Electrospray Mass Spectrometry of Toxin from Complete and Incomplete Monocellated Hood Marking Thai Cobra (*Naja naja kaouthia*) Venoms

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Crude Thai Cobra (*Naja naja kaouthia*) venoms from snakes with complete and incomplete monocellated hood markings were fractionated and fractions were collected using C-18 HPLC. Venoms from the two sources show similar chromatographic patterns. Each fraction was tested for its LD_{50} value in mice. Electrospray mass spectrometry reveals the molecular weight of 7820 Da for the most toxic fraction of venom from Thai cobras with the complete and incomplete monocellated hood markings. This toxin accounts for about 20-30% of the total protein in the venom.

Key words: Toxin, *Naja naja kaouthia*, venoms, molecular weight.

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อิเล็กโทรสเปรย์แมสสเปกโทรเมตรีของสารพิษจากพิษของงูเห่าไทยที่มีดอกจันสมบูรณ์และไม่สมบูรณ์

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วารสารวิจัยวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย, **23 (2)**

เมื่อนำพิษงูเห่าไทยซึ่งรีดได้จากงูเห่าไทย (*Naja naja kaouthia*) ที่มีดอกจันสมบูรณ์และชนิดที่มีดอกจันไม่สมบูรณ์ มาทำการแยกโดยวิธี C-18-HPLC พบว่าได้โครมาโตแกรมที่คล้ายคลึงกัน ผลการทดสอบค่าความเป็นพิษในหนูทดลอง (LD_{50}) ของแต่ละส่วนแยกที่เก็บได้จากโครมาโตกราฟี พบว่าส่วนแยกที่ให้พิษสูงสุด คิดได้เป็นปริมาณ 20-30% ของปริมาณโปรตีนทั้งหมดในพิษงูที่แยกได้ เมื่อนำส่วนแยกที่ให้พิษสูงสุดนี้มาหาน้ำหนักโมเลกุลโดยวิธีอิเล็กโทรสเปรย์แมสสเปกโทรเมตรี พบว่าส่วนแยกดังกล่าวมีความบริสุทธิ์สูงมาก ส่วนโปรตีนที่มีพิษสูงสุดที่ได้จากงูเห่าไทยที่มีดอกจันสมบูรณ์และไม่สมบูรณ์มีน้ำหนักโมเลกุลเท่ากันคือ 7820 ดาลตัน

คำสำคัญ พิษงู, งูเห่าไทยที่มีดอกจันสมบูรณ์และไม่สมบูรณ์, น้ำหนักโมเลกุล

INTRODUCTION

The Siamese cobra subspecies *(Naja naja kaouthia)* is the dominant cobra of family Elapidae in Thailand.(1) Various biological activities identified in cobra venom include phospholipase A2 activity, cardiotoxicity, haemolysis activity, acetylcholinesterase activity and hyaluronidase activity. These activities result from the complex composition of the venom, which is usually composed of water, buffer, various enzymes and many toxins.

Attempts to elucidate chemical components in venoms have been made. Studies of neurotoxins in venom from some Asian cobras such as *Naja naja siamensis* (Thai cobra), *Naja naja naja* (Indian cobra), *Naja naja oxiana* (Taiwan cobra) and *Naja naja atra* (Chinese cobra) revealed the presence of small peptide toxins of molecular weight of 7000-8000 Da in each venom.(2,3) One of the toxins in venom from *Naja naja siamensis* was identified and structurally characterized as a single chain polypeptide with seventy - one amino acid residues and a molecular weight of 7820 Da. A similar neurotoxin was also found in venom from the Indian cobra, *Naja naja naja*, with only three differing amino acid residues (from the total of 71 residues).(4,5) Although investigation of the composition of the venoms of *Naja naja kaouthia* had suggested that this venom contained similar major toxin components to that of *Naja naja siamensis,*(6) none of the neurotoxins from *Naja naja kaouthia* has been characterized previously.

In this study, venom from *Naja naja kaouthia* was fractionated by reverse phase HPLC. Each collected fraction was tested for the LD_{50} value in mice. The molecular mass of the most lethal fraction was then determined by electrospray ionization mass spectrometry (ESI-MS). Moreover, since there are some variations of the hood-marking pattern among Siamese cobras,(1) experiments were performed in parallel for 1) venom obtained from Siamese cobras wsith complete monocellated hood markings and 2) venom obtained from Siamese cobras with incomplete hood markings.

MATERIALS AND METHODS

Materials

Venoms from both complete and incomplete monocellated hood marking cobras were obtained from the corresponding types of Thai cobras (*Naja naja kaouthia*) raised at the snake farm of the Queen Saovabha Memorial Institute. Venom was obtained by hand pressure on the venom glands of the snakes. Collected venom was kept at -24°C until needed. All solvents used were HPLC grade purchased from J.T. Baker Inc., Phillipsburg, NJ, USA. All other chemicals were analytical grade.

Liquid Chromatography

The HPLC system consisted of a SP Thermo Separation unit from Spectra Physics (Paloalto, CA, USA) connected with the HPLC pump P4000 (Spectra Physics) and programmable UV detector model 1000 (Spectra Physics). The HPLC column used was a C-18 Hypersil ODS column (250 mm × 4.6 mm ID, 10 μm particle, Shandon Scientific Ltd., Runcorn, UK).

The crude venom was diluted with one volume of 0.15 M ammonium formate buffer, pH 2.7 and then centrifuged at 10,000 g. The supernatant was filtered through 0.22 μm pore size Millipore filter paper before it was fractionated with the C-18 HPLC system (20 μl injected), using a two-solvent system, aqueous 0.15 M ammonium formate of pH 2.7 (A) and acetonitrile (B). The gradient elution was carried out at 25°C with an isocratic flowrate of 1.5 ml/min as follows: 0-10 min with 100% A, then linearly increased to A:B = 55:45 during 10 to 100 min. The UV detector was set to a wavelength of 280 nm. During the fractionation, collections were made into glass vials at 1 min intervals (1.5 ml per vial). Those collections were then tested for their LD_{50} values using laboratory mice as described by Theakston and Reid.(7) Quantification of the protein in each fraction was done by the Lowry method.(8)

Electrospray Mass Spectrometry

The most toxic fraction (lowest LD_{50} value) was dialyzed against water and then dried by lyophilization. The dried sample was dissolved in 50% acetonitrile/water to the concentration of 40 µg/ml. Eight µl of this solution was then injected into the mass spectrometer. The mass spectrometer used was a VG Trio 2000 (VG Biotech, Altrincham, UK) equipped with an electrospray ionization source. The solvent used for ESI-MS was 50% (V/V) acetronitrile/water with 1% (V/V) formic acid. The flow rate was 8µl/min, controlled by the Waters 626 HPLC pump (Waters Corp. Milford, MA, USA). Analysis was performed under positive ion mode, using Masslynx software (VG Biotech, Manchester, UK). Mass calibration was done with standard myoglobin from m/z of 700 to 1700.

Determination of LD_{50} Values

Determination of the LD_{50} value for each protein fraction was done using laboratory mice as described by Theakston and Reid(7). Each protein fraction was diluted with 0.9% (W/V) sodium chloride solution to appropriate concentrations. Intravenous injection into mice was done through the mice's tails. Five mice, each of weight 18 g, were used for each concentration tested. After 24 hr, the number of dead mice was counted and the LD_{50} value was then calculated as described by Theakston and Reid.(7)

RESULTS AND DISCUSSION

The chromatographic pattern of the C-18 HPLC fractionation of venom from Siamese cobras with complete monocellated hood markings is quite similar to that from Siamese cobras with incomplete hood markings (Figure 1a and b). The results (Table 1) show that the fraction eluted at 58 min retention time was the most lethal fraction for both snake groups, as indicated by the lowest LD_{50} value. Although the most toxic fractions from the two snake groups gave different LD_{50} values (0.1 and 0.26 mg/kg in mice), electrospray mass spectrometry analysis of this fraction indicated a molecular mass of 7820 ± 1 Da for the venoms from both kinds of snakes (Figure 2). The similarity in both the chromatographic pattern and the molecular mass of the most toxic component between snakes of different hood marking patterns indicates that the Thai cobras with different hood marking patterns actually have venom with a very close or similar chemical composition.

This paper described a one - step chromatographic separation of crude venom and the step chromatographic separation of crude ven om and the use of ESI-MS to quickly determine the molecular mass of the fraction of interest. The results provide good evidence of the similarity between venoms from Thai cobras of different hood marking patterns. The size of the peptide isolated at 58 min retention time as determined by the ESI-MS suggests the possibility that this 7820 Da peptide might be the same neurotoxin found previously in other Asian snakes.(2,3) Further structure elucidation of this peptide needs to be done to confirm this suggestion. The method has proven to be a very quick and useful procedure to quickly isolate and scan for some interesting components in biological samples. In this case the HPLC procedure provides a good separation protocol for the most toxic peptide and specific molecular mass information gained from ESI-MS suggests that this peptide might be the same neurotoxin found previously in some other Asian snakes.

Figure 1. Fractionation of Thai cobra's venom using C-18 HPLC. Chromatograms of venoms from snakes with a) complete monocellated hood markings and b) incomplete monocellated hood markings.

Figure 2. Electrospray ionization (ESI) mass spectrometry of the most lethal fraction of venom isolated by C-18 HPLC: a) mass spectrum of multiply charged ions and b) resolved spectrum showing the molecular mass of 7820 ± 1 Da.

Table 1. LD_{50} value of fractions collected from C-18-HPLC fractionation of venom from Siamese cobras (*Naja naja kaouthia*) with complete or incomplete monocellated hood markings.

Retention Time (min)	Complete monocellated hood markings		Incomplete monocellated hood markings	
	% of total protein content (by weight)	LD_{50} (mg/kg mice)	% of total protein content (by weight)	LD_{50} (mg/kg mice)
9	1.0	> 17.32	3.2	> 25.00
18	5.8	> 28.10	4.9	> 38.79
45	17.2	0.49	6.3	0.45
58	33.4	0.26	20.1	0.10
67	2.3	1.93	4.8	1.97
70	6.6	1.83	7.6	1.70
71	15.7	3.19	21.7	4.23
78	10.9	3.41	19.3	2.16
83	4.4	1.97	8.4	2.86
85	1.3	4.21	0.9	7.15
89	1.4	7.84	1.0	9.65

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Received: March 8, 1998
Accepted: July 3, 1998