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Biochemical and toxinological characterization of *Naja sumatrana* (Equatorial spitting cobra) venom

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Abstract: The lethal and enzymatic activities of venom from *Naja sumatrana* (Equatorial spitting cobra) were determined and compared to venoms from three other Southeast Asian cobras (*Naja sputatrix, Naja siamensis* and *Naja kaouthia*). All four venoms exhibited the common characteristic enzymatic activities of Asiatic cobra venoms: low protease, phosphodiesterase, alkaline phosphomonoesterase and L-amino acid oxidase activities, moderately high acetylcholinesterase and hyaluronidase activities and high phospholipase A_2. Fractionation of *N. sumatrana* venom by Resource® S cation exchange chromatography (GE Healthcare, USA) yielded nine major protein peaks, with all except the acidic protein peak being lethal to mice. Most of the protein peaks exhibit enzymatic activities, and L-amino acid oxidase, alkaline phosphomonoesterase, acetylcholinesterase, 5'-nucleotidase and hyaluronidase exist in multiple forms. Comparison of the Resource® S chromatograms of the four cobra venoms clearly indicates that the protein composition of *N. sumatrana* venom is distinct from venoms of the other two spitting cobras, *N. sputatrix* (Javan spitting cobra) and *N. siamensis* (Indochinese spitting cobra). The results support the revised systematics of the Asiatic cobra based on multivariate analysis of morphological characters. The three spitting cobra venoms exhibit two common features: the presence of basic, potentially pharmacologically active phospholipases A_2 and a high content of polypeptide cardiotoxin, suggesting that the pathophysiological actions of the three spitting cobra venoms may be similar.

Key words: *Naja,* cobra venom, enzyme activity, cardiotoxin.

INTRODUCTION

Snake envenomation is a major public health problem in tropical and subtropical countries. Recent studies indicated that globally, there are more than 400,000 envenomation cases annually of which at least 20,000 are fatal (1). Cobra (*Naja* sp.) is one of the commonest causes of snake envenomations in Asia, including Malaysia (2). Envenomation by *Naja* sp. is characterized by local necrosis, neurological paralysis and cardiotoxicity (3). Snake venoms are complex mixtures containing predominantly proteins and polypeptides and small amount of organic compounds and minerals. Many of the proteins exhibit enzymatic activities, whereas the polypeptides include neurotoxins, cardiotoxins, myotoxins and cytotoxins.

The systematics of the Asiatic cobra (formerly *Naja naja*) has recently been revised and many subspecies have been elevated to the status of full species (4). A number of the cobras have also been more appropriately renamed. According to Wűster (4), the spitting cobras of Southeast Asia, once known collectively as *Naja sputatrix* (formerly known as Malayan cobra) actually consist of three separate species: *Naja siamensis* (Indochinese spitting cobra) in Thailand, *Naja sumatrana* (Equatorial spitting cobra) in Peninsular Malaysia and Sumatra, and

Naja sputatrix (Javan spitting cobra) in Java and southern Indonesia.

Despite some early reports on the toxinological properties of the spitting cobra in Malaysia, it is not known whether the venom samples used for the investigations, labeled as *N. sputatrix,* were from *N. sumatrana* or *N. sputatrix* (5). This was due to insufficient understanding of the systematics of the Southeast Asian spitting cobra prior to the 1990's and it was assumed at that time that *N. sputatrix* refers to the Malaysian spitting cobra. In view of the medical importance of spitting cobras in Southeast Asia, it is important to re-investigate their venom properties. In this paper, we report the biochemical and toxinological properties of venom from *N. sumatrana* captured in central Malaysia, and compared with the biochemical and toxinological properties of venoms from the other two Southeast Asian spitting cobras (*N. sputatrix* and *N. siamensis*) as well as *N. kaouthia* (monocellate cobra), also a common cobra in Malaysia. Knowledge of the toxinological properties of these venoms is important in order to understand the pathophysiological effects of cobra bites in Southeast Asia.

MATERIALS AND METHODS

N. sumatrana venom was a pooled venom sample obtained from spitting cobras captured in central Malaysia (Snake Valley, Malaysia) and identified by one of the authors (Tan NH). Venoms of *N. sputatrix*, *N. siamensis* and *N. kaouthia* (Thailand) were purchased from Latoxan (France). All chemicals and reagents used in this study were of analytical grade and purchased from Sigma-Aldrich (USA). Resource® S ion exchange column (1 mL) was purchased from GE Healthcare (USA). Albino mice (ICR strain) were supplied from Laboratory Animal Center, Faculty of Medicine, University of Malaya. The animals were handled according to the CIOMS guidelines (6).

Fractionation of *Naja* sp. Venoms by Resource® S Ion Exchange Chromatography

Ten milligram of the venom dissolved in 200 µL of start buffer (20 mM MES, pH 6.0) was subjected to Resource® S cation exchange chromatography using the Shimadzu LC-20AD HPLC system (Japan). The column was first pre-equilibrated with the same start buffer, followed by elution by a linear gradient (0.0-0.8 M NaCl) of 0-30% from 5 to 30 minutes, followed by 30-100% from 30-55 minutes, at a flow rate of 1 mL/minute. Absorbance at 280 nm was measured.

Determination of Protein Concentration by Bradford Method

Protein content was determined according to the Bradford method (7). A standard curve was constructed using bovine serum albumin (0-10 µg of protein).

Enzyme Assays

Venom was dissolved in physiological saline (1 mg/mL) and used for enzyme assays and lethality determinations.

Protease assay

Protease activity was determined according to the method described by Kunitz (8) with slight modifications. One milliliter of 1% casein in 0.25 M sodium phosphate buffer, pH 7.75, and 50 µL of venom solution/fraction were incubated at 37°C for 30 minutes. The enzyme reaction was stopped and undigested casein was precipitated by adding 1 mL of 5% trichloroacetic acid. The mixture was then centrifuged at 10,000 x g for 10 minutes. The absorbance of the supernatant at 280 nm was then measured. Protease activity was arbitrarily defined as an increase of one absorbance unit at 280 nm per hour.

Phosphodiesterase assay

Phosphodiesterase activity was determined by a method modified from Lo *et al.* (9). First, 0.1 mL of venom solution/fraction was added to an assay mixture containing 0.5 mL of 0.0025 M Ca-bis-p-nitrophenyl phosphate, 0.3 mL of 0.01 M $MgSO_4$ and 0.5 mL of 0.17 M Veronal buffer, pH 9.0. The reaction was monitored by absorbance measurement at 400 nm. Phosphodiesterase activity was expressed in nanomole of product released/minute. Molar extinction coefficient at 400 nm was 8100 $cm^{-1}M^{-1}$.

Alkaline phosphomonoesterase assay

Alkaline phosphomonoesterase assay was carried out by a method modified from Lo *et al.* (9). A substrate mixture consisting of 0.5 mL of 0.5 M glycine buffer, pH 8.5; 0.5 mL of 0.01 M p-nitrophenylphosphate and 0.3 mL

of 0.01 M $MgSO_4$ was prepared. Then, 0.1 mL of venom solution/fraction was added to the substrate mixture, and incubated at 37°C for 30 minutes. After 20 minutes, 2 mL of 0.2 M sodium hydroxide was added, and absorbance at 400 nm was measured. Alkaline phosphomonoesterase activity was expressed as nanomole of product released/minute. The molar extinction coefficient at 400 nm was 18500 $cm^{-1}M^{-1}$.

5'-Nucleotidase assay

The 5'-nucleotidase activity was measured according to Heppel and Hilmore (10). First, 0.1 mL of venom solution/fraction was added to an assay mixture containing 0.5 mL of 0.02 M 5'-AMP (pre-adjusted to pH 8.5), 0.5 mL of 0.2 M glycine buffer, pH 8.5 and 0.1 mL of 0.1 M $MgSO_4$. The mixture was incubated at 37°C for 10 minutes, and the reaction was terminated by adding 1.5 mL of 10% trifluoroacetic acid. The quantity of inorganic phosphate released was determined by ascorbic acid method (11). Briefly, 1 mL of ascorbic acid reagent (equal parts of 3 M sulfuric acid, 2.5 % ammonium molibdate, 10% ascorbic acid and water) was added to the above mixture. The mixture was left at room temperature for 30 minutes and the absorbance at 820 nm was then determined. A standard curve of known phosphate concentrations was constructed. The enzyme activity was expressed as nanomole of phosphate/minute.

Hyaluronidase assay

Hyaluronidase activity was determined turbidimetrically as described by Dorfman (12). First, 0.1 mL of venom solution/fraction was added to an assay mixture containing 1 mL of 0.03 % (w/v) hyaluronic acid in 0.3 M sodium phosphate pH 5.35, and 1 mL of 0.02 M sodium phosphate, pH 7.0 containing 0.077 M sodium chloride. The mixture was incubated at 37°C for 45 minutes. Then 2.5 mL of acid albumin reagent [0.024 M sodium acetate, pH 3.75 containing 0.1% (w/v) bovine serum albumin] was added to 0.5 mL of the above reaction mixture and incubated at room temperature for another 10 minutes. The absorbance at 600 nm was then measured. A standard curve was constructed using a standard hyaluronidase (760 National Formulary units/ mg solid). Hyaluronidase activity was expressed in National Formulary Unit/mg (NFU/mg).

Phospholipase A_2 assay

Phospholipase A_2 assay was determined according to the acidimetric method of Tan and Tan (13). Briefly, an egg yolk suspension was prepared by mixing three equal parts consisting of one part chicken egg yolk, one part 18 mM calcium chloride, and one part 8.1 mM sodium deoxycholate. The pH of the egg yolk suspension was adjusted to 8.0 with 1 M sodium hydroxide, and stirred for 10 minutes to ensure homogenous mixing. Next, 0.1 mL of venom solution/fraction was added to 15 mL of egg yolk suspension to initiate the hydrolysis. The initial decrease in pH was measured by a pH meter. A decrease of 1 pH unit corresponds to 133 µmoles of fatty acid released. Enzyme activity was expressed as µmoles of fatty acid released/minute.

L-amino acid oxidase assay

L- amino acid oxidase activity was determined using L-leucine as substrate as described by Decker (14). First, 50 µL of 0.0075% 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-dianisidine, and incubated for 3 minutes at room temperature. Then, 50 µL of venom solution/fraction was added and the increase in absorbance at 436 nm was measured. The molar extinction coefficient was 8.31 x 10^{-3} $cm^{-1}M^{-1}$. L- amino acid oxidase activity was expressed as µmole of L-leucine oxidized/minute.

Acetylcholinesterase assay

Acetylcholinesterase activity was determined according to the method described by Ellman *et al.* (15). One hundred milliliters of venom solution/ fraction was added to an assay mixture contained 0.8 mL of 0.025 M sodium phosphate buffer, pH 7.5, 0.05 mL of 0.0125 M acetylthiocholine iodide and 0.05 mL of 6.66 mM dithionitrobenzoate (DTNB) containing 1 mg/mL sodium bicarbonate. The hydrolysis of the substrate was measured by the increase in absorbance at 412 nm. The molar extinction coefficient was 13600 $cm^{-1}M^{-1}$. The enzyme activity was expressed as µmole of product released/minute.

Determination of the Median Lethal Dose (LD_{50})

The median lethal dose (LD_{50}) of the venoms and venom fractions obtained from Resource®

S chromatography was determined by injecting appropriate dilutions of the sample intravenously into the caudal vein of mice (n = 4, 18-20 g). The survival ratio was determined after 24 hours. The LD_{50} (95% confidence interval) was then calculated by the Probit analysis (16).

Confirmation of the Identity of Fractions 8 and 9 as Cardiotoxins by Mass Spectrometry

The cardiotoxic fractions 8 and 9 isolated from Resource® S ion exchange chromatography were further purified by C-18 reverse-phase HPLC using a Shimadzu LC-20AD HPLC system (Japan), eluted with 0 to 100 % acetonitrile gradient. In both cases, there was only one major protein peak and the protein was subjected to tryptic digestion. The digested peptides were then analyzed by mass spectrometry (MALDI-TOF/TOF); ion spectra of peptides generated were interpreted using MASCOT database so that they could be assigned to known protein families. The deduced peptide ion sequence was submitted to BLAST to search for protein sequence similarity.

RESULTS AND DISCUSSION

Enzymatic and Lethal Activities of Venoms from *N. sumatrana* and Three Other Southeast Asian Cobras

The enzymatic activities of venoms from *N. sumatrana*, *N. sputatrix*, *N. siamensis* and *N. kaouthia* are shown in Table 1. All four venoms exhibited the common characteristic enzymatic activities of Asiatic cobra venoms: low protease, phosphodiesterase, alkaline phosphomonoesterase and L-amino acid oxidase activities, moderately high acetylcholinesterase and hyaluronidase activities and high phospholipase A_2 (17). Venoms from the two cobras of Thailand (*N. kaouthia* and *N. siamensis*) were highly lethal (with comparable intravenous (IV) LD_{50} of 0.22 µg/g mouse and 0.28 µg/g mouse respectively). *N. sputatrix* (Javan spitting cobra) was the least lethal (IV LD_{50} of 0.90 µg/g mouse) whereas the spitting cobra in Malaysia, *N. sumatrana*, presented a moderate IV LD_{50} of 0.50 µg/g mouse.

Table 1. Lethal and enzymatic activities of four Asiatic cobra venoms

Lethality/enzyme activity	Venom			
	N. sumatrana	*N. sputatrix*	*N. siamensis*	*N. kaouthia*
IV LD_{50} µg/g mouse	0.50 (0.40-0.62)	0.90 (0.59-1.36)	0.28 (0.18-0.42)	0.23 (0.115-0.34)
L-Amino acid oxidase (µmole/min/mg)	0.0059 ± 0.0002	0.0098 ± 0.0011	0.0119 ± 0.0006	0.0057 ± 0.0018
Phospholipase A_2 (µmole/min/mg)	311.2 ± 23.9	275.3 ± 0.0	239.4 ± 0	278.0 ± 43.9
Protease (unit/mg)	0.40 ± 0.07	0.83 ± 0.27	0.67 ± 0.03	0.91 ± 0.39
Phosphodiesterase (nmole/min/mg)	17.9 ±0.8	20.9 ± 1.9	11.2 ± 0.9	14.8 ± 0.1
Alkaline phosphomonoesterase (nmole/min/mg)	1.15 ±0.23	1.95 ± 0.11	4.47 ± 0.06	4.19 ± 0.69
5′-Nucleotidase (nmole/min/mg)	981.3 ± 277.1	589.7 ± 82.7	459.8 ± 17.6	1061.9 ± 45.9
Hyaluronidase (NFU/mg)	28.4 ± 0.9	84.7 ± 3.2	112.6 ± 9.8	31.5 ± 3.9
Acetylcholinesterase (µmole/min/mg)	1.59 ± 0.23	6.02 ± 0.30	2.85 ± 0.00	3.77 ± 0.43

Values in bracket for LD_{50} indicate 95% confidence intervals. To determine enzymatic activities, a 1 mg/mL saline venom stock solution was used. All enzymatic activity determinations were carried out in triplicate.

Fractionations of the *N. sumatrana* Venom by Resource® S Cation Exchange Chromatography

Figure 1 shows the elution profile of Resource® S cation exchange chromatography of *N. sumatrana* venom. There were nine major peaks with a number of minor peaks. The enzymatic activities, relative protein percentage and IV LD_{50} of the nine major fractions are shown in Table 2 and Figure 1. Of the nine major peaks, only peak 1, which contains acidic proteins, was not lethal to mice. Based on IV LD_{50} and relative elution volumes (slightly to moderately basic), peaks 2, 6 and 7 were probably the polypeptide neurotoxins. Peak 2 probably consisted of only polypeptide neurotoxin as it was devoid of any enzymatic activity. Their IV LD_{50}s were < 0.1 µg/g in mice and together they constituted about 23% of venom protein. Peak 8 and 9, the two very basic protein fractions, are presumably the polypeptide cardiotoxins, as it is well established that cobra venom cardiotoxins are highly basic polypeptides (18).

The cardiotoxins (fractions 8 and 9) together constituted about 40% of the venom protein. Similarly high contents of cardiotoxins in cobra venoms have been reported (19). To confirm their identity, fractions 8 and 9 were further purified by reverse-phase HPLC and the purified polypeptides were subjected to MALDI-TOF-TOF. Both polypeptides possessed the consensus sequence of cardiotoxin RGCIDVCPK, thereby confirming that fractions 8 and 9 were indeed cardiotoxin (20). Three peaks exhibited phospholipase A_2 activity: peak 1 (acidic, non-lethal), peak 4 (7% of venom protein, IV LD_{50} 0.8 µg/g) and peak 5, the major phospholipase A2 (17% of venom protein, IV LD_{50} 0.91 µg/g). Both the two lethal phospholipases A_2 were basic proteins.

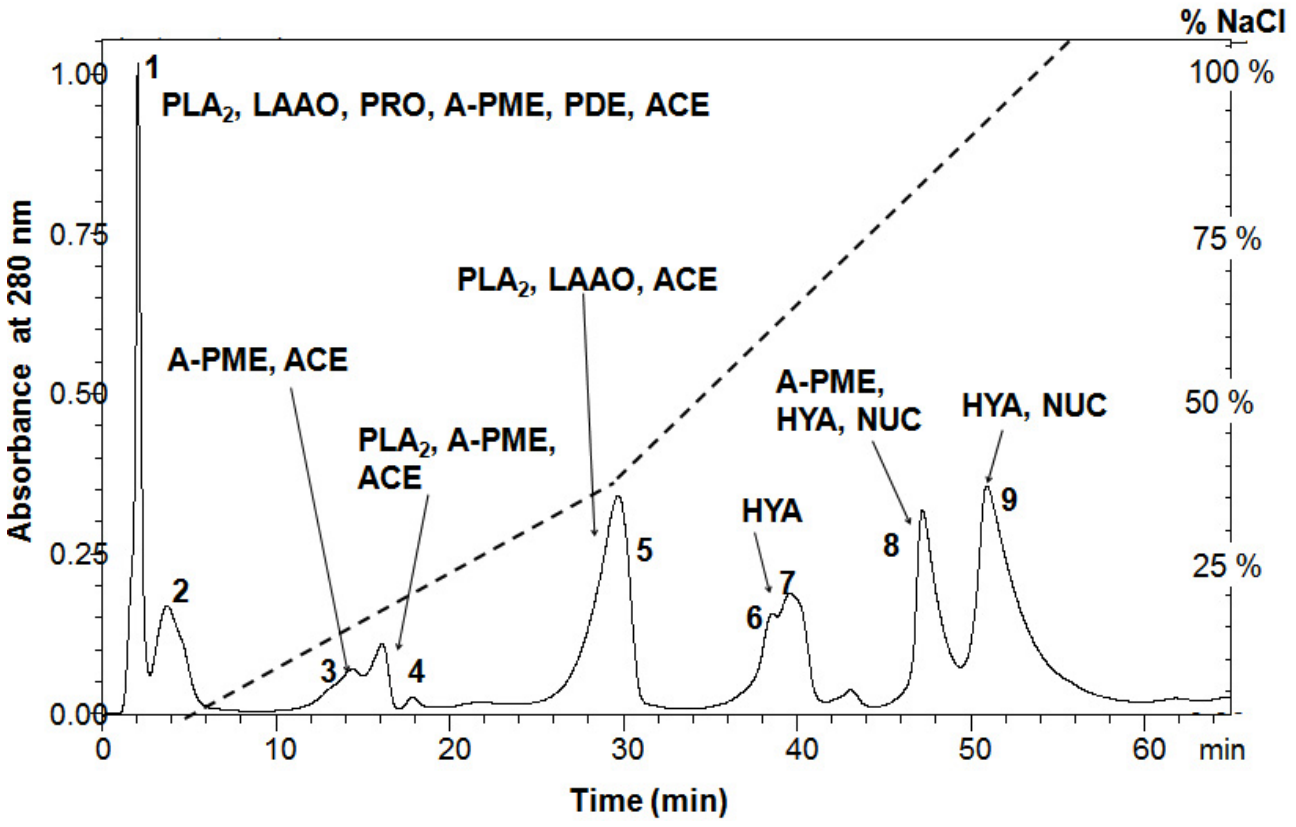

Figure 1. Resource® S cation exchange chromatography of *N. sumatrana* venom. Ten milligrams of *N. sumatrana* venom was injected into the column equilibrated with 20 mM MES, pH 6.0, and eluted by a linear gradient (0 to 0.8 M NaCl of 0-30% from 5 to 30 minutes, followed by 30-100% from 30 to 55 minutes). Flow rate was 1 mL/min. Dotted line: elution gradient. LAAO: L-amino acid oxidase; PLA_2: phospholipase A_2; PRO: protease; PDE: phosphodiesterase; NUC: 5'-nucleotidase; APME: alkaline phosphomonoesterase; HYA: hyaluronidase; ACE: acetylcholinesterase.

Table 2. Enzymatic and lethal activities of *Naja sumatrana* venom fractions obtained from Resource S ion exchange chromatography

	***N. sumatrana* venom fractions**							
	1	**2**	**3**	**4**	**5**	**6 & 7**	**8**	**9**
Protein content (%)	**4%**	**6%**	**4%**	**7%**	**17%**	**17%**	**19%**	**21%**
L-amino acid oxidase (µmole/min/mL)	0.0021	0	0	0	0.0022	0	0	0
Phospholipase A_2 (µmole/min/mL)	46.6	0	0	106.4	271.3	0	0	0
Protease (unit/mL)	0.53	0	0	0	0	0	0	0
Phosphodiesterase (nmole/min/mL)	0.35	0	0	0	0	0	0	0
Alkaline phosphomonoesterase (nmole/min/mL)	0.29	0	0.67	0.28	0	0	0.93	0
5′-Nucleotidase (nmole/min/mL)	0	0	0	0	0	0	16.9	529.6
Hyaluronidase (NFU/mL)	0	0	0	0	0	41.6	46.2	73.6
Acetylcholinesterase (µmole/min/mL)	5.29	0	1.49	6.38	1.47	0	0	0
IV LD_{50} (µg/g)	Non-toxic	0.08 (0.02-0.35)	0.87 (0.43-1.48)		0.91 (0.81-1.03)	0.07 (0.05-0.11)	0.43 (0.35-0.53)	0.22 (0.08-0.62)

Values in bracket for LD_{50} indicate 95% confidence intervals. To determine enzymatic activities, 50-100 µL of the protein fractions obtained from Resource® S cation exchange chromatography of *Naja sumatrana* venom (10 mg) was used. All enzymatic activity determinations were carried out in triplicate.

All major protein peaks except peak 2 exhibited enzymatic activities; and L-amino acid oxidase, alkaline phosphomonoesterase, acetylcholinesetrase, 5'-nucleotidase and hyaluronidase were observed in multiple forms. Figure 1 shows that all the major lethal protein fractions, except peak 2, contained two to three different types of enzymatic activities. To isolate the pure polypeptide toxins or phospholipases A_2, additional chromatographic steps were therefore necessary to remove the enzymes.

Fractionation of the *N. sputatrix, N. siamensis* and *N. kaouthia* Venom by Resource® S Cation Exchange Chromatography

Figure 2 (B, C and D) show the Resource® S cation exchange chromatographic fractionation of *N. sputatrix*, *N. siamensis and N. kaouthia* venoms under the same conditions as the fractionation of *N. sumatrana* venom (Figure 2 – A). The phospholipase A_2 activity of the peaks is also shown. It is interesting to note that the chromatographic pattern of *N. sumatrana* venom is similar to, yet distinct from, *N. sputatrix* venom, but differs greatly from that of *N. kaouthia* venom, in agreement with the findings of Vejayan *et al.* (21). On the other hand, the chromatographic pattern of *N. siamensis* venom is similar to that of *N. kaouthia* venom.

Comparison of the chromatograms clearly indicates that the composition of the *N. sumatrana* venom is definitely different from venoms obtained from the other two spitting cobras, *N. sputatrix* and *N. siamensis*. This supports the revised systematics of the Asiatic cobra based on multivariate analysis of morphological

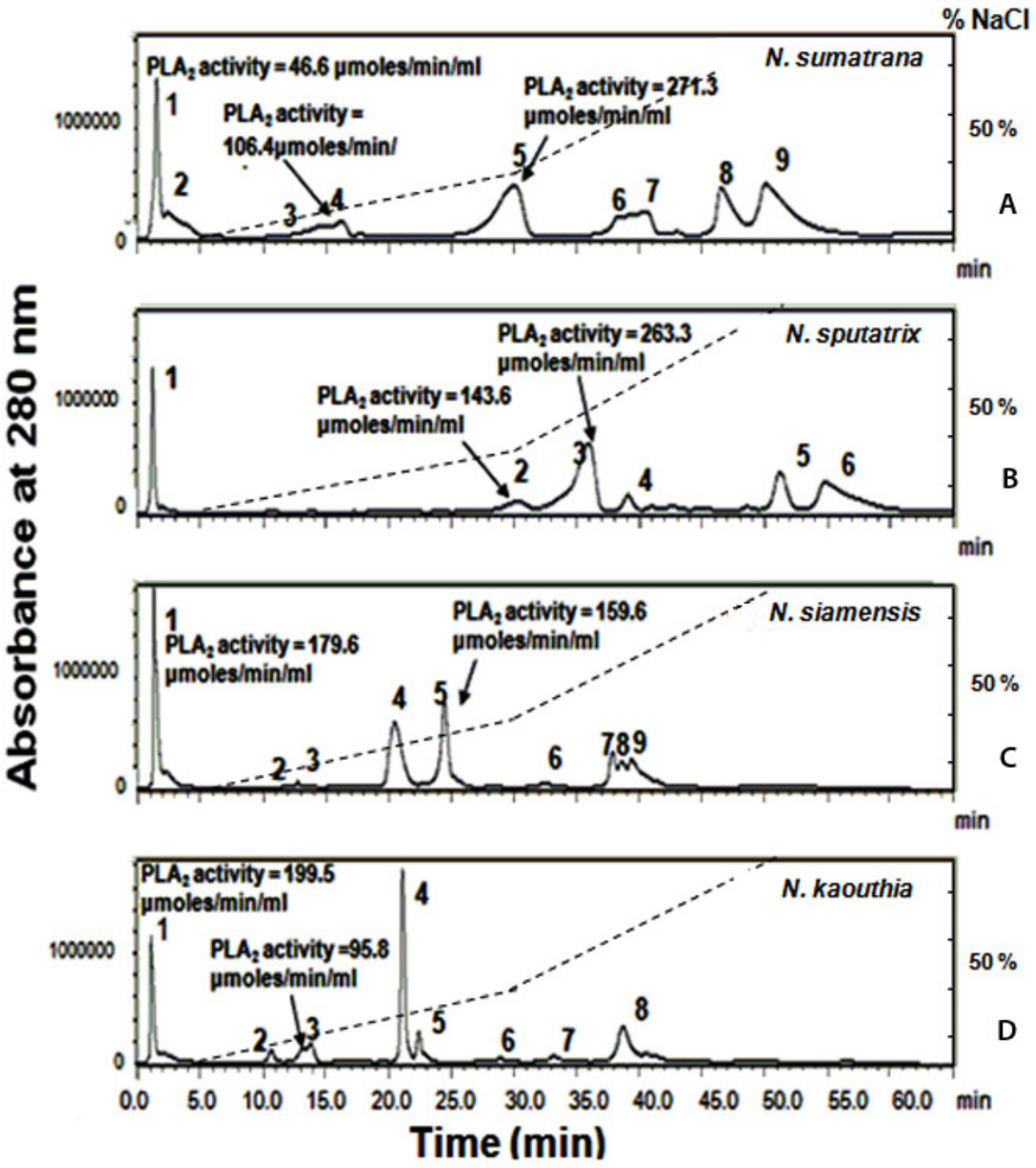

Figure 2. Fractionation of four Southeast Asian cobra venoms by Resource® S cation exchange chromatography. Ten milligrams of the venom was injected into the column equilibrated with 20 mM MES, pH 6.0, and eluted by a linear gradient (0 to 0.8 M NaCl of 0-30 % from 5 to 30 minutes, followed by 30-100% from 30-55 minutes). Flow rate was 1 mL/minute. Dotted line: gradient of elution. The venoms were from: (**A**) *N. sumatrana*; (**B**) *N. sputatrix*; (**C**) *N. siamensis* and (**D**) *N. kaouthia*.

characteristics (22). Thus, the results and data in all previous publications on Malaysian spitting cobra (also known as Malayan cobra) based on venom labelled as *N. sputatrix* should be re-interpreted as properties of Javan spitting cobra venom instead.

Examination of the chromatograms in Figure 2 also reveals an interesting common feature of the venoms from the Southeast Asian spitting cobras – all three spitting cobra venoms contained relatively basic phospholipases A_2 (peak 5 in *N. sumatrana*, peak 2 and 3 in *N. sputatrix*, peak

Table 3. Cardiotoxin contents in the four Southeast Asian cobra venoms.

Venom	Cardiotoxin peaks in chromatogram	Percentage of total venom protein
Naja sumatrana	8, 9	40%
Naja sputatrix	5, 6	35%
Naja siamensis	7, 8, 9	30%
Naja kaouthia	8	18%

Percentage of total venom protein was estimated from total area under the various peaks (Figure 2).

5 in *N. siamensis*), whereas there was only one (minor) neutral phospholipase A_2 (peak 3) in the non-spitting *N. kaouthia* venom. Basic phospholipases A_2 are known to be involved in many pharmacological actions of cobra venoms including myotoxicity, cytotoxicity, cardiotoxicity and edema-inducing activities (23-28). Also, we note that all three Southeast Asian spitting cobra venoms contained a substantial amount (30-40%) of polypeptide cardiotoxins (Table 3). This would suggest that the pathophysiological actions of the three spitting cobra venoms may be similar. On the other hand, *Naja kaouthia* venom did not possess basic phospholipase A_2 and presented a much lower cardiotoxin content (18%) but very high neurotoxin content (peak 4, 40% of venom protein). This is consistent with the clinical reports that a high percentage of patients of *N. kaouthia* envenomation in Thailand experienced neurotoxic symptoms (29).

CONCLUSIONS

N. sumatrana venom exhibits the common characteristic enzymatic activities of Asiatic cobra venoms: The protein composition of *N. sumatrana* venom is distinct from venoms of the other two regional spitting cobras, *N. sputatrix* and *N. siamensis*. However, all three spitting cobra venoms contain basic phospholipases A_2 and a high content of polypeptide cardiotoxin. Our results support the revised systematics of the Asiatic cobra based on multivariate analysis of morphological characteristics.

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CONFLICTS OF INTEREST

There is no conflict.

FINANCIAL SOURCE

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ETHICS COMMITTEE APPROVAL

The present study was approved by the Animal Care and Use Committee of the University of Malaya [PM/03/03/2010/FSY(R)]. Moreover, the study animals were handled following the CIOMS guidelines on animal experimentation.

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