J. Biochem. **134**, 799–804 (2003)
DOI: 10.1093/jb/mvg187

Isolation, Toxicity and Amino Terminal Sequences of Three Major Neurotoxins in the Venom of Malayan Krait (*Bungarus candidus*) from Thailand

Orawan Khow[1], Lawan Chanhome[1], Tamotsu Omori-Satoh[*,1], Yuko Ogawa[2], Ryohei Yanoshita[2], Yuji Samejima[2], Ulrich Kuch[3], Dietrich Mebs[3] and Visith Sitprija[1]

[1]Queen Saovabha Memorial Institute, The Thai Red Cross Society, 1871 Rama IV Rd., Bangkok 10330, Thailand; [2]The Institute of Medicinal Chemistry, Hoshi University, Shinagawa-ku, Tokyo 142-8501; and [3]Zentrum der Rechtsmedizin, Klinikum der Johan Wolfgang Goethe-Universität, Kennedyallee 104, 60596 Frankfurt, Germany

Received June 27, 2003; accepted August 5, 2003

We isolated the most lethal toxins in the venom of the Malayan krait (*Bungarus candidus*), one of the medically most important snake species in southeast Asia. Three β-BTx like basic neurotoxins, T1-1, T1-2, and T2, with PLA_2 activity were isolated from pooled venom of eight *B. candidus* from southern Thailand by cation-exchange chromatography, followed by adsorption chromatography on hydroxylapatite and RP-HPLC, with 14-, 16-, and 4-fold increases in toxicity compared to crude venom. The LDs_{50} determined in mice weighing 18–20 g were 0.26, 0.22, and 0.84 μg per mouse with i.v. injection. T1-1 and T1-2 possessed comparable lethal toxicities to those of β1-BTx, the most toxic neurotoxin in *B. multicinctus* venom, and the major neurotoxin in *B. flaviceps* venom. The apparent molecular weights of the native toxins were approximately 25–25.5 kDa. They consist of two polypeptide chains with apparent molecular weights of 15.5–16.5 and 8–8.5 kDa, respectively. The amino terminal sequences of the two chains of each of the toxins determined by Edman degradation exhibited considerable similarity with those of the A-chains and B-chains of β-BTxs in the venom of *Bungarus multicinctus*.

Key words: *Bungarus candidus*, β-bungarotoxin, snake venom, neurotoxin, Thailand.

Abbreviations; β-BTx, β-bungarotoxin; CBB, Coomassie Brilliant Blue; HLD, hemolytic dose; PLA_2, phospholipase A_2; PTH, phenylthiohydantoin; PVD, polyvinylidene difluoride; RP-, reverse phase-; TFA, trifluoroacetic acid.

The venomous snake fauna of Thailand is legendary and includes several of the world's most dangerous species. The medical importance of species like the monocellate cobra (*Naja kaouthia*), the Malayan pitviper (*Calloselasma rhodostoma*), the Siamese Russell's viper (*Daboia russellii siamensis*), and several green pitvipers of the genus *Trimeresurus* is widely recognized. Other dangerous snakes include numerous species of sea snakes, the king cobra (*Ophiophagus hannah*), three species of kraits (genus *Bungarus*), and the Asian coral snakes of the genera *Calliophis* and *Sinomicrurus* (*1–3*). This second group has traditionally been regarded as one of lesser concern epidemiologically because the incidence of bites by these species, particularly in densely populated metropolitan areas, is low (*4*).

Among the kraits, the large and conspicuously colored banded krait (*Bungarus fasciatus*) has been associated with a low incidence and mortality of bites in Thailand (*5*), and no published information exists in bites by the red-headed krait (*Bungarus flaviceps*). However, the third species, the Malayan krait (*Bungarus candidus*), has in the course of the past 20 years emerged as one of the medically most important venomous snakes in southeast Asia. The first clinical data on bites by this species were provided by Warrell *et al*. (*6*), who reported on five patients from eastern Thailand and northwestern Malaysia. Loathong and Sitprija (*7*) presented the clinical details of three additional *B. candidus* bite victims in Thailand and suggested that envenoming by Malayan kraits was clinically dominated by presynaptically acting toxins. Through hospital-based surveys, Looareesuwan *et al*. (*8*) and Viravan *et al*. (*9*) found that *B. candidus*, for which no specific antivenom is available, is one of the three most important species in Thailand with respect to snake bite mortality, being responsible for as many fatalities as the monocellate cobra and the Malayan pitviper, and that the number of hospitalized bite cases for this species was much higher than previously believed. Moreover, fatalities caused by *B. candidus* are probably still underreported because most bites occur in rural areas, and the time to death is short due to the extremely high lethal toxicity and neurotoxic mode of action of its venom. By way of comparison, *B. candidus* venom (LD_{50}, 3.5 μg per mouse) is much more toxic than the venom of its larger congener *B. fasciatus* (LD_{50}, 61.7 μg), more toxic than *N. kaouthia* venom (LD_{50}, 6.5 μg), and together with the venom of *B. flaviceps* (LD_{50}, 3.4 μg) must be considered the most toxic of all terrestrial snake venoms from Thailand studied to date (*10*, *11*).

The purpose of this study was to isolate and characterize the components of the venom of *B. candidus* that have the highest toxicity and are thus likely to play a predom-

[*]To whom correspondence should be addressed. Tel: +66-2-252-0161 (Ext. 151), Fax: +66-2-254-0212, E-mail: tamotsu@asianet.co.th

inant role in human envenomation by this species. Previously, Tan *et al*. (*12*) identified two highly lethal toxin fractions with PLA_2 activity and two polypeptide toxins in a commercial venom sample of *B. candidus* of unknown geographical origin, and Tsai *et al*. (*13*) discussed the amino acid sequence of an acidic group 1B PLA_2 of unknown biological function deduced from venom gland cDNA of a *B. candidus* from Bali, Indonesia. Based on the results of studies on the venom of the Taiwanese many-banded krait, *B. multicinctus* (*14*, *15*), and the work by Tan *et al*. (*12*), the most lethal toxins of *B. candidus* venom were expected to be presynaptically acting toxins like the β-BTxs from *B. multicinctus* venom, *i.e.*, heterodimers with a PLA_2 (A-chain) covalently linked with a Kunitz type protease inhibitor (B-chain). To gain a first impression of the similarity of such toxins from *B. candidus* venom to those of *B. multicinctus*, we compared their amino terminal sequences with the published sequences of β-BTxs (*16–22*).

MATERIALS AND METHODS

Snake Venom, Chemicals, and Reagents—Bungarus candidus venom was obtained by milking from eight adult male *B. candidus* from southern Thailand kept at the Queen Saovabha Memorial Institute, and was lyophilized immediately. The following liquid chromatography columns were used: for cation-exchange chromatography, Resource™ S (6 ml, Pharmacia); and for RP-HPLC Capcell-Pak C-18 type SG 300, 15 × 250 mm (Shiseido, Tokyo). Hydroxylapatite, the kaleidoscopic prestained molecular weight standards for SDS-PAGE, and the PVD membranes were purchased from Bio-Rad Laboratories (Richmond, CA, USA), and the 4–20% polyacrylamide gradient gel for SDS-PAGE (MULTIGEL 4/20) from Daiichi Pure Chemicals (Tokyo).

Chromatographic Procedures—Cation-exchange chromatography: Fifty milligrams of crude *B. candidus* venom was dissolved in 2 ml of 50 mM phosphate buffer (pH 6.25), applied to the Resource™ S column equilibrated with the buffer, and then eluted with a linear gradient of 0–0.3 M NaCl at the flow rate of 20 ml/h. Absorbance was measured at 280 nm, and fractions of 2 ml were collected.

Adsorption chromatography on hydroxylapatite: The major toxic fractions obtained on cation-exchange chromatography were pooled (fraction XI in the previous step), concentrated in a collodion bag *in vacuo*, and then dialyzed against the phosphate buffer. In each of two runs, a half volume of the dialyzed solution was applied to a hydroxylapatite column (1 × 8 cm) equilibrated with the buffer. The column was eluted with a linear gradient of 0.05–0.5 M phosphate buffer (pH 6.25). Fractions of 1.5 ml were collected at the flow rate of 12 ml/h. The identical toxic fractions obtained in the two runs were then pooled and dialyzed against distilled water. The dialyzed samples were concentrated by lyophilization.

RP-HPLC: The samples obtained on adsorption chromatography were dissolved in water and then chromatographed on the Capecll-Pak C-18 type SG 300 column equilibrated with 10% acetonitrile in 0.1% TFA. Protein was eluted with a linear gradient of 10 to 80% acetonitrile in 0.1% TFA over 60 min at the flow rate of 1.0 ml/min. Fractions were collected based on absorbance units (AU) at 220, 254, and 280 nm.

Analytical Procedures—SDS-PAGE was performed according to Laemmli (*23*) using the MULTIGEL 4/20 containing 0.1% SDS. A constant current of 30 mA/cm gel was applied. Gels were stained with 0.1% CBB R-250 in 25% methanol–10% acetic acid or by the silver staining method of Oakley *et al*. (*24*).

Amino terminal sequencing was carried out on a PVD-membrane as described by Iwamatsu (*25*), but without *S*-carboxymethylation: the purified toxins were subjected to SDS-PAGE in a 15% gel and then transferred to a PVD-membrane using the semi-dry transfer method. After staining with Ponceau S, the bands corresponding to the A- and B-chains were cut out and subjected to automated Edman degradation with a gas-phase amino acid sequencer (Model 477A, Applied Biosystems) and an on-line PTH-derivative analyzer, Model 120A.

Assays—For toxicity assays, venom fractions or purified toxins were dissolved in 1 ml of 0.1 M phosphate-buffered saline (pH 7.2), and then the photometric absorbance at 280 nm was recorded in a 1 cm cell. Protein concentrations were determined on the assumption that an absorbance value of 1.47 (that of crude venom of Thai *B. candidus*) corresponds to 1 mg toxin in 1 ml of solvent. Outbreeding mice of both sexes weighing 18–20 g were injected intravenously with 0.2 ml of sample solutions. Death and survival were recorded for 24 h, and venom fractions killing with 7 μg (corresponding to $2LD_{50}$ of crude venom) were regarded as toxic. After preliminary experiments involving two mice per dose, dilution at two-fold intervals, the LDs_{50} of the toxins were determined by testing five doses of serially 1.4-fold diluted solutions in four mice per dose. The LD_{50} and its confidence limits at 95% probability were calculated by the methods of Reed-Muench (*26*) and Pizzi (*27*), respectively. Animal experiments were performed under supervision of the ethics committee of the institute. PLA_2 activity was determined by the indirect hemolytic method (*28*) on erythrocyte-agarose gel plates according to Gutierrez *et al.* (*29*), with the use of human erythrocytes instead of sheep ones. The method provided a straight calibration curve for the log dose-diameter relationship between 9 and 23 mm cross diameter of the hemolytic zones produced by 0.03–5 μg of crude venom. To each plate of 135 mm diameter, a series of 3-fold diluted crude venom solutions (5 μl) was introduced to obtain a calibration curve, and the parallel line analysis recommended by WHO (*30*) was adopted to determine the HLD, in micrograms, causing a hemolytic zone of 10 mm in diameter.

RESULTS AND DISCUSSION

Isolation, Toxicity, and PLA_2 Activity—On cation-exchange chromatography of the crude venom, 94 fractions were obtained and pooled into 13 fractions (the chromatogram is not shown). Significant lethal toxicity was observed for the last five pooled fractions (IX–XIII). Although PLA_2 activity was detectable in all fractions, significant activity was confined to pooled fractions I–III and X–XIII. Table 1 summarizes the distributions of protein, lethal toxicity and PLA_2 activity. Among them, frac-

Table 1. **Distributions of protein, lethal toxicity and PLA_2 activity in the pooled fractions obtained on Resource™ S column chromatography.**

Fraction (tube no.)	Protein (mg)	Lethal toxicity		PLA_2 activity	
		LD_{50} (μg)	Total LDs_{50}	HLD (μg)	Total HLDs
I (3–8)	30.36	>7	–	0.054	562,200
II (9–14)	5.52	>7	–	0.045	122,700
III (15–22)	2.03	>7	–	0.16	12,690
IV (23–26)	0.82	>7	–	0.25	3,280
V (27–32)	0.71	4.9	145	0.25	2,840
VI (33–38)	0.86	>7	–	0.72	1,194
VII (39–46)	1.84	>7	–	0.84	2,190
VIII (47–51)	2.02	>7	–	0.90	2,244
IX (52–56)	1.81	1.8	1,006	0.67	2,700
X (57–61)	1.15	0.70	1,643	0.060	19,170
XI (62–77)	3.43	0.40	8,575(59)[a]	0.060	57,167(6.9)[b]
XII (78–88)	0.97	0.52	1,865	0.054	17,960
XIII (89–94)	2.10	1.60	1,313	0.10	21,000
Total	54.41		14,547		827,335
Crude venom	50.0	3.5	14,285	0.045	1,111,000

[a, b]Figures in parentheses are the recoveries (%) calculated to the total toxicity and PLA_2 activity obtained in the first step of purification, respectively.

tion XI contained 59% of the recovered lethal toxicity and thus was chosen for further purification.

Adsorption chromatography of fraction XI on a hydroxylapatite column resulted in an elution pattern comprising six peaks (Fig. 1). Lethal toxicity was detected in the incompletely separated second and third peaks, which were combined and designated as T1, and the fourth peak, designated as T2. RP-HPLC of T1 resulted in four peaks (Fig. 2A). The first and second of these contained lethal toxicity, and were designated as T1-1 and T1-2. In contrast, T2 was eluted essentially as one peak (Fig. 2B).

Table 2 summarizes the purification of the three neurotoxins from *B. candidus* venom. For T1-1, T1-2, and T2, 14-, 16- and 4-fold purification was achieved, respectively. The lethal toxicity of these neurotoxins amounted to 30.5% of that of crude venom. Although the purified neurotoxins exhibit PLA_2 activity, no correlation was observed between the increase in lethal toxicity in the course of purification and the detected enzymatic activity. We interpret these findings as indicative of another PLA_2 in an adjacent fraction on cation-exchange chromatography, which exhibits high enzymatic activity but low toxicity, and was consequently eliminated during the purification process.

Table 3 compares the lethal toxicities of the three purified neurotoxins from Thai *B. candidus* venom with those of several neurotoxins hitherto isolated from *Bungarus* snake venoms. Comparison was made by means of LDs_{50}, defined as μg per gram of mouse. The lethal toxicities of T1-1 and T1-2 are comparable to those of β_1-BTx from *B. multicinctus* venom (*18*) and the major neurotoxin from *B. flaviceps* venom (*31*) recently isolated in our laboratory. They are slightly stronger than β_2-BTx and toxic fraction F6A of *B. candidus* venom of unknown geographical origin (*12*), and much stronger than β_3-, β_4-, and β_5-

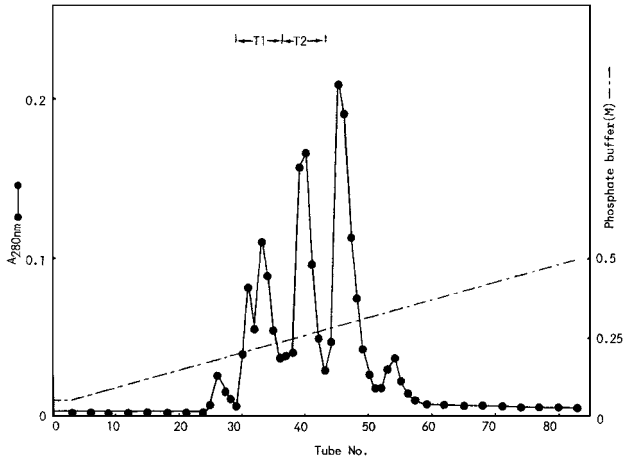

Fig. 1. **Adsorption chromatography on hydroxylapatite.** The experimental conditions are given in section "*Adsorption chromatography on hydroxylapatite*."

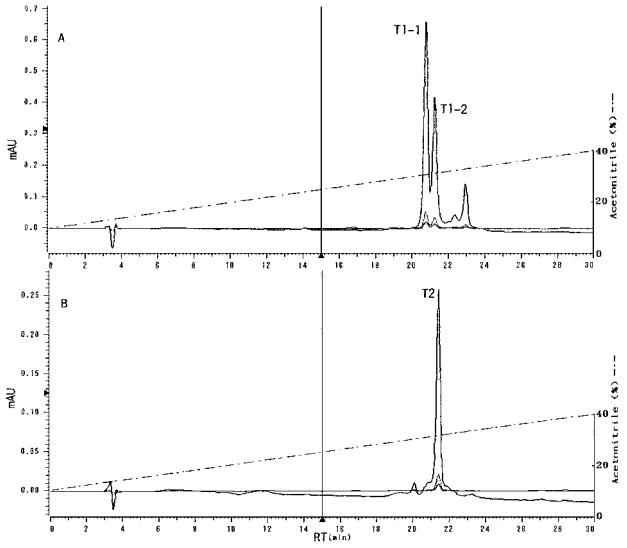

Fig. 2. **RP-HPLC of *B. candidus* venom fractions. (A) Fraction T1 in Fig. 1; (B) fraction T2 in Fig. 1.** The experimental conditions are given in section "*RP-HPLC*."

Table 2. **Summary of the purification of the three major neurotoxins from Thai *B. candidus* venom.**

Step	Protein (mg)	Lethal toxicity		PLA_2 activity
		LD_{50} (μg)	Total LDs_{50}	HLD (μg)
Crude venom	50	3.50 (2.50–4.19)	14,285	0.045
Resource™ S	3.43	0.40 (0.29–0.50)	8,575	0.06
Hydroxylapatite				
T1	1.07	0.20 (0.15–0.29)	5,385	0.037
T2	0.92	0.77 (0.55–0.96)	1,195	0.19
RP-HPLC				
T1-1	0.53	0.26 (0.18–0.31) (13.5)[a]	1,923 (13.5)[b]	0.045
T1-2	0.30	0.22 (0.15–0.28) (15.9)[a]	1,364 (9.5)[b]	0.12
T2	0.90	0.84 (0.60–1.01) (4.2)[a]	1,071 (7.5)[b]	0.42

[a, b]Figures in parentheses are the purification factors and the percent recoveries calculated to crude venom, respectively.

Table 3. **Comparison of the lethal toxicities of the purified neurotoxins with those of several neurotoxins hitherto isolated from *Bungarus* snake venoms.**

Venom	LD_{50} (μg/g of mouse)	Injection route	Reference
B. candidus			
T1-1	0.014 (0.0095–0.016)[a]	i. v.[b]	present study
T1-2	0.012 (0.0078–0.015)[a]	i. v.[b]	*ibid.*
T2	0.044 (0.032–0.053)[a]	i. v.[b]	*ibid.*
F6A	0.02	i. v.[b]	*12*
F4A	0.18	i. v.[b]	*12*
B. multicinctus			
β_1-BTx	0.019 (0.010–0.035)	i. p.[c]	*18*
β_2-BTx	0.028 (0.017–0.043)	i. p.[c]	*18*
β_3-BTx	0.066 (0.059–0.074)	i. p.[c]	*18*
β_4-BTx	0.072 (0.060–0.086)	i. p.[c]	*18*
β_5-BTx	0.130 (0.063–0.252)	i. p.[c]	*18*
B. flaviceps	0.013 (0.010–0.017)[a]	i. v.[b]	*31*
B. fasciatus			
Ceruleotoxins	0.03–0.07	i. v.[b]	*32*

[a]Converted from the LDs_{50} given in Table 2 and the previous paper (*31*) divided by 19, the mean body weight of mice used in this study, to obtain LDs_{50} defined as μg per gram of mouse. [b,c]Intravenous and intraperitoneal, respectively.

BTxs (*18*), and F4A (*12*). T2 exhibits comparable lethal toxicity of β_2-BTx (*18*) and ceruleotoxins from *B. fasciatus* venom (*32*).

Mice injected with variable doses of the toxins from *B. candidus* venom showed no abnormal behavior within the first 60 min after injection. After that time, slow movement and inactivity were followed by paralysis, dyspnoea and death in a dose-independent manner. The toxic symptoms observed in mice correspond to those induced by β-BTxs, *e.g.*, delayed onset of paralysis (*15*, *33*).

Homogeneity and Molecular Weight—The homogeneity of the neurotoxins purified from *B. candidus* venom was examined by SDS-PAGE in gradient gels (4–20%) stained with CBB R-250 (Fig. 3A) or by the silver staining method (Fig. 3B). In the absence of mercaptoethanol, the neurotoxins migrated as single bands to positions corresponding to apparent molecular weights of about 25, 25.5, and 25 kDa (T1-1: Fig. 3A, lane 1; T1-2: Fig. 3A, lane 2; and T2: Fig. 3B, lane 1), respectively. In the presence of mercaptoethanol, they migrated as two bands corresponding to apparent molecular weights of about 16.5 and 8 kDa (T1-1: Fig. 3A, lane 4), 15.5 and 8.5 kDa (T1-2: Fig. 3A, lane 5), and 15.5 and 8 kDa (T2: Fig. 3B, lane 3), respectively.

The apparent molecular weights of *B. multicinctus* β-BTxs were estimated to be 21.2 kDa under non-reducing conditions, and 13.5 (A-chain) plus 7 kDa (B-chain) in the presence of mercaptoethanol (*32*), which are comparable to the apparent molecular weights of the toxins we isolated from *B. candidus* venom (the calibration curve for molecular weight estimation is not shown) and the highly toxic fraction (F6A) with PLA_2 activity found by Tan *et al.* (*12*), which had an apparent molecular weight of 21 kDa.

Amino Terminal Sequences—For the neurotoxins from *B. candidus* venom, amino terminal sequences were determined up to residue 14 for the A-chains and 15 for the B-chains (Fig. 4). In this amino terminal part, the A-chain of toxin T2 is identical to those of β_{1-3}BTxs from *B. multicinctus* venom, but T2 differs from the latter in its B-chain. Likewise, toxins T1-1 and T1-2 are identical to each other in the amino terminus of their A-chains but differ in that of their B-chains. The available data suggest a variable degree of similarity between the neurotoxins from *B. candidus*, and published A- and B-chains of β-BTxs in *B. multicinctus* venom (*16–22*, *34*).

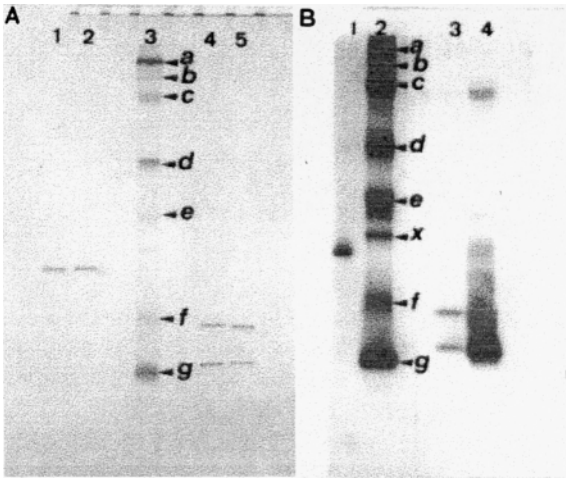

Fig. 3. **SDS-PAGE of the neurotoxins purified from Thai *B. candidus* venom.** The gels, and electrophoretic and staining conditions are described in section "*Analytical Procedures*." (A) Lanes 1 and 2, 4 μg of T1-1 and T1-2, respectively, without mercaptoethanol; lane 3, 4 μl of kaleidoscopic prestained standards comprising (a) myosin (208 kDa), (b) β-galactosidase (132 kDa), (c) bovine serum albumin (91 kDa), (d) carbonic anhydrase (45.2 kDa), (e) soybean trypsin inhibitor (35.1 kDa), (f) lysozyme (18 kDa), and (g) aprotinin (7.6 kDa); lanes 4 and 5, 4 μg of T1-1 and T1-2, respectively, with mercaptoethanol. (B) Lane 1, 1 μg of T2 without mercaptoethanol; lane 2, 1 μl of kaleidoscopic prestained standards; lanes 3 and 4, 1 μg of T2 and 5 μg of crude venom, respectively, with mercaptoethanol. An unidentified standard protein exhibiting strong reactivity on silver staining is indicated by x in lane 2.

CONCLUSION

One of the most prominent examples of natural engineering by through an accelerated rate of molecular evolution of peptide sequences is that of snake venom gland PLA_2, including the group 1 PLA_2 genes of elapid snakes (*35*, *36*). The potential implications of a likely accelerated mode of molecular evolution of β-BTx genes for krait venom variability should therefore be considered when the production of a specific antivenom is attempted. Beta-BTxs are the most lethal toxins in *Bungarus* snake venom, and variability in these toxins may warrant the inclusion of snakes from several populations in the production of regional antivenom (*37*). Warrell *et al*. (*6*) found Taiwanese *B. multicinctus* antivenom to be ineffective in neutralizing Thai *B. candidus* venom in mouse-protection tests. This could be due to possible differences in the antigenic properties of *B. candidus* β-BTx like neurotoxins. Studies in progress, including snakes from other populations and species of *Bungarus,* hopefully will resolve this problem and reveal the evolutionary relationships of the β-BTx family.

T. O. S. wishes to thank Dr. M. Takahashi, National Institute of Infectious Diseases, Tokyo, Japan, for the helpful advice on the bioassays. This work was supported in part by the Japan Science and Technology Corporation, Ministry of Education, Science and Technology of Japan.

		1 10	
A-chain	T1-1;	N-L-Y-Q-F-K-E-M-I-R-Y-T-I-P-	(79)[a]
	T1-2;	N-L-Y-Q-F-K-E-M-I-R-Y-T-I-P-	(79)[a]
	T2;	N-L-I-N-F-M-E-M-I-R-Y-T-I-P-	(100)[a]
	β_{1-3}BTxs;	N-L-I-N-F-M-E-M-I-R-Y-T-I-P-	
B-chain	T1-1;	R-K-R-H-P-D-C-D-K-A-A-D-T-R-I-	(53, 80 and 53)[b]
	T1-2;	R-Q-R-H-P-D-C-D-K-P-P-D-T-G-N-	(87, 80 and 87)[b]
	T2;	R-Q-R-H-R-D-C-D-K-P-P-D-K-T-N-	(93, 67 and 93)[b]
	β_1-BTx;	R-Q-R-H-R-D-C-D-K-P-P-D-K-G-N-	
	β_2-BTx;	R-K-R-H-P-D-C-D-K-P-P-D-T-K-I-	
	β_3-BTx;	R-Q-R-H-R-D-C-D-K-P-P-D-K-G-N-	

Fig. 4. **Amino terminal sequences of the purified neurotoxins in comparison with those of β-BTxs from *B. multicinctus* venom.** Beta$_{1-3}$BTxs denote β_1-, β_2-, and β_3-bungarotoxins, respectively. [a]Figures in parentheses are percent homologies with the A-chains of β_{1-3}BTxs, respectively. [b]Figures in parentheses are percent homologies with the B-chains of β_{1-3}BTxs, respectively. The presence of cysteine at the seventh position in the B-chains of the purified neurotoxins was deduced from the deletion of the PTH-amino acid on automated sequencing.

REFERENCES

1. Taylor, E.H. (1965) The serpents of Thailand and adjacent waters. *Univ. Kansas Sci. Bull.* **45**, 609–1096
2. Chanhome, L., Cox, M.J., Wilde, H., Jintakoon, P., Chaiyabutr, N., and Sitprija, V. (1998) Venomous snakebite in Thailand I: medically important snakes. *Military Medicine* **163**, 310–317
3. Slowinski, J.S., Boundy, J., and Lawson, R. (2001) The phylogenetic relationships of Asian coral snakes (Elapidae: *Calliophis* and *Maticora*) based on morphological and molecular characters. *Herpetologica* **57**, 233–245
4. Pochanugool, C., Wilde, H., Bhanganada, K., Chanhome, L., Cox, M.J., Chaiyabutr, N., and Sitprija, V. (1998) Venomous snakebite in Thailand II: clinical experience. *Military Medicine* **163**, 318–323
5. Sawai, Y., Mishima, S., Tseng, C.S., Toriba, M., Lim, B.L., Ramachandran, C.P., Devaraj, T., Phongaksara, S., and Romer, J.D. (1978) Study on seasnakes and the bites in Malaysia, Thailand and Hong Kong. *The Snake* **9**, 48–62
6. Warrell, D.A., Looareesuwan, S., White, N.J., Theakston, R.D.G., Warrell, M.J., Kosakarn, W., and Reid, H.A. (1983) Sever neurotoxic envenoming by the Malayan krait *Bungarus candidus* (Linnaeus): response to antivenom and anticholinesterase. *Brit. Med. J.* **286**, 678–680
7. Laothong, C. and Sitprija, V. (2001) Decreased parasymphathetic activities in Malayan krait (*Bungarus candidus*) envenoming. *Toxicon* **39**, 1353–1357
8. Looareesuwan, S., Viravan, C., and Warrell, D.A. (1988) Factors contributing to fatal snake bite in the rural tropics: analysis of 46 cases in Thailand. *Trans. R. Soc. Trop. Med. Hyg.* **82**, 930–934
9. Viravan, C., Looareesuwan, S., Kosakarn, W., Wuthiekanun, V., McCarthy, C.J., Stimson, A.F., Bunnag, D., Harinasuta, T., and Warrell, D.A. (1992) A national hospital-based survey of snakes responsible for bites in Thailand. *Trans. R. Soc. Trop. Med. Hyg.* **86**, 100–106
10. Ganthavorn, S. (1969) Toxicities of Thailand snake venoms and neutralization capacity of antivenom. *Toxicon* **7**, 239–241
11. Chanhome, L., Wongtongkam, N., Khow, O., Pakmanee, N., Omori-Satoh, T., and Sitprija, V. (1999) Genus specific neutralization of *Bungarus* snake venoms by Thai Red Cross banded krait antivenom. *J. Nat. Toxins* **8**, 135–140

12. Tan, N.H., Poh, C.H., and Tan, C.S. (1989) The lethal and biochemical properties of *Bungarus candidus* (Malayan krait) venom and venom fractions. *Toxicon* **27**, 1065–1070
13. Tsai, I.H., Hsu, H.Y., and Wang, Y.M. (2002) A novel hospholipase A_2 from the venom glands of *Bungarus candidus*: cloning and sequence-comparison. *Toxicon* **40**, 1363–1367
14. Chang, C.C. and Lee, C.Y. (1963) Isolation of neurotoxins from the venom of *Bungarus multicinctus* and their mode of neuromuscular blocking action. *Archs. Int. Pharmacodyn. Ther.* **144**, 241–257
15. Chang, C.C., Chen, T.F., and Lee, C.Y. (1973) Studies on the presynaptic effect of β-bungarotoxin on neuromuscular transmission. *J. Pharmac. exp. Ther.* **184**, 339–345
16. Kondo, K., Narita, K., and Lee, C.Y. (1978) Amino acid sequences of two polypeptide chains in beta 1-bungarotoxin from the venom of *Bungarus multicinctus*. *J. Biochem.* **83**, 101–115
17. Kondo, K., Toda, H., Narita, K., and Lee, C.Y. (1982a) Amino acid sequence of beta 2-bungarotoxin from *Bungarus multicinctus* venom. The amino acid substitutions in the B chain. *J. Biochem.* **91**, 1519–1530
18. Kondo, K., Toda, H., Narita, K., and Lee, C.Y. (1982b) Amino acid sequences of three beta-bungarotoxins (beta 3-, beta 4-, and beta 5-bungarotoxins) from *Bungarus multicinctus* venom. Amino acid substitutions in the A chains. *J. Biochem.* **91**, 1531–1548
19. Danse, J.M., Toussaint, J.L., and Kempf, J. (1990) Nucleotide sequence encoding beta-bungarotoxin A2-chain from the venom glands of *Bungarus multicinctus*. *Nucleic Acids Res.* **18**, 4609
20. Chu, C.C., Chu, S.T., Chen, S.W., and Chen, Y.H. (1994) The non-phospholipase A_2 subunit of beta-bungarotoxin plays an important role in the phospholipase A_2 independent neurotoxic effect: characterization of three isotoxins with a common phospholipase A_2 subunit. *Biochem. J.* **303**, 171–176
21. Wu, P.F., Wu, S.N., Chang, C.C., and Chang, L.S. (1998) Cloning and functional expression of B chains of beta-bungarotoxins from *Bungarus multicinctus* (Taiwan banded krait). *Biochem. J.* **334**, 87–92
22. Wu, P.F. and Chang, L.S. (2000) Genetic organization of A chain and B chain of beta-bungarotoxin from Taiwan banded krait (*Bungarus multicinctus*): A chain genes and B chain genes do not share a common origin. *Eur. J. Biochem.* **267**, 4668–4675
23. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**, 680–685
24. Oakley, B.R., Kirsch, D.R., and Morris, N.R. (1980) A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. *Anal. Biochem.* **105**, 361–363
25. Iwamatsu, A. (1992) S-carboxymethylation of proteins transferred onto polyvinylidene difluoride membranes followed by in situ protease digestion and amino acid microsequencing. *Electrophoresis* **13**, 142–147
26. Reed, L.J. and Muench, H. (1938) A simple method of estimating fifty percent end- points. *Amer. J. Hyg.* **27**, 493–497
27. Pizzi, M. (1950) Sampling variation of the fifty per cent endpoint, determined by the Reed-Muench (Behrens) method. *Hum. Biol.* **22**, 151–190
28. Marinetti, G.V. (1965) The action of phospholipase A on lipoproteins. *Biochim. Biophys. Acta* **98**, 554–565
29. Gutierrez, J.M., Avila, C., Rojas, E, and Cerdas, L. (1988) An alternative *in vitro* method for the testing potency of the polyvalent antivenom produced in Costa Rica. *Toxicon* **26**, 411–413
30. World Health Organization (1995) Parallel line analysis in Manual of Laboratory Methods for Potency Testing of Vaccines Used in the WHO Expanded Programme on Immunization (WHO, ed.) pp. 206–212
31. Khow, O., Chanhome, L., Omori-Satoh, T., and Sitprija, V. (2002) Isolation of the major lethal toxin in the venom of *Bungarus flaviceps*. *Toxicon* **40**, 463–469
32. Bon, C. and Saliou, B. (1983) Ceruleotoxin: Identification in the venom of *Bungarus fasciatus*, molecular properties and importance of phospholipase A_2 activity for neurotoxicity. *Toxicon* **21**, 681–698
33. Chang, C.C. (1979) The action of snake venoms on nerve and muscle in *Snake Venoms* (Lee, C.Y., ed.) pp. 309–376, Springer, Berlin
34. Mebs, D. and Claus, I. (1991) Amino acid sequences and toxicities of snake venom components in *Snake Toxins*: *International Encyclopedia of Pharmaceutical Therapeutics* (Harvey, A.L., ed.) Section 134, pp. 425–447, Pergamon Press, New York
35. Chuman, Y., Nobuhisa, I., Ogawa, T., Deshimaru, M., Chijiwa, T., Tan, N.H., Fukumai, Y., Shimohigashi, Y., Ducancel, F., Boulain, J.C., Menez, A., and Ohno, M. (2000) Regional and accelerated molecular evolution in group I snake venom gland phospholipase A_2 isoenzymes. *Toxicon* **38**, 449–462
36. Fujimi, T.J., Kariya, Y., Tsuchiya, T., and Tamiya, T. (2002) Nucleotide sequence of phospholipase A_2 gene expressed in snake pancreas reveals the molecular evolution of toxic phospholipase A_2 genes. *Gene* **292**, 225–231
37. Chippaux, J.P., Williams, V., and White, J. (1991) Snake venom variability: methods of study, results and interpretation. *Toxicon* **29**, 1279–1303