Biochemical Pharmacology 88 (2014) 229–236

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Isolation and characterization of α-elapitoxin-Bf1b, a postsynaptic neurotoxin from Malaysian *Bungarus fasciatus* venom

Muhamad Rusdi Ahmad Rusmili [a,b], Ting Yee Tee [b], Mohd Rais Mustafa [c], Iekhsan Othman [b], Wayne C. Hodgson [a,*]

[a] Monash Venom Group, Department of Pharmacology, Faculty of Medicine, Nursing and Health Sciences, 3168 Clayton, Victoria, Australia
[b] Jeffrey Cheah School of Medicine and Health Sciences, Monash University Sunway Campus, 46150 Bandar Sunway, Malaysia
[c] Department of Pharmacology, Faculty of Medicine, University of Malaya, 59100 Kuala Lumpur, Malaysia

ARTICLE INFO

Article history:
Received 22 October 2013
Received in revised form 3 January 2014
Accepted 7 January 2014
Available online 16 January 2014

Keywords:
Bungarus fasciatus
Short-chain neurotoxin
Antivenom
Skeletal muscle
Proteomics

ABSTRACT

Bungarus fasciatus is one of three species of krait found in Malaysia. Envenoming by *B. fasciatus* results in neurotoxicity due to the presence of presynaptic and postsynaptic neurotoxins. Antivenom, either monovalent or polyvalent, is the treatment of choice in systemically envenomed patients. In this study, we have isolated a postsynaptic neurotoxin which we named α-elapitoxin-Bf1b. This toxin has an approximate molecular weight of 6.9 kDa, with LCMS/MS data showing that it is highly homologous with Neurotoxin 3FTx-RI, a toxin identified in the *Bungarus fasciatus* venom gland transcriptome. α-Elapitoxin-Bf1b also shared similarity with short-chain neurotoxins from *Laticauda colubrina* and *Pseudechis australis*. α-Elapitoxin-Bf1b produced concentration- and time-dependent neurotoxicity in the indirectly-stimulated chick biventer cervicis muscle preparation, an effect partially reversible by repetitive washing of the preparation. The pA_2 value for α-elapitoxin-Bf1b of 9.17 ± 0.64, determined by examining the effects of the toxin on cumulative carbacol concentration-response curves, indicated that the toxin is more potent than tubocurarine and α-bungarotoxin. Pre-incubation of *Bungarus fasciatus* monovalent and neuro polyvalent antivenom failed to prevent the neurotoxic effects of α-elapitoxin-Bf1b in the chick biventer cervicis muscle preparation. In conclusion, the isolation of a postsynaptic neurotoxin that cannot be neutralized by either monovalent and polyvalent antivenoms may indicate the presence of isoforms of postsynaptic neurotoxins in Malaysian *B. fasciatus venom*.

1. Introduction

Bungarus fasciatus, commonly known as the banded krait, is a widely distributed species of venomous snake found from India to China and some countries in South East Asia. It is easily recognized by its triangular shaped body and alternating white or yellow and black bands on its body [1]. *B. fasciatus* is considered a medically important snake even though envenoming by this species is relatively rare compared with envenoming by *Bungarus candidus* or *Bungarus multicinctus* in South East Asia [2].

Systemic envenoming by *B. fasciatus* can be fatal in the absence of urgent medical treatment [2,3] with respiratory paralysis due to the presence of highly potent neurotoxins [3]. Envenomed victims may present with drowsiness, headache, vomiting, abdominal pain, and ptosis which may progress to flaccid paralysis. In Malaysia, neuro polyvalent antivenom is the treatment of choice for *B. fasciatus* envenoming. This antivenom has been found to neutralize not only Asian elapid snake venom but also African elapid snake venom in experimental envenomed animals [4].

Postsynaptic (α) neurotoxins are major components of *B. fasciatus* venom [4,5]. These neurotoxins bind to the postsynaptic nicotinic receptors and prevent binding of the neurotransmitter, acetylcholine. Postsynaptic neurotoxins can be divided into several groups based on their amino acid length and the number of their cysteine residues. Short-chain neurotoxins have approximate molecular weights of 6–8 kDa, 8–9 cysteine residues and bind strongly to muscle-type nicotonic receptors but with less affinity to neuronal acetylcholine receptors as compared with muscle-type acetylcholine nicotinic receptors [6,7].

Several short-chain postsynaptic neurotoxins have been isolated from *B. fasciatus* venom and pharmacologically characterized [5,8,9]. Most of the postsynaptic toxins that have been isolated from *B. fasciatus* venom have been found to be orphan group neurotoxins that are weakly neurotoxic when tested in isolated tissue preparations [5]. One type I neurotoxin, called short neurotoxin 1, has been isolated and characterized from *B. fasciatus* venom [9]. There have been no studies on the prevention and/or

* Corresponding author.
E-mail address: wayne.hodgson@monash.edu (W.C. Hodgson).

0006-2952/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2014.01.004

reversibility of the neurotoxic effects caused by short-chain neurotoxins from *B. fasciatus* venom by antivenoms.

In this study, we describe the isolation and characterization of α-elapidtoxin-Bf1b, a short-chain neurotoxin that has been isolated from Malaysian *B. fasciatus* venom. We have also tested the effectiveness of *B. fasciatus* monovalent and neuro polyvalent antivenoms against the *in vitro* neurotoxicity.

2. Materials and methods

2.1. Collection of Bungarus fasciatus venom

B. fasciatus venom was donated by Mr. Zainuddin Ismail (Perlis, Northwest of Peninsular Malaysia). All snakes originated from Northwest of Peninsular Malaysia and were milked by positioning the fangs on a plastic container, with a parafilm cover, while the venom glands were externally massaged. Fresh crude venom was transported on ice to the laboratory at Sunway campus and frozen at −80 °C before being freeze-dried and stored at -20 °C until required.

2.2. Drugs and chemicals

The following were purchased from Sigma–Aldrich (St. Louis, Missouri, USA); D-tubocurarine, proteomics grade bovine trypsin, carbachol, ammonium bicarbonate, acetylcholine and mass spectrometry grade formic acid. The following were purchased from Merck (Darmstadt, Germany); glycine, tris base, acrylamide, bis-polyacylamide, bromophenol blue, acetic acid, trifluroacetic acid, butanol, methanol, sodium chloride (NaCl), potassium chloride (KCl), and dithiothretiol (DTT). The following were purchased from Fisher Scientific (Loughborough, Leicestershire, UK); glucose, calcium chloride ($CaCl_2$), potassium di-hydrogen phosphate ($KHPO_4$), sodium hydrogen carbonate ($NaHCO_3$) ammonium acetate, high pressure chromatography (HPLC) grade acetonitrile, HPLC grade water, MS grade acetonitrile, and MS grade water. The following were purchased from GE Healthcare (Uppsala, Sweden); iodoacetamide and PhastGel Blue tablets. All chemicals were dissolved or diluted in Milli-Q water for experiments.

2.3. Purification of α-elapitoxin-Bf1b

Freeze-dried venom (100 mg) was dissolved in Milli-Q water and centrifuged at 14,000 rpm before being loaded into a Superdex G75 gel filtration column (GE Healthcare, Uppsala, Sweden) mounted on an Akta Purifier system (GE Healthcare, Uppsala, Sweden). The column was equilibrated with 0.01 M ammonium acetate buffer pH 6.8 at a flow rate of 0.5 mL/min and fractions collected at 0.5 mL/tube during the run. Fractions of the crude venom were later pooled according to the peaks in the gel filtration chromatogram monitored at 280 nm before being frozen and freeze-dried. The pooled fractions corresponding to the gel filtration peak containing the toxin were later dissolved in Milli-Q water before being loaded onto a Mono S strong cationic ion-exchange chromatography column mounted on an Akta Purifier System (GE Healthcare, Uppsala, Sweden). The column was equilibrated with 0.05 M sodium phosphate buffer pH 6.8 at a flow rate of 2 mL/min. The peaks were monitored at 280 nm and eluted with a linear gradient up to 0.5 M sodium chloride in 0.05 M sodium phosphate buffer using the following gradient; 0% for 6.5 min, 0–20% over 2 min, 20–50% over 9.5 min, 50–100% over 3 min and 100% for 10 min. Fractions were automatically collected at 1 mL/tube during the run. The fractions containing the toxin were pooled and freeze-dried. The pooled fractions were desalted using a HiTrap desalting column attached to an Akta Prime Plus (GE Healthcare, Uppsala, Sweden). The desalting column was equilibrated with 0.01 M ammonium acetate pH 6.8 and run at 0.5 mL/min. The desalted fractions were pooled and freeze-dried. Fractions collected after size-exclusion and cation-exchange chromatography were screened by LCMS/MS to identify a toxin homologous to Neurotoxin 3FTX-RI. Neurotoxicity was confirmed using the indirectly-stimulated chick biventer cervicis muscle. The ion-exchange chromatography peak containing the toxin was later dissolved in Milli-Q water before being loaded onto a C18 300 Å Jupiter reverse phase column (Phenomenex, USA) mounted on an Agilent high pressure liquid chromatography system (Agilent Technologies, Santa Clara, USA). The column was equilibrated with 5% acetonitrile (ACN) with 0.1% trifluoroactic acid (TFA) in water and was run at a flow rate of 0.2 mL/min. Elution of the peak containing the toxin was monitored at 214 nm and eluted with 90% ACN in 0.1% TFA in water by using the following gradient; 5% for 5 min, 3–35% over 5 min, 35–40% over 30 min, 40–100% over 10 min, 100% for 5 min and 100–5% over 5 min. The peak containing the toxin was pooled into a single vial using the fraction collector.

2.4. Protein quantification by bicinchoninic acid assay (BCA)

Protein content was determined using a commercial BCA kit (Pierce Biotechnology, Illinois, USA) according to manufacturer's instruction.

2.5. Tricine SDS-PAGE

A 10% Tricine SDS-PAGE gel was cast and run according to the protocol previously described [10]. The gel was stained with Coomassie blue and destained by using 5% acetic acid in water. Broad range protein marker (Bio-Rad, Hercules, USA) was used for molecular protein weight marker. The gel was scanned using the ChemiDoc XRS Imaging System (BioRad, Hercules, USA).

2.6. Intact protein analysis with ESI-LCMS

The toxin was loaded onto a Agilent SPQ-105 Protein Chip mounted on an Agilent 1200 HPLC-Chip/MS Interface, coupled with an Agilent 6520 Accurate-Mass Q-TOF LC/MS system (Agilent, USA). The chip was run at 4 μL/min from an Agilent 1200 Series Capillary pump and 0.4 μL/min from an Agilent 1200 Series Nano Pump with 0.1% formic acid in water (solution A) and 90% acetonitrile in water with 0.1% formic acid (solution B). The chip was equilibrated with 3% solution B and the gradient used during the run was 3–70% solution B from 0 to 15 min, 70–97% from 15 to 15.1 min, 97% from 15.1 to 22 min, and 97–3% from 22 to 22.1 min. The polarity of the Q-TOF was set at positive, capillary voltage at 1850 V, fragmentor voltage at 300 V, drying gas flow of 5 L/min and gas temperature of 350 °C. The intact protein spectrum was analyzed in MS only mode from range of 100–3200 *m/z*. The spectrum was then deconvulated by using Agilent BioConfirm MassHunter Qualitative Analysis software (Agilent Technologies, Santa Clara, USA).

2.7. Protein identification with ESI-LCMS/MS

The freeze-dried toxin was dissolved in Milli-Q water before being digested using in-solution trypsin digestion protocol as supplied by the manufacturer (Agilent Technologies, Santa Clara, USA). The digested peptides were loaded onto an Agilent C18 300 Å Large Capacity Chip (Agilent Technologies, Santa Clara, USA). The column was equilibrated with 0.1% formic acid in water (solution A) and peptides were eluted with an increasing gradient of 90% ACN in water with 0.1% formic acid (solution B) with the following

Table 1
List of toxins with homology with α-elapitoxin-Bf1b.

Entry	Toxin names	Species	% Coverage
P0C555	Neurotoxin 3FTx-RI	*Bungarus fasciatus*	72.3
P10808	Short neurotoxin 1 (Toxin V-II-1)	*Bungarus fasciatus*	71.9
P10456	Short neurotoxin D	*Laticauda colubrina*	50.6
P10455	Short neurotoxin C	*Laticauda colubrina*	50.6
P25497	Short neurotoxin 1 (Neurotoxin PA A)	*Pseudechis australis*	50.6

gradient; 3–50% from 0 to 30 min, 50–95% from 30 to 32 min, 95% from 32 to 37 min and 95–3% from 37 to 38 min. The polarity of the Q-TOF was set at positive, capillary voltage at 2050 V, fragmentor voltage at 300 V, drying gas flow of 5 l min and gas temperature of 350 °C. The intact protein spectrum was analyzed in MSMS only mode over a range of 50–3000 *m/z*. The spectrum was analyzed by using Agilent MassHunter data acquisition software (Agilent Technologies, Santa Clara, USA). *De novo* sequencing was conducted using PEAK 6.0 (Bioinformatics Solution Inc., Waterloo, ON, Canada) by using the same LCMS/MS data to identify and detect amino acid modification of the toxin based on the homologous proteins and peptides from the SWISS-PROT protein database. Searches by PEAK 6.0 were conducted by setting the parent and fragment mass tolerance at 0.1 Da, maximum 3 missed cleavage, carbamidomethylation as fixed modifications, trypsin as digestion enzyme and false discovery rate at 0.1%. Only proteins with at least 2 peptides and a unique peptide were accepted.

2.8. N-Terminal amino acid sequence determination

Purified toxin was loaded onto the sequencing chamber of a Procise N-terminal acid sequencer (Applied Biosystem, Foster City, CA, USA) and the amino acid sequence was determined (Edman degradation, phenylthiohydantoin derivatization chemistry and separation of derivatized amino acids) using the manufacturer's recommended methods and reagents.

2.9. Chick biventer cervicis nerve-muscle preparation

Male chicks (5–12 days old) were killed by CO_2 inhalation before a pair of chick biventer muscles were dissected and mounted in 5 mL organ baths. The organ baths were filled with physiological salt solution of the following composition; NaCl, 118.4 mM; KCl, 4.7 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_3$, 25 mM and glucose, 11.1 mM. The tissues in the organ baths were constantly aerated with carbogen (5% CO_2 and 95% O_2). The tissues were stimulated every 10 s with pulses of 0.2 ms duration at a supramaximal voltage (10–20 V) using a Grass S88 stimulator attached to ring electrodes. Indirect stimulation of the tissues was confirmed by the abolishment of twitches by tubocurarine (10 μM), which was then washed out until twitches were restored. In the absence of electrical stimulation, responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s) and KCl (40 mM for 30 s) were obtained. Responses were recorded on a PowerLab (ADInstruments, Australia). In order to examine the reversibility of the toxin, the toxin (0.1 μg/mL) was left in contact with the tissue for 40 min before washing every 10 min for at least 60 min. For antivenom studies, antivenom was added into the organ bath 10 min prior to the addition of toxin.

For carbachol concentration-response curves, the isolated chick biventer cervicis muscle was mounted in an organ bath. The toxin was added and allowed to equilibrate for 1 h prior to obtaining a cumulative concentration-curve to carbachol (1 μM to 10 mM).

2.10. Statistics

All statistics (Student t test, one way ANOVA, two-way ANOVA, paired *t*-test) where indicated) were performed using Prism version 6.0 (GraphPad, San Diego, CA, USA). Responses are expressed as mean ± the standard error of the mean.

3. Results

3.1. Purification of α-elapidtoxin-Bf1b

α-Elapitoxin-Bf1b was isolated from *B. fasciatus* venom using the following steps. Fractionation of venom by size-exclusion chromatography on a Superdex G75 column yielded 10 peaks that were individually collected. This fraction was further fractionated using a strong cation-exchange column. LCMS/MS analysis showed that peak 3 from size-exclusion and peak 1 from the cation-exchange chromatograms (Fig. 1a and b) contained peptides with homology to Neurotoxin 3FTx-RI. Initial screening of the peaks in the chick biventer cervicis nerve-muscle preparation indicated marked postsynaptic neurotoxicity in peak 3 from size-exclusion and peak 1 from the cation exchange chromatography. Further fractionation using reverse-phase chromatography isolated a pure toxin, which shared homology with Neurotoxin 3FTx-RI, which we

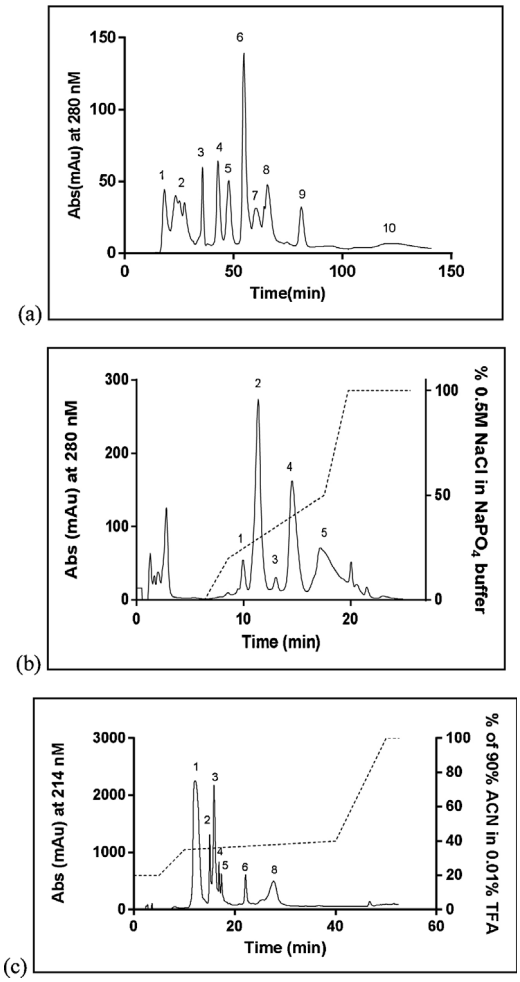

Fig. 1. Chromatograms of Malaysian *B. fasciatus* venom showing the fractionation steps to purify α-elapitoxin-Bf1b. (a) Superdex G75 size-exclusion of *B. fasciatus* venom; (b) Fractionation of peak 3 from size-exclusion by Mono S cation-exchange; (c) further purification of peak 1 from Mono S cation-exchange using a C18 reverse phase column. Peak 1 in the reverse phase chromatogram contained α-elapitoxin-Bf1b.

named α-elapitoxin-Bf1b (based on the nomenclature proposed by King et al. [11]) from peak 1 (Fig. 1c). The homogeneity of the toxin was confirmed by tricine SDS-PAGE gel and intact protein LCMS analysis. α-Elapitoxin-Bf1b is estimated to constitute 0.15% of the total venom protein content.

3.2. Tricine SDS-PAGE

From the standard curve graph and bands in the tricine gel, reduced α-elapitoxin-Bf1b was estimated to have an R_f value of 0.875 with log MW value of 0.958 and non-reduced α-elapitoxin-Bf1b an R_f value of 0.852 with log MW value of 0.983. The estimated molecular weight of the toxin from the gel was 9.08 kDa when reduced and 9.62 kDa when non-reduced (Fig. 2a).

3.3. Intact protein analysis by LCMS

Intact protein analysis by using ESI-LCMS showed α-elapitoxin-Bf1b has a molecular weight of 6982.12 Da and the sample did not have any prominent protein contaminants within the range of 5000–24,000 Da when stained with Coomassie blue (Fig. 2b). This was also confirmed by LCMS/MS analysis with only one venom protein detected.

3.4. Identification and de novo sequencing by LCMS/MS and partial N-terminal sequencing

Protein identification and *de novo* sequencing with PEAKS 6 *de novo* sequencing software (BSi, Canada) generated the following sequence for α-elapitoxin-Bf1b based on the sequence obtained from the most homologous protein, Neurotoxin 3FTx-RI [POC555]:

1 50
MKTLLLTLVV LTIVCLDLGH TRICLNQQSS EPQTTELCPN GEDTCYNKTW

51 88
NTHRGSRTDR GCGCPKVKPG INLRCCKTDK CNQ

Partial N-terminal analysis by Edman degradation for 25 amino acid residues was conducted to confirm the presence of the sequence that was determined by LCMS/MS. The first 25 amino acids sequence was:

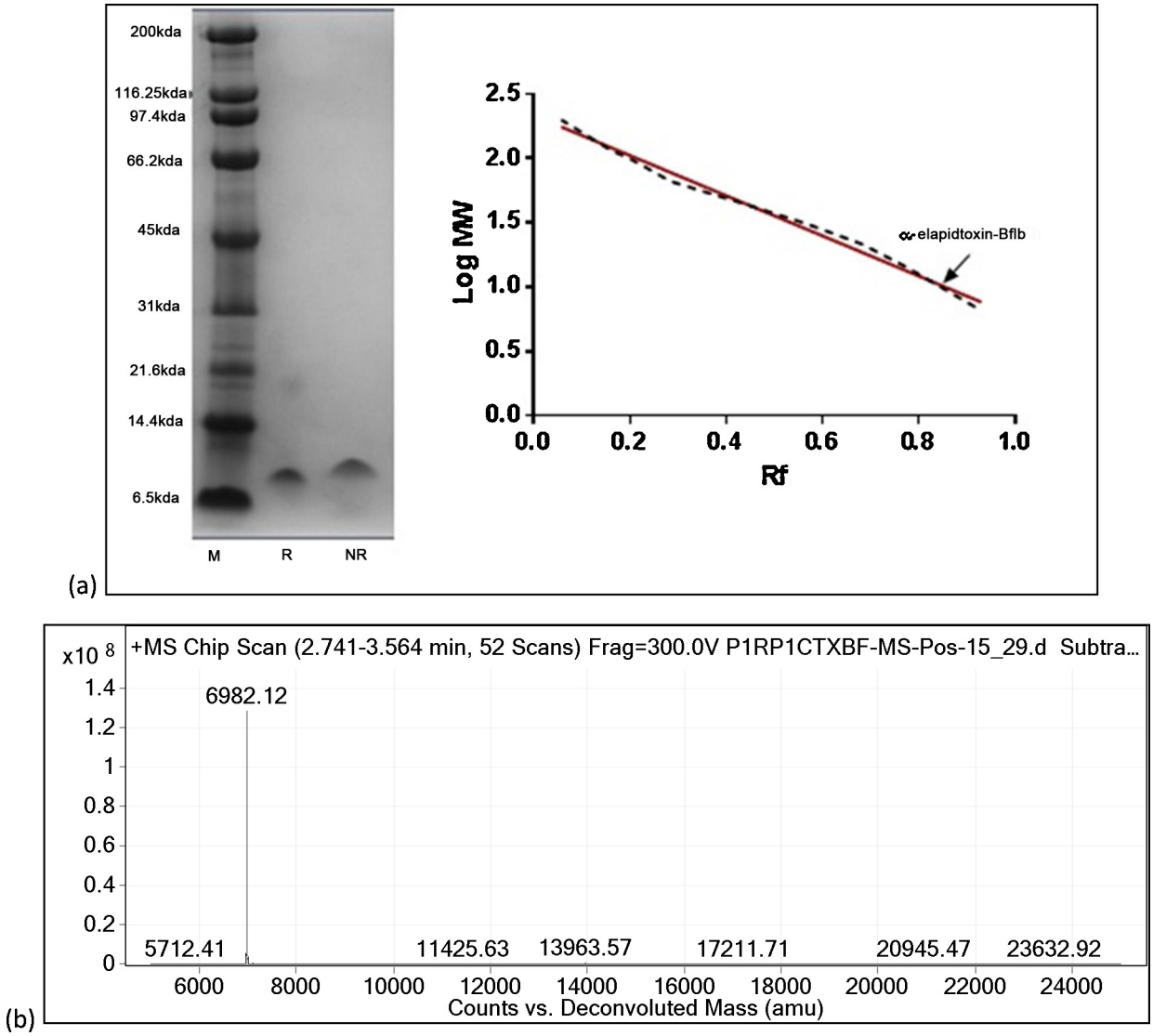

Fig. 2. Molecular weight determination of α-elapitoxin-Bf1b on (a) 10% Tricine SDS-PAGE gel loaded with marker (M), line R was loaded with α-elapitoxin-Bf1b treated with reducing SDS-PAGE sample buffer (with DTT) and line NR was loaded with α-elapitoxin-Bf1b treated with non-reducing SDS PAGE sample buffer (without DTT). (b) Intact protein analysis chromatogram by LCMS for α-elapitoxin-Bf1b. α-Elapitoxin-Bf1b has a MW of 6982.12 Da.

```
α-elapitoxin-Bf1b   ----------RICLNQQSSEPQTTEIC--PDGEDTCYNKTWNTHRGSRT 37
NTXRI_BUNFA[P0C555] MKTLLLTLVVLTIVCLDLGHTRICLNQQSSEPQTTETC--PNGEDTCYNKTWNTHRGSRT 58
NXS1_BUNFA [P10808] ----------RICLNQQQSTPEDQPTNGQCYIKTDCQNKTWNTHRGSRT 39
NXSD_LATCO [P10456] MKTLLLTLVVVTMVCLDLGYTRRCFNQQSSQPKTTKSC--PPGENSCYNKQWRDHRGSIT 58
NXSC_LATCO [P10455] MKTLLLTLVVVTMVCLDLAYTRRCYNQQSSQPKTTKSC--PPGENSCYNKQWRDHRGSIT 58
NXS1_PSEAU [P25497] MKTLLLTLVVVTIVCLDLGYTMTCCNQQSSQPKTTTIC--AGGESSCYKKTWSDHRGSRT 58
                                  * ***.* *:          :  * :* *  **** *

α-elapitoxin-Bf1b   DRGCGCPKVKPGINLRCCKTDKCNQ 62
NTXRI_BUNFA[P0C555] DRGCGCPKVKPGINLRCCKTDKCNQ 83
NXS1_BUNFA [P10808] DRGCGCPKVKPGINLRCCKTDKCNE 64
NXSD_LATCO [P10456] ERGCGCPKVKPGIKLRCCESEDCNN 83
NXSC_LATCO [P10455] ERGCGCPKVKPGIKLRCCESEDCNN 83
NXS1_PSEAU [P25497] ERGCGCPHVKPGIKLTCCKTDECNN 83
                    :******:*****:* **::::.**:
```

Fig. 3. Alignment of α-elapitoxin-Bf1b with homologous short-chain neurotoxins from Table 1. The shaded amino acids are homologous amino acids with α-elapitoxin-Bf1b and amino acids with (*) are fully conserved in all toxins. Conserved amino acids with (.) are weakly similar properties group and (:) are strongly similar properties group.

RICLNQQSSEPQTTEICPDGEDTCY

The toxin partial N-terminal sequence search and alignment using UniProt (http://www.uniprot.org/) confirmed that α-elapitoxin-Bf1b is highly homologous to Neurotoxin 3Ftx-RI (POC555) with a 92% identity match. Partial N-terminal sequencing showed two amino acid substitutions at amino acid position 37 and 40 of the sequence obtained from *de novo* sequencing and leucine at position 37 in the sequence generated by LCMS/MS, was found to be substituted with isoleucine and asparagine was substituted aspartic acid at position 40 in partial N-terminal sequence. It also showed that the signal peptides, which are the first 21 amino acids as predicted from *de novo* sequencing, are not present in the toxin. Based on these results, the deduced sequence of α-elapitoxin-Bf1b is:

```
1                                   40
RICLNQQSSE PQTTEICPDG EDTCYNKTWN THRGSRTDRG

41                          62
CGCPKVKPG I NLRCCKTDKC NQ
```

Alignment of the deduced sequence from *de novo* sequencing and partial N-terminal sequencing showed that the toxin has 72% sequence homology with Neurotoxin 3FTx-RI (Fig. 3 and Table 1). Further search and alignment using the UniProt database indicates that α-elapitoxin-Bf1b is similar to short-chain neurotoxin 1 from *Bungarus fasciatus* venom, short-chain neurotoxin 1 found in *Pseudechis australis* venom and short-chain neurotoxin D and C from *Laticauda colubrina* venom (Table 1).

3.5. Indirectly stimulated chick biventer cervicis nerve-muscle

α-Elapitoxin-Bf1b (0.1–1 μg/mL) abolished indirect twitches in the chick biventer cervicis nerve-muscle preparation (one-way ANOVA, $P < 0.05$ compared to vehicle; Fig. 4). The time required to produce 90% inhibition of twitches (i.e. t_{90}) for 1 μg/mL, 0.3 μg/mL and 0.1 μg/mL was 18.9 ± 0.7 min, 24.3 ± 1.0 min and 64.8 ± 6.5 min, respectively. In comparison, the t_{90} values for α-bungarotoxin (1 μg/mL) and *B. fasciatus* venom (1 μg/mL) were 31.4 ± 3.8 min and 54.9 ± 5.7 min, respectively (Fig. 5). α-Elapitoxin-Bf1b significantly inhibited contractile responses to ACh and CCh, but not KCl, similar to α-bungarotoxin and *B. fasciatus* venom (Fig. 5b). The inhibition of nerve-mediated twitches by α-elapitoxin-Bf1b (0.1 μg/mL) was partially reversed after washing every 10 min, commencing approximately 90 min after addition of α-elapitoxin-Bf1b, with 41 ± 7% recovery of the initial twitch height (Fig. 9).

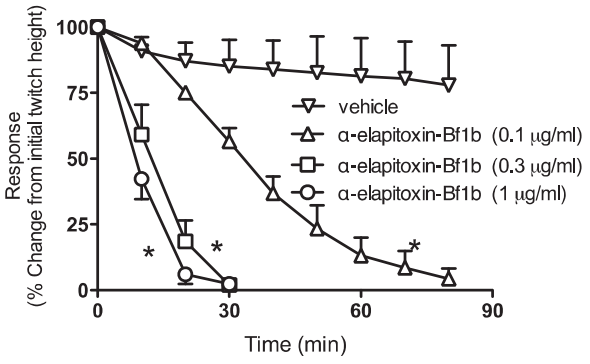

Fig. 4. The effect of α-elapitoxin-Bf1b (0.1–1 μg/mL) on indirect twitches in the chick biventer cervicis nerve-muscle preparation. *$P < 0.05$, significantly different from vehicle control ($n = 3$–4, one-way ANOVA).

3.6. Antivenom studies

Pre-incubation (10 min) of monovalent *Bungarus fasciatus* antivenom (Thai Red Cross Society, Thailand) at 1, 3 or 10 times the recommended titre (i.e. 1 mL antivenom/0.6 mg of venom) did not prevent the inhibition of twitches by α-elapitoxin-Bf1b (Fig. 6). The t_{90} values for the tissues treated with antivenom were as follows: 1 ×, 24.8 ± 0.9 min; 3 ×, 26.3 ± 1.5; and 10 ×, 17.0 ± 5.6 min, respectively. Contractile responses to ACh and CCh were still

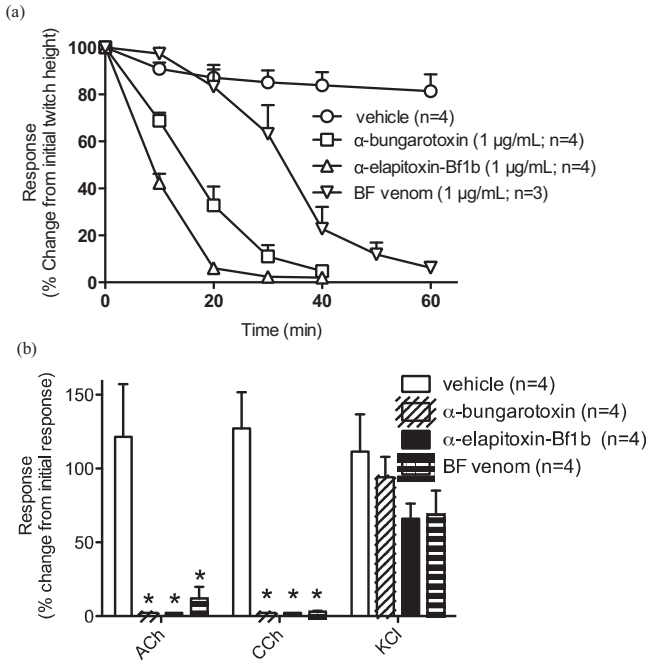

Fig. 5. The effect of α-elapitoxin-Bf1b (1 μg/mL) compared to *B. fasciatus* venom (BF; 1 μg/mL) and α-bungarotoxin (1 μg/mL) on (a) indirect twitches or (b) contractile response to exogenous ACh, CCh, or KCl in the chick biventer cervicis nerve-muscle preparation. *$P < 0.05$, significantly different from vehicle control ($n = 4$, one-way ANOVA for (a) and two-way ANOVA for (b)).

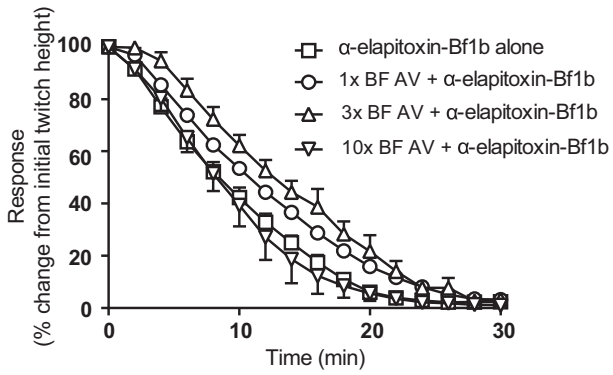

Fig. 6. The effect of preincubation of *Bungarus fasciatus* monovalent antivenom (BF AV; 1–10× concentration) on the effects of α-elapitoxin-Bf1b (1 μg/mL) on indirect twitches in the chick biventer cervicis nerve-muscle preparation (n = 3-4).

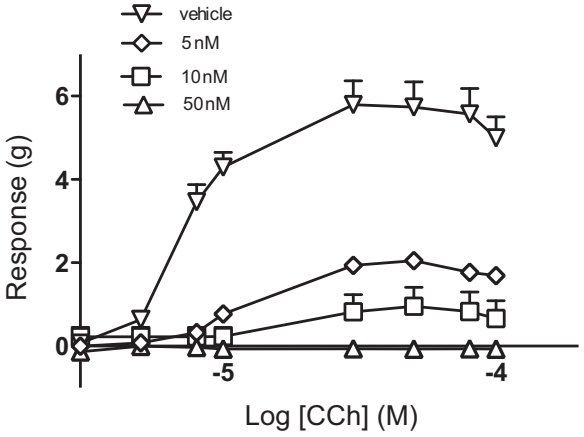

Fig. 8. Cumulative concentration–response curves to carbachol in the unstimulated chick biventer cervicis nerve-muscle preparation in presence and absence of increasing concentrations of α-elapitoxin-Bf1b (n = 4).

significantly inhibited in the combined presence of antivenom and α-elapitoxin-Bf1b (data not shown).

Similarly, pre-incubation (10 min) with neuro polyvalent antivenom (Thai Red Cross Society, Thailand) at 10 × the recommended titre (i.e. 1 mL antivenom/0.6 mg of venom) did not prevent the inhibition of twitches by α-elapitoxin-Bf1b (Fig. 7). However, prior incubation with neuro polyvalent antivenom significantly increased the t_{90} value in comparison with the t_{90} value obtained in the absence of antivenom. Contractile responses to ACh and CCh were still significantly inhibited in the combined presence of antivenom and α-elapitoxin-Bf1b (data not shown).

3.7. Cumulative carbachol concentration-response curves

α-Elapitoxin-Bf1b (5–50 nM) caused a marked reduction in the maximum response of tissues to cumulative additions of carbachol (Fig. 8). Using the modified Lew and Angus method, the pA_2 was calculated as 9.17 ± 0.64.

4. Discussion

Envenoming by *Bungarus spp.* causes considerable morbidity and mortality in victims. *Bungarus spp.* venom contains a complex mixture of toxins and enzymes. Some of the venom components have been isolated and characterised but the functions of many of the toxins are unknown. *B. fasciatus* is one species of the *Bungarus* genus found in Malaysia, and is considered to be medically important. Despite this, only one type-1 short-chain neurotoxin has been isolated and partially characterized from *B. fasciatus* venom [9]. In addition, several orphan group 'neurotoxins' have been isolated but were found to lack activity [5].

Short-chain neurotoxins are usually characterized by the presence of 62–64 amino acids in their primary structure with four conserved disulfide bonds. They have high affinity to neuronal Torpedo and muscle type nicotinic acetylcholine receptors [6,12]. Short-chain neurotoxins have been found to have similar functional sites as long-chain neurotoxins [12,13]. However, unlike the long-chain neurotoxins, short-chain neurotoxins lack the fifth disulfide bond at the tip of the second loop which has been shown to increase the affinity of long-chain neurotoxins towards neuronal nicotinic acetylcholine receptors in comparison to short-chain neurotoxins [7,14–16].

α-Elapitoxin-Bf1b was purified using size-exclusion, cation-exchange and reverse-phase chromatography. The calculated yield of α-elapitoxin-Bf1b, based on area under the curve, is 0.15% of the total venom protein. The very low yield of the toxin indicates that α-elapitoxin-Bf1b is not a major toxin in the venom and its role in envenoming is unclear. Molecular weight determination by intact protein analysis of α-elapitoxin-Bf1b showed that the toxin was within the molecular weight range of other short-chain neurotoxins from *B. fasciatus* venom and the venoms of other elapid species that have been previously isolated [9]. Protein molecular weight determination by intact protein analysis using mass spectrometry has been shown to produce a more accurate determination of

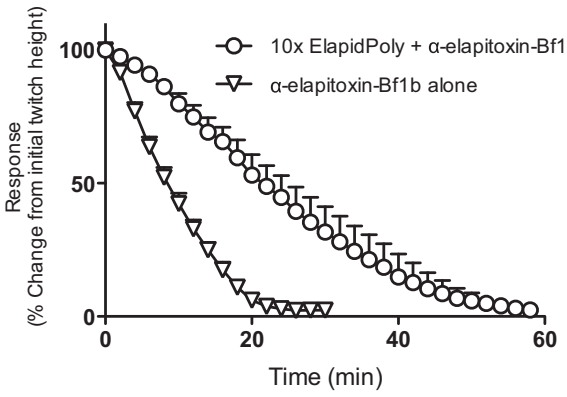

Fig. 7. The effect of α-elapitoxin-Bf1b alone (1 μg/mL), or in the presence of Thailand's neuro polyvalent antivenom (ElapidPoly; 10 × concentration) on indirect twitches in the chick biventer cervicis nerve-muscle preparation. *P < 0.05, significantly different compared to α-elapitoxin-Bf1b alone (n = 3).

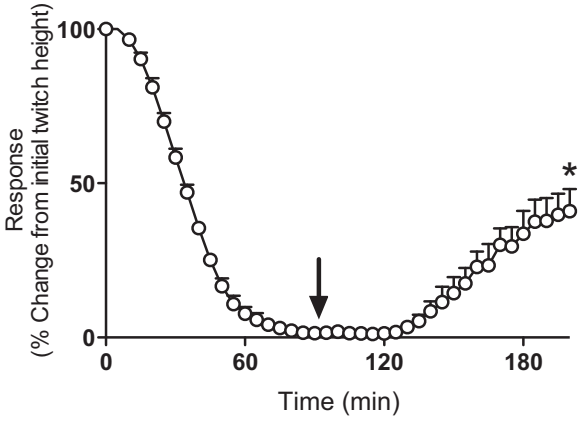

Fig. 9. The effect of washing the tissue at 10 min intervals after exposure to α-elapitoxin-Bf1b (0.1 μg/mL). Washing started at min 90 (indicated by arrow). *P < 0.05, significantly different compared with the 90 min time point (n = 3, paired t-test).

molecular weight than gel-based estimation [17]. The higher molecular weight of the toxin in tricine-PAGE compared to the value obtained from intact protein analysis by LCMS is probably due to the formation of short-chain neurotoxin complexes [4] or gel-induced modification during electrophoresis [17].

Protein identification of α-elapitoxin-Bf1b using LCMS/MS indicates that the toxin is a short-chain neurotoxin with 72% sequence homology with Neurotoxin 3FTx-RI [P0C555], which was previously detected in the *Bungarus fasciatus* gland transcriptome but not isolated from *B. fasciatus* venom [5]. The differences between α-elapitoxin-Bf1b and Neurotoxin 3FTx-RI [P0C555] were confirmed with N-terminal partial sequencing by Edman degradation. α-Elapitoxin-Bf1b was also found to be homologous with other short-chain neurotoxins from *B. fasciatus* [P10808], *Laticauda colubrina* [P10456] [P10455] and *Pseudechis australis* [P25497] (Fig. 3 and Table 1). Based on the Neurotoxin 3FTx-RI [P0C555] sequence, there are two amino acids substitutions in the α-elapitoxin-Bf1b sequence. At position 37, where isoleucine replaced the thronine and at position 40 where aspartic acid replaced aspargine. However, the significance of these two substitutions in the deduced sequence of α-elapitoxin-Bf1b is unknown. Isoleucine and leucine are isomers and are not easily differentiated by using mass spectrometry unless a specialized method is used [18]. Therefore, partial N-terminal sequencing was used to confirm the substitution at position 37.

Alignments with homologous short-chain neurotoxins (Table 1) indicate that α-elapitoxin-Bf1b has 7 out of its 8 cysteine residues conserved in all sequences. In addition to the conserved cysteine residues, α-elapitoxin-Bf1b has conserved functional amino acids at Lys-27, Trp-29, Arg-33, and Lys-47 that are essential for the short-chain neurotoxin binding to produce its neurotoxic effects [6,19,20]. However, one of the amino acids which is important for short-chain neurotoxin receptor binding, i.e. Asp-31 is substituted with threonine (Fig. 3). Substitution of Asp-31 with Gly-31 has been reported to reduce neurotoxicity [21]. It is possible that substitution with threonine may also make α-elapitoxin-Bf1b less neurotoxic compared to short-chain neurotoxins with Asp-31.

The *in vitro* neurotoxic effects of α-elapitoxin-Bf1b in skeletal muscle were concentration-dependent. Inhibition of contractile responses to exogenous nicotinic receptor agonists, but not KCl, indicate that α-elapitoxin-Bf1b acts postsynaptically and appears to be devoid of marked myotoxic activity. The t_{90} value, which indicates the time taken to cause 90% inhibition of twitches in the nerve-muscle preparation, is a useful parameter to compare the rate of the neurotoxic activity of venoms/toxins [22]. Based on the t_{90} values, α-elapitoxin-Bf1b was more neurotoxic than α-bungarotoxin at the same concentration. Unlike the previously isolated reversible short-chain neurotoxin from *B. fasciatus* venom [8], the inhibitory effects of α-elapitoxin-Bf1b (0.1 μg/mL) were partially reversed by repeated washing. The absence of Asp-31 has been postulated to be essential for the reversibility of short-chain neurotoxins [6]. Even though, α-elapitoxin-Bf1b is devoid of Asp-31, the neurotoxic effects of the toxin are only partially reversible indicating that α-elapitoxin-Bf1b may have other functional sites that prevent complete reversibility. This lack of reversibility is supported by the effects of α-elapitoxin-Bf1b on concentration-response curves to carbachol, where the maximum effects of the agonist were reduced in a concentration-dependent manner, indicating an irreversible or, at least, 'pseudo-irreversible' inhibitory effect at the nicotinic receptor. The lack of full reversibility of pseudo irreversible post-synaptic snake toxins, in skeletal muscle preparations over a similar time course, is well established and is related to the binding kinetics. Pseudo-irreversible antagonism occurs when the agonist (e.g. acetylcholine) and antagonist (e.g. α-elapitoxin-Bf1b) compete for the receptor (e.g. skeletal muscle nicotinic receptor) but the antagonist dissociates from the receptor so slowly (i.e. slow off-rate antagonist) that the agonist cannot achieve equilibrium with the receptor. Using the modified Lew and Angus method, α-elapitoxin-Bf1b was determined to have a pA_2 value of 9.17 ± 0.64, indicating a highly potent inhibitory effect. This pA_2 value indicates that the potency of α-elapitoxin-Bf1b is nearly 10 times more potent than α-bungarotoxin [23], α-scutoxin 1 [24] and α-hostoxin-1 [25]

Monovalent *Bungarus fasciatus* antivenom has been previously shown to have a neutralizing effect against *B. fasciatus* venom from Thailand [26]. It has also been shown to have *in vitro* cross-reactivity and cross genus neutralization against *B. candidus* and *B. flaviceps* venoms [26]. However, administration of *Bungarus fasciatus* antivenom to patients following envenoming by *B. candidus* did not show any benefits in clinical outcomes [27,28]. The neuro polyvalent antivenom has been recently reported to neutralize the effects of Asian krait species [29]. In the current study, pre-incubation with monovalent *Bungarus fasciatus* or neuro polyvalent antivenoms was unable to prevent the *in vitro* inhibitory effects of α-elapitoxin-Bf1b even when ten times the recommended titer for both antivenoms was used. However, the t_{90} values obtained in the presence of antivenoms (i.e. increased time to inhibition) indicated that the neuro polyvalent antivenom may be more effective that monovalent *Bungarus fasciatus* antivenom in neutralizing the neurotoxic effect of α-elapitoxin-Bf1b. The pre-incubation of an appropriate antivenom prior to the addition of crude venom or toxins has been shown to effectively neutralize neurotoxicity in the chick biventer cervicis muscle [30,31]. This data suggests that the antivenoms do not neutralize all short-chain neurotoxins in Malaysian *B. fasciatus* venom. However, the estimated quantity of α-elapitoxin-Bf1b in the venom is only 0.15%. Therefore, the role of the toxin in prey capture and the clinical effects observed in envenomed humans is unclear. To help clarify this role, studies examining the potency of α-elapitoxin-Bf1b in human and rodent skeletal muscle nicotinic receptors would be beneficial as we have previously shown species differences in the neurotoxic activity of short-chain neurotoxins and whole venoms [32–34].

In conclusion, we have isolated a potent short-chain post-synaptic neurotoxin, α-elapitoxin-Bf1b, from Malaysian *B. fasciatus* venom. The neurotoxic effect of α-elapitoxin-Bf1b was not prevented by either *Bungarus fasciatus* monovalent or neuro polyvalent antivenoms. This likely indicates that the composition of the non-enzymatic toxin in Malaysian *B. fasciatus* is slightly different to the composition of venom used in antivenom production. The results also showed that there are multiple isoforms of short-chain neurotoxins in *B. fasciatus* venom which may have cause variation in efficacy of antivenoms and management of envenoming.

Acknowledgements

We would like to thank Shane Reeves, Biomedical Proteomic Facility, Monash University, for conducting partial N-terminal sequencing and Zainuddin Ismail for donating Malaysian *B. fasciatus* venom from his collection. Muhamad Rusdi Ahmad Rusmili is a recipient of a SLAB scholarship from the Ministry of Higher Education, Government of Malaysia.

References

[1] Cox MJ. A photographic guide to snakes and other reptiles of Thailand and Southeast Asia. Asia Books; 1998.
[2] Fry BG, Winkel KD, Wickramaratna JC, Hodgson WC, Wüster W. Effectiveness of snake antivenom: species and regional venom variation and its clinical impact. Toxin Rev 2003;22:23–34.

[3] Pe T, Myint T, Htut A, Htut T, Myint AA, Aung NN. Envenoming by Chinese krait (*Bungarus multicinctus*) and banded krait (*B. fasciatus*) in Myanmar. Trans R Soc Trop Med Hyg 1997;91:686–8.
[4] Kruck TP, Logan DM. Neurotoxins from *Bungarus fasciatus* venom: a simple fractionation and separation of alpha- and beta-type neurotoxins and their partial characterization. Biochemistry 1982;21:5302–9.
[5] Tsai IH, Tsai HY, Saha A, Gomes A. Sequences, geographic variations and molecular phylogeny of venom phospholipases and three finger toxins of eastern India *Bungarus fasciatus* and kinetic analyses of its Pro31 phospholipases A_2. FEBS J 2007;274:512–25.
[6] Nirthanan S, Gwee MC. Three-finger alpha-neurotoxins and the nicotinic acetylcholine receptor, forty years on. J Pharmacol Sci 2004;94:1–17.
[7] Barber CM, Isbister GK, Hodgson WC. Alpha neurotoxins. Toxicon 2013;66:47–58.
[8] Liu CS, Hsiao PW, Chang CS, Tzeng MC, Lo TB. Unusual amino acid sequence of fasciatoxin, a weak reversibly acting neurotoxin in the venom of the banded krait, *Bungarus fasciatus*. Biochem J 1989;259:153–8.
[9] Liu CS, Chen JP, Chang CS, Lo TB. Amino acid sequence of a short chain neurotoxin from the venom of banded krait (*Bungarus fasciatus*). J Biochem 1989;105:93–7.
[10] Schägger H. Tricine–SDS-PAGE. Nat Protoc 2006;1:16–22.
[11] King GF, Gentz MC, Escoubas P, Nicholson GM. A rational nomenclature for naming peptide toxins from spiders and other venomous animals. Toxicon 2008;52:264–76.
[12] Teixeira-Clerc F, Ménez A, Kessler P. How do short neurotoxins bind to a muscular-type nicotinic acetylcholine receptor. J Biol Chem 2002;277:25741–47.
[13] Kini RM. Molecular moulds with multiple missions: functional sites in three-finger toxins. Clin Exp Pharmacol Physiol 2002;29:815–22.
[14] Servent D, Antil-Delbeke S, Gaillard C, Corringer PJ, Changeux JP, Menez A. Molecular characterization of the specificity of interactions of various neurotoxins on two distinct nicotinic acetylcholine receptors. Eur J Pharmacol 2000;393:197–204.
[15] Servent D, Lam Thanh H, Antil S, Bertrand D, Corringer P-J, Changeux J-P <ET-Al>. Functional determinants by which snake and cone snail toxins block the $\alpha 7$ neuronal nicotinic acetylcholine receptors. J Physiol Paris 1998;92:107–11.
[16] Servent D, Winckler-Dietrich V, Hu H-Y, Kessler P, Drevet P, Bertrand D <ET-Al>. Only snake curaremimetic toxins with a fifth disulfide bond have high affinity for the neuronal $\alpha 7$ nicotinic receptor. J Biol Chem 1997;272:24279–86.
[17] Jeannot MA, Zheng J, Li L. Observation of gel-induced protein modifications in sodium dodecylsulfate polyacrylamide gel electrophoresis and its implications for accurate molecular weight determination of gel-separated proteins by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. J Am Soc Mass Spectrom 1999;10:512–20.
[18] Armirotti A, Millo E, Damonte G. How to discriminate between leucine and isoleucine by low energy ESI-TRAP MSn. J Am Soc Mass Spectrom 2007;18:57–63.
[19] Trémeau O, Lemaire C, Drevet P, Pinkasfeld S, Ducancel F, Boulain J-C, Ménez A. Genetic engineering of snake toxins the functional site of erabutoxin a, as delineated by site-directed mutagenesis, includes variant residues. J Biol Chem 1995;270:9362–9.
[20] Endo T, Inagaki F, Hayashi K, Miyazawa T. Proton-nuclear-magnetic-resonance study on molecular conformations of long neurotoxins. alpha-Bungarotoxin from *Bungarus multicinctus* and Toxin B from *Naja naja*. Eur J Biochem 1981;120:117–24.
[21] Endo T, Nakanishi M, Furukawa S, Joubert FJ, Tamiya N, Hayashi K. Stopped-flow fluorescence studies on binding kinetics of neurotoxins with acetylcholine receptor. Biochemistry 1986;25:395–404.
[22] Hodgson WC, Wickramaratna JC. *In vitro* neuromuscular activity of snake venoms. Clin Exp Pharmacol Physiol 2002;29:807–14.
[23] Wickramaratna JC, Fry BG, Loiacono RE, Aguilar MI, Alewood PF, Hodgson WC. Isolation and characterization at cholinergic nicotinic receptors of a neurotoxin from the venom of the *Acanthophis sp.* Seram death adder. Biochem Pharmacol 2004;68:383–94.
[24] Kornhauser R, Hart AJ, Reeve S, Smith AI, Fry BG, Hodgson WC. Variations in the pharmacological profile of post-synaptic neurotoxins isolated from the venoms of the Papuan (*Oxyuranus scutellatus canni*) and coastal (*Oxyuranus scutellatus scutellatus*) taipans. Neurotoxicology 2010;31:239–43.
[25] Tan LC, Kuruppu S, Smith AI, Reeve S, Hodgson WC. Isolation and pharmacological characterisation of hostoxin-1, a postsynaptic neurotoxin from the venom of the Stephen's banded snake (*Hoplocephalus stephensi*). Neuropharmacology 2006;51:782–8.
[26] Chanhome L, Wongtongkam N, Khow O, Pakmanee N, Omori-Satoh T, Sitprija V. Genus specific neutralization of *Bungarus* snake venoms by Thai Red Cross banded krait antivenom. J Nat Toxins 1999;8:135–40.
[27] Laothong C, Sitprija V. Decreased parasympathetic activities in Malayan krait (*Bungarus candidus*) envenoming. Toxicon 2001;39:1353–7.
[28] Warrell DA, Looareesuwan S, White NJ, Theakston RD, Warrell MJ, Kosakarn W <ET-Al>. Severe neurotoxic envenoming by the Malayan krait *Bungarus candidus* (Linnaeus): response to antivenom and anticholinesterase. Br Med J (Clin Res Ed) 1983;286:678–80.
[29] Leong PK, Sim SM, Fung SY, Sumana K, Sitprija V, Tan NH. Cross neutralization of afro-asian cobra and asian krait venoms by a Thai polyvalent snake antivenom (neuro polyvalent snake antivenom). PLoS Neglect Trop Dis 2012;6:e1672.
[30] Isbister GK, O'Leary MA, Hagan J, Nichols K, Jacoby T, Davern K <ET-Al>. Cross-neutralisation of Australian brown snake, Taipan and death adder venoms by monovalent antibodies. Vaccine 2010;28:798–802.
[31] Kornhauser R, Isbister GK, O'Leary MA, Mirtschin P, Dunstan N, Hodgson WC. Cross-neutralisation of the neurotoxic effects of Egyptian cobra venom with commercial tiger snake antivenom. Basic Clin Pharmacol Toxicol 2013;112:138–43.
[32] Hart AJ, Isbister GK, O'Donnell P, Williamson NA, Hodgson WC. Species differences in the neuromuscular activity of post-synaptic neurotoxins from two Australian black snakes (*Pseudechis porphyriacus* and *Pseudechis colletti*). Toxicol Lett 2013;219:262–8.
[33] Hart AJ, Isbister GK, Hodgson WC. *In vitro* neurotoxic effects of *Pseudechis* spp. venoms: a comparison of avian and murine skeletal muscle preparations. Toxicon 2013;63:112–5.
[34] Hart AJ, Scott-Davey T, Harris JB. Venom of Collett's snake (*Pseudechis colletti*) blocks the binding of alpha-bungarotoxin to acetylcholine receptors at chick but not human neuromuscular junctions: a histochemical study. Toxicon 2008;52:647–50.