. rhodostoma* venom (Table 1), which are quite typical of pit viper venoms, notably the presence of arginine ester hydrolase and potent protease activities. Particularly noteworthy is the substantially higher phospholipase A_2 activity in *H. hypnale* venom; in comparison, *C. rhodostoma* venom usually exhibits rather / very low phospholipase A_2 activity. Phospholipases A_2 are known to exhibit multiple pharmacological activities, including lipid membrane damage, myotoxicity and myonecrosis (42, 43). High amounts of phospholipase A_2, proteases and L-amino acid

oxidase, known for their cytotoxic and tissue-damaging properties, are likely responsible for the local envenoming features, e.g. edema, necrosis and hemorrhagic blistering commonly seen after *H. hypnale* bites (11, 12, 43-45). In addition, hyaluronidase that presents substantially in the venom can synergistically potentiate the venom propagation and its local effects, contributing to severe local tissue destruction (46).

H. hypnale venom also showed potent procoagulant or thrombin-like enzyme activities (lower than that of *C. rhodostoma*), as well as hemorrhagic and necrotic properties (higher than or similar to that of *C. rhodostoma*). These activities are hallmark of the pathophysiology induced by pit viper venoms. The general similarities in the biological activities of *H. hypnale* venom and *C. rhodostoma* venom are expected as they are closely related phylogenetically, hence the possibility of sharing similar venom antigens (47, 48).

Fractionation of *H. hypnale* venom by Resource® Q Anion Exchange Chromatography

Ion exchange chromatography separates proteins based on their ionic charges, and has been useful in snake venom fractionations as it does not affect the biological activity of eluted proteins, hence allowing their characterizations (33, 38). With Resouce® Q column, an anion exchanger, *H. hypnale* venom was fractionated yielding twelve major fractions (Figure 1). Protein estimation by the Bradford method (25) showed that fraction 12 has very little protein content. Fractions 1 and 2 are unbound fractions and contain basic proteins, while the remaining fractions generally contain proteins of an increasingly acidic nature.

Biochemical Properties of the Protein Fractions of *H. hypnale* venom

Table 2 shows the biological properties of the 12 fractions obtained from Resource® Q chromatography of *H. hypnale* venom. Fraction 1, containing the basic proteins, was certainly heterogeneous, as it possessed multiple enzyme activities including proteases, phospholipase A_2, alkaline phosphomonoesterase, phosphodiesterase and 5'-nucleotidase activities. It was also the only fraction that exhibited hemorrhagic and necrotic activities, and both of these toxic effects were likely associated with the proteolytic activity, as reported in viperid metalloproteinases (1). Both fractions 1 and 3 exhibited phospholipase A_2 activity, suggesting that the venom contains at least two forms of phospholipase A_2. This finding is in accord with a previous report (49) where only two out of four PLA_2 isoforms isolated from *H. hypnale* venom, using combined gel filtration and reversed-phase chromatography, exhibited enzymatic activities. We suggest that these two reported enzymes belong to a basic form (fraction 1) and an acidic form (fraction 3), respectively.

Hydrolytic enzymes – such as phosphodiesterase (a nuclease), 5'-nucleotidase

Table 1. Enzymatic, hemorrhagic and necrotic activities of *H. hypnale* and *C. rhodostoma* venoms

Activity / Species	PRO	PLA₂	LAO	AEH	PME	PDE	5'-Nuc	HYA	TLE	HEM	NEC
H. hypnale[#]	15.8	191.5	191.7	10.05	4.8	4.1	1.8	115.5	20	23	7.5
C. rhodostoma[#]	18.5	79.8	165.4	8.96	4.6	18.5	1.9	76.6	10	16	8.0
H. hypnale[@]	7.8	126	110	15.2	12	6	2.7	85	22	1.6*	ND

PRO: protease, unit/mg; PLA₂: phosphopholipase A₂, µmol/min/mg; LAO: L-amino acid oxidase, nmol/min/mg; AEH: arginine ester hydrolase, µmol/min/mg; PME: alkaline phosphomonoesterase, nmol/min/mg; PDE: phosphodiesterase, nmol/min/mg; 5'-Nuc: 5'-nucleotidase, µmol/min/mg; HYA: hyaluronidase, NFU/mg; TLE: thrombin-like enzyme (procoagulant enzyme), s/50 µg venom; Hem: hemorrhagin, mm/50 µg venom; NEC: necrosis, mm/50 µg venom; ND: not determined. Values were the average results from two independent experiments.
[#] Present study; [@] reference to Tan and Ponnudurai (41); * the unit was expressed as the product of two perpendicular diameters of the lesion, which is different from the unit defined in the present study.
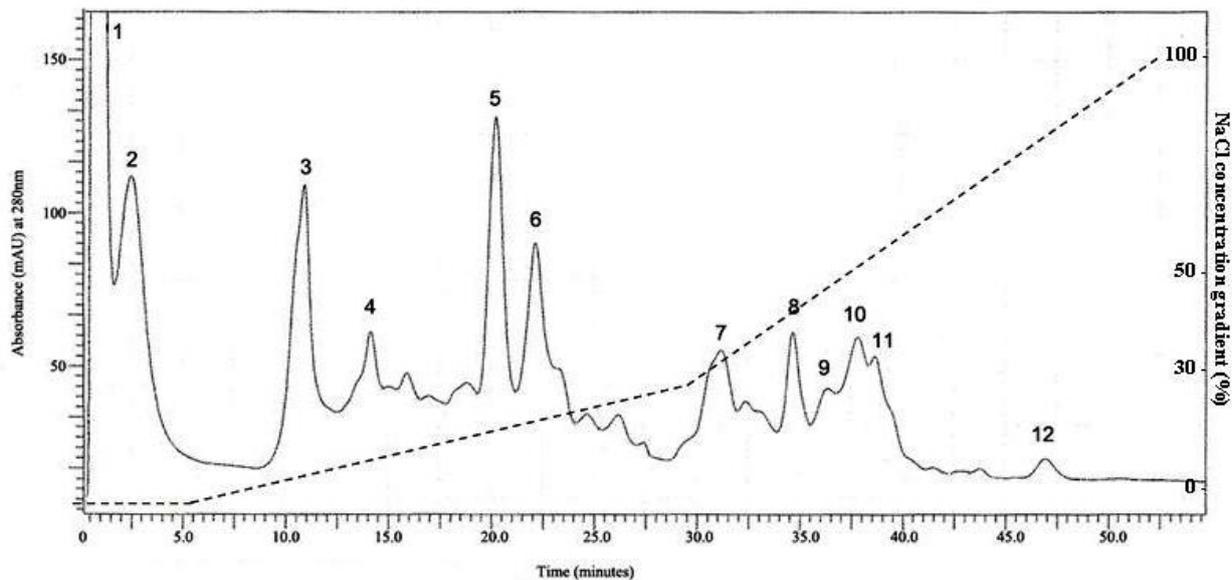
Figure 1. Fractionation of *Hypnale hypnale* venom with Resource® Q anion exchange chromatography. The venom (5 mg) was injected into the column equilibrated with 20 mM Tris-HCl, pH 8.5, and eluted by a linear, 0.0 to 0.5 M NaCl gradient (0-30% from 5 to 30 minutes, followed by 30-100% from 30 to 55 minutes. Flow rate was 1 mL/min. (-----): NaCl concentration gradient (%)

and alkaline phosphomonoesterase – are high molecular weight proteins known to be widely distributed across many snake taxa, but their toxic effects have been less extensively characterized partly due to the fact that these enzymes share similar substrates and biochemical properties (50). In general, our results showed that all three enzymes were found in *H. hypnale* venom in appreciable amounts, particularly phosphodiesterase, which is usually abundant in crotalid venoms. Phosphodiesterase had been associated with a drop in mean arterial pressure and locomotor depression, presumably due to reduced cAMP levels (51). This feature, however, has not been reported in systemic envenomation by *H. hypnale*, implying that its function might be more digestive than purely toxinological. The chromatography result also showed that 5'-nucleotidase and phosphomonoesterase from *H. hypnale* venom exist in both basic and acidic isoforms. It is intriguing to note the existence of acidic phosphomonoesterase in this venom, as this enzyme, contrary to the basic/alkaline phosphomonoesterase, has so far only been purified from sea snake venoms and rarely been reported in other snake venoms (52).

Thrombin-like activity was found to be widespread from fraction 2 to fraction 8,

indicating that the enzyme likely existed in multiple isoforms. The two fractions (fraction 5, 6) with the strongest thrombin-like activity were also the two fractions that hydrolyzed arginine esters, suggesting that the thrombin-like enzymes exhibited arginine ester hydrolase activity, a property similar to that of ICR-50 arvin, a coagulant enzyme derived from *C. rhodostoma* venom (28). The enzyme esterolytic activity towards *N*-α-benzoyl-*l*-arginine ethyl ester indicated its specific binding to the arginine residues in its substrate. The venom also contained one or more forms of L-amino acid oxidase which appeared more acidic than many other enzymes, and its activity was noted to be lost by freezing. Such freeze-labile activity had been reported and the mechanism is presumably due to a limited conformational change of the enzyme structure (53, 54). Fraction 12, which contained few proteins, was found to be devoid of enzymatic activities. As demonstrated, venom fractionation helps elucidate the different components in venoms allowing their toxic characterizations for pathophysiological correlations. Nonetheless, it also opens the door to manipulating specific toxins in search of potential therapeutic compounds, as already observed in drug discoveries of ancrod, captopril etc. (55).

Table 2. Enzymatic, hemorrhagic and necrotic activities of major protein fractions of *H. hypnale* venom obtained from Resource Q ion-exchange chromatography

Test \ Fraction	AMT	PRO	PLA$_2$	LAO	AEH	PME	PDE	5'-Nuc	HYA	TLE	HEM	NEC
1	240	8.3	79.8	–	–	0.5	9.8	0.18	–	–	9	6
2	59	–	–	–	–	–	–	–	–	470	–	–
3	77	–	159.6	–	–	–	–	–	–	212	–	–
4	32	–	–	–	–	–	–	0.15	–	115	–	–
5	47	–	–	–	9.8	–	–	0.30	–	84	–	–
6	58	–	–	–	2.5	–	–	0.66	–	77	–	–
7	28	–	–	–	–	–	–	–	–	146	–	–
8	18	–	–	–	–	1.7	–	–	–	610	–	–
9	12	–	–	13.8	–	0.9	–	–	6.9	–	–	–
10	41	–	–	11.3	–	0.1	–	–	46.9	–	–	–
11	10	–	–	–	–	–	–	–	6.6	–	–	–
12	4	–	–	–	–	–	–	–	–	–	–	–

AMT: Total protein amount recovered in each respective fraction, µg; PRO: protease, unit/mL; PLA$_2$: phosphopholipase A$_2$, µmol/min/mL; LAO: L-amino acid oxidase, nmol/min/mL; AEH: arginine ester hydrolase, µmol/min/mL; PME: alkaline phosphomonoesterase, nmol/min/mL; PDE: phosphodiesterase, nmol/min/mL; 5'-Nuc: 5'-nucleotidase, µmol/min/mL; HYA: hyaluronidase, NFU/mL; TLE: thrombin-like enzyme (procoagulant enzyme), s/100 µL; HEM: hemorrhagic activity, mm/50 µL; NEC: necrotic activity, mm/50 µL, –: absent activity. Values were the average results from two independent experiments.

Fibrinogen Clotting Activity

The clotting activity of *H. hypnale* venom showed distinct species differences (Table 3). The clotting time of mammalian fibrinogens by *H. hypnale* venom was in the increasing order as follows: bovine < rabbit < goat < human < horse < < dog. The clotting activity of *C. rhodostoma* venom exhibited a similar pattern (most and least active towards the bovine and dog, respectively) indicating that the thrombin-like activity of the venoms of these two phylogenetically related crotalids exhibited similar specificity towards fibrinogens. On the other hand, the specificity of the clotting activity of *C. purpureomaculatus* venom (bovine < goat < human < horse < < rabbit, dog) was rather different, and generally showed a lower clotting potency for bovine, goat and human fibrinogens ($p < 0.05$).

The clotting activities of the three venoms were also monitored spectrophotometrically whereby the turbidity of the reaction mixture was continuously monitored for absorbance at 450 nm. The investigation of the time course of clot assembly had also been described by Pirkle *et al.* (56). Generally, the appearance of fibrin or clots would result in turbidity, while degradation of the clots would reduce this property. Figure 2 (A to D) shows that both *H. hypnale* and *C. rhodostoma* venoms caused rapid clotting with fibrinogens from bovine, goat, horse and rabbit specimens. Dissolution of clots was rapid in the case of goat (Figure 2 – B) and horse fibrinogens (Figure 2 – C) (turbidity reduced from 30 seconds). Both venoms were active in the clotting of human fibrinogen (Figure 2 – E), showing a slow but prolonged clotting effect. *C. purpureomaculatus* venom generally demonstrated moderate to low clotting activities, except for the case of dog fibrinogen (Figure 2– F) where it appeared more active than the venoms of *H. hypnale* and *C. rhodostoma*.

We suggest that a rapid clotting profile implies the possibility that the venom may precipitate acute thrombotic syndrome that can be instantly

Table 3. Procoagulant effects of venoms from three pit vipers on mammalian fibrinogens

Venom	*H. hypnale*	*C. rhodostoma*	*C. purpureomaculatus*
Source of fibrinogen	Clotting time* (s) (mean ± SEM)	Clotting time (s) (mean ± SEM)	Clotting time (s) (mean ± SEM)
Rabbit	18.3 ± 0.9	30.7 ± 1.2	Turbidity > 120 s
Horse	42.2 ± 1.0	40.1 ± 0.6	Turbidity > 60 s
Dog	Turbidity** > 180 s	Turbidity > 180 s	Turbidity > 80 s
Goat	30.3 ± 0.9	33.0 ± 0.6	71.0 ± 0.6
Bovine	14.3 ± 0.3	11.3 ± 0.9	56.7 ± 0.7
Human	32.7 ± 0.7	28.4 ± 0.8	75.0 ± 0.6

* Clotting time is defined as the time (s) for apparent fibrin strands to appear in the mixture of venom and fibrinogen.** Turbidity refers to cloudiness appearing in the mixture of venom and fibrinogen, which may be interpreted as the formation of fibrin clots. Results are expressed as the mean ± SEM from three experiments.

fatal, as observed in an experiment where a rabbit was injected with *H. hypnale* venom intravenously (unpublished data). In cases where clot formation is less rapid, or is accompanied by a concurrent degradation, the most likely cause of death would be a consumptive coagulopathy that gradually affects hemostasis, causing prolonged bleeding and a "slower" death. This is consistent with main clinical features reported in cases of envenomation by *C. rhodostoma* (57, 58) and *H. hypnale* (11, 12). Deranged hemostasis leading to systemic bleeding has been the main feature in systemic envenomation by these crotalids. This severe, potentially fatal complication might only manifest itself temporarily following the bite as the pre-existing coagulant factors get depleted or rendered inactive by the venom more rapidly than their production, hence tilting the hemostatic balance towards a tendency to bleed. A recent example was that of an extensive retroperitoneal hemorrhage only detected five weeks after a bite by *H. hypnale* in an adult patient (59).

Nephrotoxicity Study

Ariaratnam *et al.* (11) reported a series of 302 hump-nosed pit viper bites in which 117 (39%) were systematically envenomed, all with hemostatic abnormalities, while 30 presented with acute renal failure. The kidney, as a highly vascularized excretory organ, is certainly vulnerable to acute injury by venom toxicity. The injury mechanisms can be either by direct effect of the toxins or secondary to hemostatic defect, intravascular hemolysis,

shock, immune response or rhabdomyolysis. Hematoxic and myotoxic venoms from viperids or crotalids are a rich source of enzymes that include phospholipases, endopeptidases, L-amino acid oxidases and metalloproteases which can directly cause cellular damage and kidney injury (60, 61). In the present study, *D. russellii* venom from Myanmar was administered to the positive control group (DV group) to produce acute kidney injury as the venom was known to be highly nephrotoxic (62-65).

Rats from both HV and DV groups treated with a sublethal dose (1/3 LD_{50}) of venoms of *H. hypnale* and *D. russelii* respectively, exhibited envenomation signs e.g. local hemorrhage, malaise, drowsiness and limping in the first few hours. All animals survived the 24-hour observation period. Gross inspection of urines showed marked hematuria among the DV group, with no inter-group difference in the urine volumes. Biochemical and microscopic urinalysis revealed significant proteinuria and hematuria in the DV group compared to the HV and NS groups ($p < 0.05$) (Table 4). The urea and creatinine levels, used as indicators for acute renal failure, were observably higher in the DV group although this elevation was insignificant in relation to the other two groups (Table 4), a finding probably attributable to the fact that the observation period was not sufficiently long. Nevertheless, the significant proteinuria and hematuria are sufficient indication that the injected *D. russelii* venom did cause acute nephrotoxicity in the animals. The absence of these features in rats
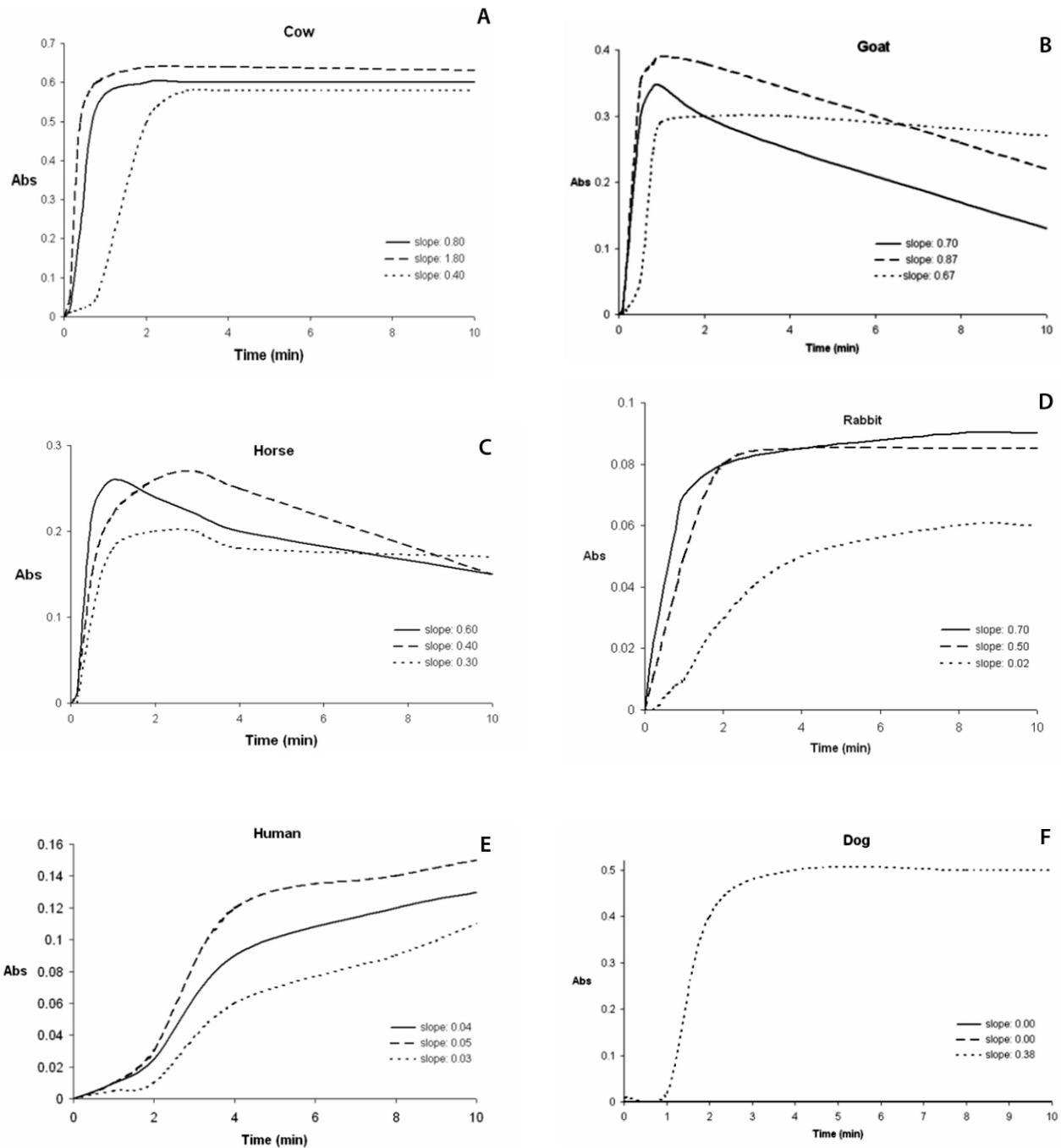
Figure 2. Time course of thrombin-like enzyme activity of three different crotalid venoms on fibrinogens from several mammals (**A**: cow; **B**: goat; **C**: horse; **D**: rabbit; **E**: human; **F**: dog). The slope (gradient) for each curve is calculated from the steepest upward portion of the graph representing the maximal clotting rate expressed as absorbance/minute. Absorbance reflects the change in mixture clarity, which is responsible for the formation of fibrin strands as well as their dissolution/degradation. "Clot" used in this discussion represents the formation of fibrins. The crotalid species are: —— *H. hypnale*; --- *C. rhodostoma*; ···· *C. purpureomaculatus*.

injected with a similar sublethal dose of *H. hypnale* venom suggested that the given dose of the venom does not cause direct nephrotoxicity. This is further demonstrated by light microscopic examination of histological cuts from the kidney (data not shown) and other vital organs (heart,

lung, liver, spleen and diaphragm) of rats injected with the sublethal dose of *H. hypnale* venom, where all tissues appeared histologically intact. Thus, a tentative explanation of nephropathy observed in some of the victims (11, 66) is that the kidney damage is likely the result of complications from

Table 4. Effects of sublethal dose (1/3 intramuscular LD$_{50}$) of *H. hypnale* and *D. russelii* venoms on blood and urine parameters (n = 3)

Parameter (mean ± SEM) / Group of rat	Normal saline	*Hypnale hypnale* venom	*Daboia russelii* venom
Urine			
Color	Straw	Straw	Reddish
Appearance	Clear	Clear	Hazy
Volume (mL)	4.2 ± 0.7	4.0 ± 0.8	3.9 ± 0.7
Protein (+)	0.0 ± 0.0	0.0 ± 0.0	2.3 ± 0.5*
Blood (hematuria) (+)	0.0 ± 0.0	0.0 ± 0.0	1.4 ± 0.8*
Microscopic red blood cell	0.0 ± 0.0	0.0 ± 0.0	3.3 ± 0.9*
Nitrite (+)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Bacteria (+)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Blood			
Urea (mmol/L)	6.0 ± 0.8	6.9 ± 0.6	14.5 ± 7.0
Creatinine (μmol/L)	18.7 ± 3.2	18.0 ± 3.6	32 ± 13.0

* Significantly different from the other two groups, $p < 0.05$, ANOVA

the venom's hematoxic effects mediated through its procoagulant and possibly fibrinolytic actions. This suggestion is in line with the view that the nephropathy was associated with disseminated intravascular coagulopathy (61).

CONFLICTS OF INTEREST
There is no conflict.

ETHICS COMMITTEE APPROVAL
The present study was approved by the Animal Care and Use Committee of the University of Malaya [ethics reference number: PM/03/03/2010/FSY(R)]. Moreover, animals were handled according to the guidelines given by CIOMS on animal experimentation.

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