December 1993 *Asiatic Herpetological Research* Vol. 5, pp. 143-146

A Study on the Purification and Pharmacological Properties of Two Neurotoxins from the Venom of the King Cobra *(Ophiophagus hannah)*

JIANXING SONG, YULIANG XIONG, WANYU WANG, AND XIAOCHUN PU

Toxinological Laboratory, Kunming Institute of Zoology, Academia Sinica, Kunming, Yunnan, China

Abstract- Using Sephadex G-50, CM-Sephadex C-25 and CM-cellulose 52 columns, two neurotoxins of *Ophiophagus hannah* were purified to be homogeneous on acidic PAGE which contain 53 and 73 amino acid residues respectively. The two neurotoxins were used as a substitute for morphine. Using a morphine withdrawal jumping model, the results demonstrated that the effects of the two neurotoxins when administered by injecting are very significant ($P<0.01$), and when administered orally are significant ($P<0.05$).

Key words: King Cobra, *Ophiophagus hannah*, neurotoxins, morphine addiction, naloxone jumping model.

Introduction

The King Cobra *(Ophiophagus hannah)* is the largest poisonous snake in the world (Tu, 1977). It is extensively distributed in southern China, India, Thailand and other Asian countries. Joubert (1973) reported on the purification and sequence determination of two toxins from the venom of King Cobras grown in Thailand. Shun (1981) reported on the purification of four postsynaptically acting toxins from the venom of the King Cobra in Guangxi, China. Our research showed that among four toxins, there is a postsynaptically acting toxin containing only 63 amino acid residues. We determined the complete amino acid sequence of neurotoxin, which contain 73 amino acid residues. This neurotoxin's sequence is analogous to that of the neurotoxins determined by Joubert (1973), but its C-terminal four amino acid residues are very similar to that of bungartoxin in hydrophobicity (Lin and Wang, 1984). Now, the sequence of the neurotoxin containing 53 amino acid residues is in the process of being determined.

Xiong and Wang (1987) reported the clinical observation results of using the neurotoxin from cobra venom to achieve better analgesic effect on morphine addicted patients. The possibility to use the neurotoxins from snake venom as a substitute for morphine was also first reported by Xiong (1990). The mechanism has been discussed in the other papers. In this paper, the research concentrated on the purifying of two neurotoxins from the venom of King Cobras from Guangxi, China and using them as a substitute for morphine during tests on the mice-jumping model.

Methods

Venom of King Cobras was purchased from Guangxi Province. Male and female mice were provided by the feed lot in our institute. Naloxone and morphine were purchased from Qinghai Medical Factory. CM-Sephadex C-25, Sephadex G-50, and CM-Cellulose 52 were provided by our pharmacy. Other chemicals of A. R. grade were produced in China.

1. The purification of two neurotoxins from the venom of King Cobras: The dry venom powder was dissolved in the buffer solution (pH 5.8, 0.05 M HAc-NaAc buffer), then the solution was centrifuged to discard the insoluble material. The supernatant was loaded on a Sephadex G-50 column (25 x 200 cm). The same buffer was used to elute the column. The fractions which contained only low molecular weight components were collected (The process was directed by acidic PAGE).

The collected material was desalted and concentrated. It was then loaded on a CM-Sephadex C-25 column (4x80 cm). The column was eluted first by the equilibrium buffer, then was eluted by the buffer

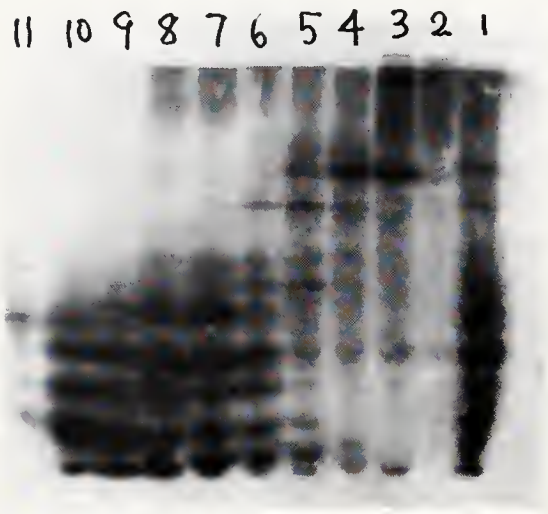

FIG. 1. The Acidic PAGE of the different fractions after separation with a Sephadex G-50 column (2.5 x 200 cm). 1: Crude venom; 2-11: Different fraction after separation with a Sephadex G- 50 column. Fractions 7-11 were collected together for the further isolation.

containing NaCl gradient (00.4) and finally was eluted by the buffer containing NaCI gradient (0.4~0.8). The two neurotoxins were eluted during the first gradient process.

The neurotoxins from CM- Sephadex C-25 still showed two other minor bands on acidic PAGE. So each of them was further purified on the CM- Cellulose 52 Column. The equilibrium buffer is 0.05M, pH 5.8 HAc-NaAc buffer. The NaCl gradient is 0~0.5M in the same buffer.

2. Determination of purities: Acidic PAGE was used. The separating gel was 15 %, and the spacal gel was 2.5 %. The gel was stained by R- 250 dissolved in 375 ml ethanol, 125 ml water and 5 ml methanol.

3. Determination of amino acid composition: The samples were analyzed with a Hitachi Model 835-50 High Speed Amino Acid Analyzer.

4. Determination of amino acid sequence of the neurotoxin: The method was in accordance with the method of Lin and Wang (1984).

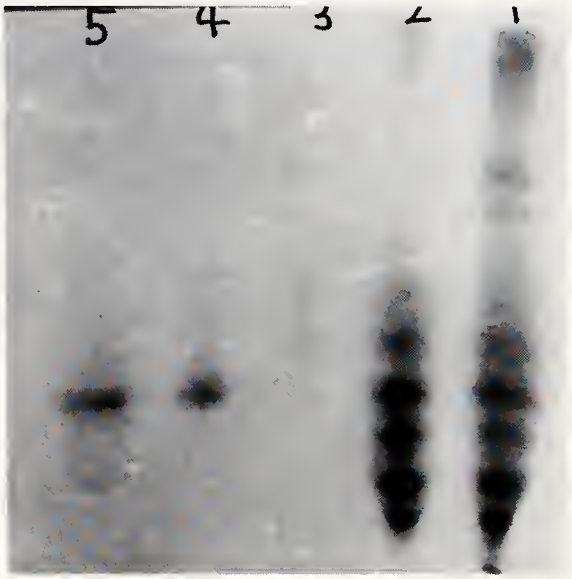

FIG. 2. The acidic PAGE of the neurotoxins from CM-Sephadex C-25 CM-Cellulose columns. 1. Crude venom; 2. The fraction containing neurotoxins from Sephadex G-50; 3. Neurotoxin 1; 4. Neurotoxin 2 from CM Sephadex C-25; 5. Neurotoxin 2 further purified on CM cellulose 52.

5. Determination of LD 50: Mice were used as the material. The method was according to Gu (1965).

6. Morphine withdrawal jumping model: Mice were divided into random groups, with each group consisting of 10 mice, half male and half female, with body weights of 20±2g. The groups were treated by injecting S. V. with morphine, for four days, three times at a dose of 10 mg/kg, three times at a dose of 20 mg/kg and 12 times at a dose of 30 mg/kg. After morphine was given the last time, the neurotoxins were administered either orally or by injecting. The control group was treated with physiological saline of 0.2 ml per mouse. Three hours later, naloxone was administered S. V. to different groups at a dose of 15 ml/kg. Then the jumping numbers of the mice during a 30 minute period were recorded. The data were analyzed with statistical methods.

Results

The lyophilized venom powder was dissolved in the buffer (0.05M, pH 5.8.

TABLE 1. The amino acid compositions of neurotoxins 1 and 2.

Amino Acid	Neurotoxin 1		Neurotoxin 2	
	Min. Residue Numbers	Residue Number 100 Residues	Min. Residue Numbers	Residue Number Number
Lye	6	6.81	4	7.55
1lis	0	0	1	1.89
Arg	4	5.26	1	1.89
Asp	8	10.53	6	11.33
Thr	9	11.84	2	3.77
Ser	3	3.95	5	9.43
Glu	5	6.58	2	3.77
Pro	7	9.21	4	7.55
Gly	4	5.26	4	7.55
Ala	4	5.26	3	5.66
Val	6	7.89	2	3.77
Met	1	1.32	0	0
1le	3	3.95	3	5.66
Leu	1	1.32	3	5.66
Try	2	2.63	0	0
Phe	2	2.63	4	7.55
1/2 Cys	8	10.53	7	13.21
Try	3	3.95	2	3.77
Total Residue	76		53	

HAc-Ac buffer), loaded on the Sephadex G-50 column, then eluted with the same buffer. The fractions containing neurotoxins were put together, m desalted, concentrated, then loaded on the CM-sephadex C-25 column, which was eluted first with equilibrium buffer, then with different NaCl gradient. After the CM-Sephadex C-25 column, the two neurotoxins were further purified on a CM-Cellulose column.

The crude venom was isolated first on a Sephadex G-50 column, then CM-Sephadex C-25, and finally, a CM-Cellulose column. Figure 1 and 2 shows the acidic PAGE of different fractions.

The amino acid sequence of neurotoxin 1 was determined by the Immobilized Phase Edman Method as: 1 Thr • Lys • Cys • Tyr • Val • Thr • Pro • Asp • Val • Lys • Ser • Glu • Thr • Cys • Pro • Ala • Gly • Gin • Asp • Leu • Cys • Tyr • Thr • Glu • Thr • Trp • Cys • Val • Ala • Trp • Cys • Thr • Val • Arg • Gal • Lys • Arg • Val • Ser • Leu • Thr • Cys • Aal • Ala • Ile • Cys • Pro • Ile • Val • Pro • Pro • Lys • Val • Ser • Ile • Lys • Cys • Cys • Ser • Thr • Asp • Aal • Cys • Gly • Pro • Phe • Pro • Thr • Trp • Pro • Asn • Val • Arg

The toxicity of neurotoxins 1 and 2 was determined by the following method: The mice, with body weight of 18-20 g, were divided at random into groups of 5 mice. The different doses of the neurotoxins were given to the groups S. V. The death numbers of mice within 24 hours were recorded and the LD 50 was determined by modified a Ginsberg method. For neurotoxin I the LD 50=0.21±0.013. For neurotoxin 2 the LD 50=0.24±0.009

For the morphine withdrawal jumping model, the effects of purified neurotoxins on mice jumping numbers and the results of statistical analysis are shown in Table 2. The results demonstrated that the two neurotoxins display very significant effects ($P<0.01$) as a substitute for morphine, by injecting S. V. and significant effects ($P<0.05$) when administered orally.

Discussion

The origination and evolutionary relationships of neurotoxins and

TABLE 2. The effects of the two neurotoxins on mice jumping test.

	Administered S. V.			Administered Orally		
Groups	Dose	No. of jumps	Statistics	Dose	No. of jumps	Statistics
Saline	0.1 ml	44.4		0.1 ml	44.4	
Neurotoxin 1	0.1μg/20 g	11.3	$p<0.01$	1.0 μg/20g	18.2	$p<0.05$
Neurotoxin 2	0.1 μg/20 g	19.3	$p<0.01$	1.0 μg/20 g	16.8	$p<0.05$

phospholipase A2 from snake venom are still disputed problems. Some experts thought that the original molecule is the postsynaptical toxins containing only 60-61 amino acid residues. By increasing the cycles in the molecules, the short-chain neurotoxins evolved into long-chain neurotoxin containing 7374 amino acid residues, then evolved into phospholipase A2. On the other hand, some experts thought Phospholipase A2 was the original molecule which diverged into neurotoxins (including postsynaptical and presynaptical toxin), proteinase inhibitor and a new neurotoxin (dendrotoxin). In King Cobra venom, both short and long chain neurotoxins were isolated. Especially, a postsynaptical toxin containing 53 amino acid residues was purified. So this neurotoxin is very important to understand the evolutionary relationship of the neurotoxin. Now, we are focused on the determination of its sequences and its conformational properties in the solution by means of 2D- NMR method (mainly by different correlated spectrum and NOSY spectrum).

By using the withdrawal jumping model, two neurotoxins display significant effects on substituting for morphine. The clinical observation in Kunming also demonstrated remarkable effects. These facts suggest that these two neurotoxins from the King Cobra venom may be a better medicine to be used as a substitute for morphine. Now, experiments have been accomplished to understand its mechanism.

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