CHAPTER 4

BIOCHEMICAL AND TOXINOLOGICAL CHARACTERIZTION OF *NAJA SUMATRANA* (EQUATORIAL SPITTING COBRA) VENOM

4.1 INTRODUCTION

Cobra (*Naja* sp.) is one of the commonest causes of snake envenomaton in Asia, including Malaysia (Warrell, 1995). In the last two decades, the systematics of the Asiatic cobra (formerly known as *Naja naja*) has been revised and many subspecies have been elevated to the status of full species (Wüster, 1996). A number of cobras have also been appropriately renamed. For example, the spitting cobras of Southeast Asia, once known collectively as *Naja sputatrix* (formerly known as Malayan cobra) has been divided into three separate species: *Naja siamensis* (Indochinese spitting cobra) in Thailand, *Naja sumatrana* (Equatorial spitting cobra) in Peninsula 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 venom 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* (Tan, 1991). This was due to the confusion of the systematics of Southeast Asian spitting cobra prior to earlier 1990's and it was then assumed that *N. sputatrix* refers exclusively to the Malayan spitting cobra (was known as Malay cobra at one time). In view of the medical importance of spitting cobras in Southeast Asia, it is important to re-investigate their venom properties. In the present study, the biochemical and toxinological properties of venom from *N. sumatrana* were examined and compared with the biochemical and toxinological properties of venoms from the other Southeast Asian spitting cobras (*N. sputatrix* and *N. siamensis*) as well as *N. kaouthia* (monocellate cobra) which is also a common cobra in Malaysia.

The knowledge of the toxinological properties of these venoms is important in order to understand the pathophysiological of cobra bites in Southeast Asia.

4.2 METHODS

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

Ten milligrams of the venom dissolved in 200 µl of start buffer (20 mM MES, pH 6.0) was subjected to Resource® S ion exchange chromatography using the Shimadzu LC-20AD HPLC system (Japan). The column was first pre-equilibrated with the start buffer, followed by elution by a linear gradient of 0-30% of 0.8 M NaCl from 5 to 30 min, followed by 30-100% of 0.8 M NaCl from 30-55 min, at a flow rate of 1 ml/min. Absorbance at 280 nm was measured using the Shimadzu SPD-20 A Prominence UV/Vis Detector (Japan). The fractions were collected manually and kept at -20°C.

4.2.2 Determination of Protein Concentration

Protein concentration was determined according to the method as described in Chapter 3, section 3.2.1.1.

4.2.3 Determination of enzymatic activities

Venom (1 mg/ml) was dissolved in phosphate buffered saline, PBS and used for enzyme assays and lethality determinations.

4.2.3.1 Protease assay

Protease activity was determined according to the method described by Kunitz (1947) with slight modifications. One milliliter of 1% (w/v) 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 min. The enzyme reaction was stopped and undigested casein was precipitated by adding 1 ml of 5% (w/v) trichloroacetic acid. The mixture was then centrifuged at 10,000 xg for 10 min. The absorbance of the supernatant was measured at 280 nm. Protease activity was arbitrarily defined as an increase of one absorbance unit at 280 nm per hour.

4.2.3.2 Phosphodiesterase assay

Phosphodiesterase activity was determined by a method modified from Lo *et al.* (1966). The venom solution/fraction (0.1 ml) 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 magnesium sulfate and 0.5 ml of 0.17 M veronal buffer (sodium barbital), pH 9.0. The reaction was monitored by absorbance measurement at 400 nm. Phosphodiesterase activity was expressed in nmole of product released/min. Molar extinction coefficient at 400 nm was 8100 $cm^{-1}M^{-1}$.

4.2.3.3 Alkaline phosphomonoesterase assay

Alkaline phosphomonoesterase assay was carried out by a method modified from Lo *et al.* (1966). 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 magnesium sulfate was prepared. The venom solution/fraction (0.1 ml) was added to the substrate mixture, and incubated at 37°C for 30 min. After 20 min, 2 ml of 0.2 M sodium hydroxide was added, and absorbance at 400 nm was measured. Alkaline phosphomonoesterase activity was expressed as nmole of product released/min. The molar extinction coefficient at 400 nm was 18500 $cm^{-1}M^{-1}$.

4.2.3.4 5'-Nucleotidase assay

The 5'-nucleotidase activity was measured according to Heppel and Hilmore (1955). The venom solution/fraction (0.1 ml) was added to an assay mixture containing 0.5 ml of 0.02 M 5'-adenosine monophosphate (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 magnesium sulfate. The mixture was incubated at 37°C for 10 min, and the reaction was terminated by adding 1.5 ml of 10% (v/v) trifluoroacetic acid. The quantity of inorganic phosphate released was determined by ascorbic acid method (Chen *et al*., 1956). Briefly, 1 ml of ascorbic acid reagent (equal parts of 3 M sulfuric acid, 2.5% (w/v) ammonium molibdate, 10% (w/v) ascorbic acid and UltraPure^{TM} water) was added to the above mixture. The mixture was incubated at room temperature for 30 min. The absorbance at 820 nm was then determined. A standard curve of known phosphate concentrations was constructed. The enzyme activity was expressed as nmole of phosphate/min.

4.2.3.5 Hyaluronidase assay

Hyaluronidase activity was determined turbidimetrically as described by Dorfman and Ott (1948). The venom solution/fraction (0.1 ml) 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 min. Subsequently, 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 min. The absorbance at 600 nm was then measured. A standard curve was constructed using a standard hyaluronidase (760 National Formulary Unit/ mg

solid). Hyaluronidase activity was expressed in National Formulary Unit/mg (NFU/mg).

4.2.3.6 Phospholipase A_2 assay

Phospholipase A_2 activity was determined according to the acidimetric method of Tan and Tan (1988a). 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 min 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 decrease in pH was measured. A decrease of 1 pH unit corresponds to 133 μmoles of fatty acid released. Enzyme activity was expressed as μmoles of fatty acid released/min.

4.2.3.7 L-amino acid oxidase assay

L-amino acid oxidase activity was determined using L-leucine as substrate as described by Decker (1977). Briefly, 50 μl of 0.0075% (w/v) horseradish peroxidase (100 purpurogalin unit/mg) was added to 0.9 ml of 0.2 M triethanolamine buffer, pH 7.6, containing 0.1% (w/v) L-leucine and 0.0075% (w/v) o-dianisidine, and incubated for 3 min 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/min.

4.2.3.8 Acetylcholinesterase assay

Acetylcholinesterase activity was determined according to the method described by Ellman *et al*. (1961). One hundred microliters 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/min.

4.2.4 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 as described in, Chapter 3, section 3.2.5.

4.2.5 Statistical analysis

LD_{50} of the venoms and venom fractions are expressed as means with 95% confidence Intervals (C.I) and were calculated using the Probit analysis method of Finney (Finney, 1952). The significance of difference in enzyme activities was determined by one-way analysis of variance (ANOVA), the level of significance was set at $p = 0.05$.

4.3 RESULTS

4.3.1 Fractionation of the *N. sumatrana* venom by Resource® S Ion Exchange Chromatography

Figure 4.1 shows the elution profile of Resource® S ion exchange chromatography of *N. sumatrana* venom. Resource® S ion exchange chromatography resolved the venom into nine major protein fractions with two minor fractions (fractions 5 and 9). The enzymatic activities, relative protein contents and LD_{50} (*i.v.*) of the nine major fractions are shown in Table 4.1 and Figure 4.1.

Of the nine major fractions, only fraction 1, which contains acidic proteins, was not lethal to mice. Fractions 2, 7 and 8 exhibited LD_{50} (*i.v.*) of 0.08 and 0.07 μg/g, respectively. They constituted about 23% of venom proteins. Fractions 10 and 11 constituted about 40% of the venom protein with LD_{50} (*i.v.*) of 1.00 μg/g. There are three fractions that exhibited phospholipase A_2 activity which are fraction 1 (acidic, non-lethal), fraction 4 (7% of venom protein, LD_{50} (*i.v.*) 2.00 μg/g) and fraction 6, the major phospholipase A_2 (17% of venom protein, LD_{50} (*i.v.*) 2.00 μg/g). Both the two lethal phospholipases A_2 were basic proteins.

All major fractions except fraction 2 possessed enzymatic activities. Fraction 1 possessed phospholipase A_2, L-amino acid oxidase, protease, phosphodiesterase, alkaline phosphomonoesterase, and acetylcholinesterase activities. Fraction 3 exhibited alkaline phosphomonoesterase and acetylcholinesterase activities. Alkaline phosphomonoesterase was also found in fractions 4 and 10. Both fractions 4 and 6 also possessed acetylcholinesterase activities besides exhibiting significantly high activities of phospholipase A_2. Fractions 10 and 11 were found to exhibit 5'-nucleotidase and hyaluronidase activities.

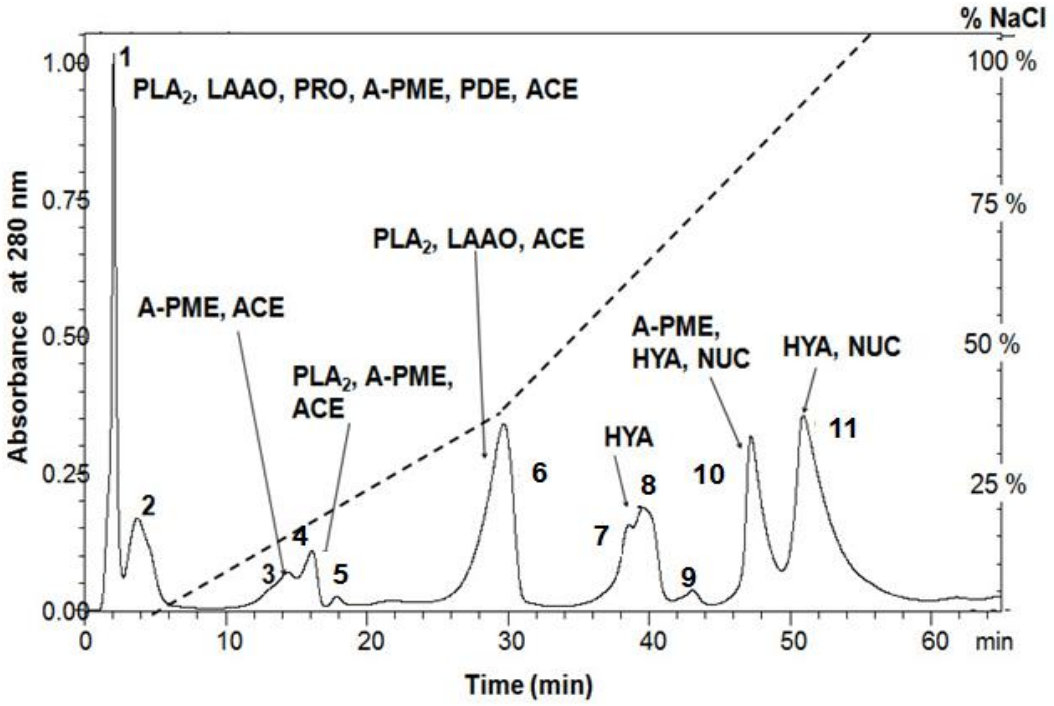

Figure 4.1: Resource® S ion exchange chromatography of *N. sumatrana* venom.

Ten milligrams of *N. sumatrana* venom was injected into the column equilibrated with 20mM MES, pH 6.0, and eluted by a linear gradient (0-30% of 0.8 M NaCl from 5 to 30 min, followed by 30-100% from 30 to 55 min). 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; A-PME: alkaline phosphomonoesterase; HYA: hyaluronidase; ACE: acetylcholinesterase.

Table 4.1: Enzymatic and lethal activities of *N. sumatrana* venom fractions obtained from Resource® S ion exchange chromatography.

	***N. sumatrana* venom fractions**							
	1	**2**	**3**	**4**	**6**	**7 & 8**	**10**	**11**
% 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
LD_{50} (*i.v.*), μg/g	Non-lethal	0.08 (0.02-0.35)	2.00 (1.09-3.40)		2.00(1.09-3.40)	0.07 (0.05-0.11)	1.00 (0.95-1.23)	1.00 (0.95-1.23)

Values in parentheses for LD_{50} indicated 95% confidence intervals. To determine enzyme activities, 50-100 μl of the protein fractions obtained from Resource S® ion exchange chromatography of *N. sumatrana* venom (10 mg) were used. Determinations of enzyme activities were carried out in triplicate.

4.3.2 A comparative study of the toxinological properties of *N. sumatrana* venom with other Southeast Asian cobra venom

4.3.2.1 Determination of the enzymatic and lethal activities of venoms from *N. sumatrana* and three other Southeast Asian cobras

The intravenous (*i.v.*) LD_{50} of venoms from *N. kaouthia* and *N. siamensis*, *N. sputatrix* and *N. sumatrana* were determined to be 0.22 μg/g, 0.28 μg/g, 0.90 μg/g and 0.50 μg/g, respectively.

The enzymatic activities of the four venoms are shown in Table 4.2.

All the four cobra venoms exhibited a comparable and high phospholipase A_2 activity and low protease activity (0.40–0.91 unit/mg). The ranges of other enzymatic activities were L-amino acid oxidase, 0.057–0.0119 μmole/min/mg; phosphodiesterase, 11.2–20.9 nmole/min/mg; alkaline phosphomonoesterase, 1.15–4.47 nmole/min/mg; hyaluronidase, 28.4–112.6 NFU/mg; acetylcholinesterase, 1.59–6.02 μmole/min/mg.

These data show that the four cobra venoms exhibited rather different enzyme contents.

Table 4.2: Lethality and enzymatic activities of four Asiatic cobra venoms.

Lethality / Enzymatic activity	Venom			
	N. sumatrana	***N. sputatrix***	***N. siamensis***	***N. kaouthia***
LD_{50} (*i.v.*) 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)	275.3 ± 0	311.2 ± 23.9	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 parentheses for LD_{50} (*i.v.*) indicated 95% confidence intervals. To determine enzymatic activities, a 1 mg/ml saline venom stock solution was used. All enzymatic activities determinations were carried out in triplicate.

4.3.3 Fractionation of the *N. sputatrix, N. siamensis* and *N. kaouthia* venom by Resource® S Ion Exchange Chromatography

Figure 4.2 (B, C and D) shows the Resource® S ion exchange chromatography fractionation of *N. sputatrix, N. siamensis and N. kaouthia* venoms under the same conditions as the fractionation of *N. sumatrana* venom (Figure 4.2A). The phospholipase A_2 activity of the fractions was also shown in the chromatogram.

Resource® S ion exchange chromatography resolved the *N. sputatrix* venom into six major fractions, *N. siamensis* venom into nine major fractions and *N. kaouthia* venom into eight major fractions.

All the Southeast Asian cobra venoms contained fractions exhibiting significantly high activities of phospholipase A_2 - fractions 4 and 6 in *N. sumatrana* venom (106.4 and 271.3 μmoles/min/ml, respectively), fractions 2 and 3 in *N. sputatrix* venom (143.6 and 263.3 μmoles/min/ml, respectively), fractions 1 and 5 in *N. siamensis* venom (179.6 and 159.6 μmoles/min/ml, respectively) and fractions 1 and 3 in *N. koauthia* venom (199.5 and 95.8 μmoles/min/ml, respectively).

It is noteworthy that all three Southeast Asian spitting cobra venoms contained 30-40% of polypeptide cardiotoxins (as shown in Table 4.3). In contrast, the non-spitting *N. kaouthia* venom has a rather low cardiotoxin content (18%). However, *N. kaouthia* venom possesses a remarkable high neurotoxin content which corresponds to fraction 4 in chromatogram (Figure 4.2D). The neurotoxin fraction constitutes about 40% of venom protein.

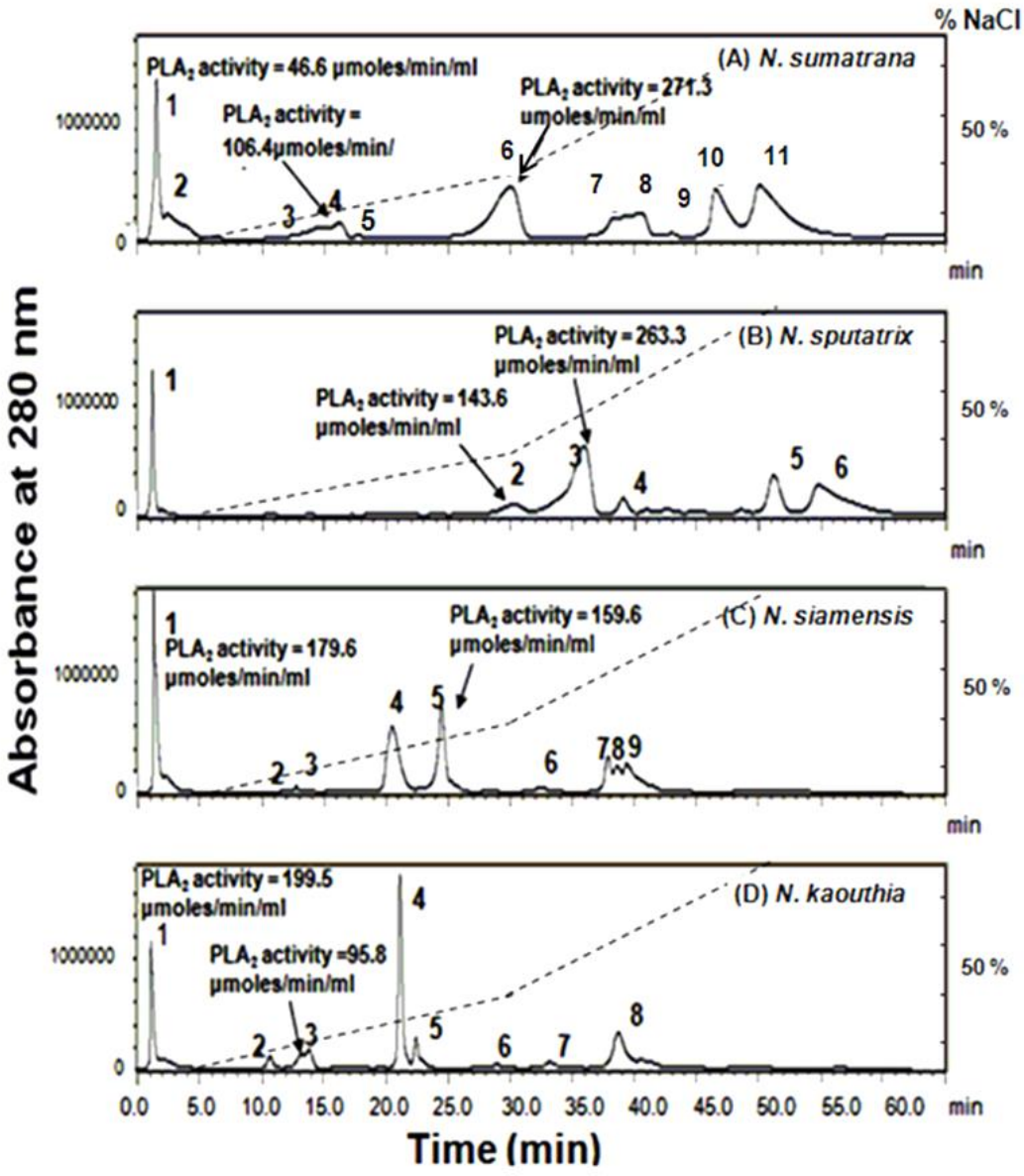

Figure 4.2: Fractionation of four Southeast Asian cobra venoms by Resource® S ion exchange chromatography.

Ten milligrams of the venom was injected into the column equilibrated with 20 mM MES, pH 6.0, and eluted by a linear gradient (0-30 % of 0.8 M NaCl from 5 to 30 min, followed by 30-100% from 30-55 min). Flow rate was 1 ml/min. Dotted line: gradient of elution. The venoms were from: (A) *N. sumatrana*; (B) *N. sputatrix*; (C) *N. siamensis* and (D) *N. kaouthia.*

Table 4.3: Cardiotoxin contents in the four Southeast Asian cobra venoms.

Venom	Cardiotoxin fractions in chromatogram	Percentage of total venom protein (%)
Naja sumatrana	10,11	40
Naja sputatrix	5,6	35
Naja siamensis	7,8,9	30
Naja kaouthia	8	18

4.4 DISCUSSION

Resource® S ion exchange chromatography of *N. sumatrana* venom resolved the venom into nine major fractions. Based on the LD_{50} (*i.v.*) (less than 0.1 μg/g in mice) and relative elution volumes (slightly to moderately basic fraction), fractions 2, 7 and 8 were presumably polypeptide neurotoxins. Fraction 2 probably consisted only of polypeptide neurotoxin as it was devoid of any enzymatic activity. On the other hand, fractions 10 and 11, the two very basic proteins, are probably polypeptide cardiotoxins, as it was well established that cobra venom cardiotoxins are highly basic polypeptides (Tu, 1977). These assignments of fractions to neurotoxins and cardiotoxins) have been proven by mass spectrometry in the later proteomic characterization of *N. sumatrana* venom (Chapter 5, section 5.3).

L-amino acid oxidase, alkaline phosphomonoesterase, acetylcholinesterase, 5'-nucleotidase and hyaluronidase occurred in multiple forms as shown in Figure 4.1. All the major lethal protein fractions, except fraction 2, contained two to three different types of enzymatic activities. Thus, in order to isolate the pure polypeptide toxins or phospholipase A_2, additional chromatographic steps would be required.

All the four Southeast Asian cobra (*N. sumtrana, N. sputatrix, N. siamensis* and *N. kaouthia*) venoms exhibited common features of enzyme activities found in other 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 (Tan and Tan, 1988b).

The Resource® S ion exchange chromatograpy of the *N. sumatrana, N. sputatrix, N. siamensis* and *N. kaouthia* venom revealed the differences in protein

composition of the Southeast Asian spitting cobra and non-spitting cobra venoms. It is interesting to note that the chromatographic pattern of *N. sumatrana* venom is similar to, yet distinct from, *N. sputatrix* venom, but differed substantially from that of *N. koauthia* venom, in agreement with the findings of Vejayan *et al*. (2010). On the other hand, the chromatographic pattern of *N. siamensis* is similar to that of *N. kaouthia* venom.

Comparison of the chromatograms clearly indicates that the composition of *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 characteristics (Wüster and Thorpe, 1991). Thus, the results and data in all previous publications on Malaysian spitting cobra (also known as Malayan cobra) venom based on venom labeled as *N. sputatrix* should be re-interpreted as properties of venom of Javan spitting cobra instead.

The chromatographic fractionation of the four Southeast Asian cobra venoms also reveals an interesting common feature of the venoms from spitting cobra: the presence of basic phospholipases A_2, which were not observed in non-spitting cobra, *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 (Bhat and Gowda, 1989; Das *et al*., 2011; Fletcher *et al.*, 1982; Mukherjee, 2008 and Yamaguchi *et al.*, 2001). Besides, the polypeptide cardiotoxin present in a substantial amount in the Southeast Asian spitting cobra venoms. This would suggest that the three spitting cobra venoms may possess similar pathophysiological actions.

On the other hand, *N. kaouthia* venom did not possess basic phospholipase A_2 and contained a lower amount of polypeptide cardiotoxins, but possessed very

high neurotoxin content. This is consistent with the clinical reports that a high percentage of patients of *N. kaouthia* envenomation in Thailand experienced neurotoxic symptoms (Viravan *et al*., 1986).