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Isolation and characterization of a presynaptic neurotoxin, P-elapitoxin-Bf1a from Malaysian *Bungarus fasciatus* venom

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ABSTRACT

Presynaptic neurotoxins are one of the major components in *Bungarus* venom. Unlike other *Bungarus* species that have been studied, β-bungarotoxin has never been isolated from *Bungarus fasciatus* venom. It was hypothesized that the absence of β-bungarotoxin in this species was due to divergence during evolution prior to evolution of β-bungarotoxin. In this study, we have isolated a β-bungarotoxin isoform we named P-elapitoxin-Bf1a by using gel filtration, cation-exchange and reverse-phase chromatography from Malaysian *B. fasciatus* venom. The toxin consists of two heterogeneous subunits, subunit A and subunit B. LCMS/MS data showed that subunit A was homologous to acidic phospholipase A_2 subunit A3 from *Bungarus candidus* and *B. multicinctus* venoms, whereas subunit B was homologous with subunit B1 from *B. fasciatus* venom that was previously detected by cDNA cloning. The toxin showed concentration- and time-dependent reduction of indirect-twitches without affecting contractile responses to ACh, CCh or KCl at the end of experiment in the chick biventer preparation. Toxin modification with 4-BPB inhibited the neurotoxic effect suggesting the importance of His-48. Tissue pre-incubation with monovalent *B. fasciatus* (BFAV) or neuro-polyvalent antivenom (NPV), at the recommended titer, was unable to inhibit the twitch reduction induced by the toxin. This study indicates that Malaysian *B. fasciatus* venom has a unique β-bungarotoxin isoform which was not neutralized by antivenoms. This suggests that there might be other presynaptic neurotoxins present in the venom and there is a variation in the enzymatic neurotoxin composition in venoms from different localities.

1. Introduction

Bungarus fasciatus is one of the species of krait found in Asia and its geographical range extends from India to Southern China and South East Asia [1–3]. It is considered to be a medically important species although cases of envenoming by this species are not as common as other species such as *Bungarus candidus* and *B. caereleus* [4]. *B. fasciatus* is unique as it has been suggested that it is a species from the *Bungarus* genus that split to its own lineage before other krait species evolved which means that its venom composition is different to other krait species [5].

Bungarus venoms have been shown to contain two important types of neurotoxins that are classified based on their site of action, *i.e.* presynaptic and postsynaptic neurotoxins [6]. There are several postsynaptic neurotoxins that have been described in, or isolated from, *B. fasciatus* crude venom [7,8] and venom gland transcripts [5]. All the postsynaptic neurotoxins that have been described in *Bungarus* spp. venoms are from the three finger toxin family that have very similar structural arrangements [5]. Unlike postsynaptic neurotoxins, presynaptic neurotoxins from *Bungarus* spp. are enzymatic toxins that have homology with phospholipase A_2 enzymes and have either a single chain or dimer arrangement [5,9]. The presynaptic neurotoxin that is found in *Bungarus* spp. venom, *i.e.* β-bungarotoxin, is a heterodimer that has a subunit A, which is homologous with phospholipase A_2 and another subunit, subunit B, that has homology with venom basic protease inhibitor [10,11]. β-Bungarotoxin was first discovered in Chinese many-banded krait (*Bungarus multicinctus*). Since then, several isoforms of this

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toxin have been isolated and characterized from other krait species [12]. This toxin has been suggested to play an important role in the irreversible and treatment resistant neurotoxic effects often observed following systemic *Bungarus* envenoming [13].

As for other krait species, the venom of *B. fasciatus* has been shown to contain unique postsynaptic and presynaptic neurotoxins [5]. A homodimer presynaptic neurotoxin, ceruleotoxin, has been isolated from *B. fasciatus* venom and found to be homologous with β-bungarotoxin and crotoxin [9,14]. Interestingly, *B. fasciatus* venom is devoid of the 'signature' toxins that are found in other *Bungarus* species, *i.e.* α-bungarotoxin and β-bungarotoxin [5]. Although several precursors of the subunit B of β-bungarotoxin have been found and described in the *B. fasciatus* venom gland by cDNA cloning [15], intact β-bungarotoxin or α-bungarotoxin have not been isolated and characterized from *B. fasciatus* venom.

In this study, we have isolated and characterized a novel β-bungarotoxin isoform, P-elapitoxin-Bf1a, from Malaysian *B. fasciatus*. This is the first β-bungarotoxin that has been isolated and described from *B. fasciatus* venom. We have also tested the neutralization capability of monovalent *B. fasciatus* antivenom and a neuro-polyvalent antivenom from Thailand against this neurotoxin.

2. Materials and methods

2.1. Bungarus fasciatus *venom*

B. fasciatus venom was milked and donated by Mr. Zainuddin Ismail. The snakes used for venom milking originated from the states of Perlis and Kedah, Northwest of Peninsular Malaysia. The venom was milked upon request and transported on ice, frozen at −80 °C and then freeze-dried. The freeze-dried venom was weighed and stored at −20 °C. When required, venom or isolated toxin were dissolved in Milli-Q water unless stated otherwise.

2.2. Drugs and chemicals

The following were purchased from Merck (Darmstadt, Germany); acrylamide, acetic acid, bis-polyacrylamide, bromophenol blue, butanol, dithiothretiol (DTT), glycine, tris base, trifluroacetic acid, sodium chloride (NaCl), potassium chloride (KCl). The following were purchased from Fisher Scientific (Loughborough, Leicestershire, UK); calcium chloride ($CaCl_2$), glucose, potassium dihydrogen phosphate ($KHPO_4$), ammonium acetate, high pressure chromatography (HPLC) grade acetonitrile, mass-spectrometry grade acetonitrile, and mass-spectrometry grade water. The following were purchased from Sigma-Aldrich (St. Louis, Missouri, USA); D-tubocurarine chloride, proteomics grade bovine trypsin, carbachol, ammonium bicarbonate, acetylcholine and mass-spectrometry grade formic acid. The following were purchased from GE Healthcare (Uppsala, Sweden); iodoacetamide and PhastGel Blue tablets. Antivenoms (monovalent *B. fasciatus* antivenom and neuro-polyvalent antivenom) were purchased from Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok. All chemicals were dissolved or diluted in Milli-Q water for experiments. Antivenoms were dissolved with the injection grade water provided by the manufacturer.

2.3. Purification of P-elapitoxin-Bf1a

Chromatographic fractionation with mass-spectrometry and bioactivity-guided screening were used for the isolation and purification of the toxin as in our previous work [16]. *B. fasciatus* 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 mounted on an Äkta Purifier system (GE Healthcare, Uppsala, Sweden), in batches. The column was equilibrated with 0.01 M ammonium acetate, pH 6.8 with flow rate of 0.5 ml/min. Fraction collection was conducted automatically at 1 ml/tube. The peak containing the toxin was further fractionated by using a Mono S strong cationic ion-exchange chromatography column (GE Healthcare, Sweden) mounted on an Äkta Purifier system (GE Healthcare, Sweden). The column was equilibrated with 0.05 M sodium phosphate buffer pH 6.8 (solution A) with a flow rate of 2 ml/min. The peaks were eluted with increasing percentage of 0.5 M sodium chloride in 0.05 M sodium phosphate buffer (solution B) 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. The fractions were collected automatically atn2 ml/tube. The fractions were later desalted by using a HiTrap Desalting column mounted to Äkta Prime that was equilibrated with 0.01 M ammonium acetate, pH 6.8 with a flow rate of 3 ml/min. Desalted fractions were collected at a flow rate of 2 ml/tube. The desalted fraction containing the toxin from cation-exchange chromatography was later dissolved in double distilled water before being loaded on a Jupiter C18 reverse column mounted on an Agilent 1200 high pressure liquid chromatography (HPLC) system (Agilent Technologies, Santa Clara, USA). The column was equilibrated with 5% acetonitrile (ACN) with 0.1% trifluoroactic acid (TFA) in water and run at a flow rate of 0.2 ml/min. The peak containing the toxin was eluted with an increasing percentage of 90% ACN in 0.1% TFA in water 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.

2.4. Intact protein analysis with electrospray ionization coupled liquid chromatography–mass spectrometry (ESI-LCMS)

The toxin was loaded onto an 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 Technologies, Santa Clara, USA). The chip was run at 4 μL/min with 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 (ACN) in 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% solution B from 15 to 15.1 min, 97% solution B from 15.1 to 22 min, and 97–3% solution B from 22 to 29 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 a range of 100–3200 *m*/*z*. The spectrum was then deconvulated by using Agilent Bioconfirm MassHunter Qualitative Analysis software (Agilent Technologies, Santa Clara, USA).

2.5. Protein identification with tandem electrospray ionization coupled liquid chromatography–mass spectrometry (ESI-LCMS/MS)

P-elapitoxin-Bf1a was digested by using the in-gel trypsin digestion protocol supplied by the manufacturer (Agilent Technologies, Santa Clara, USA). The digested peptides were loaded onto an Agilent C18 300 Å Large Capacity Chip (Agilent, USA). The column was equilibrated by 0.1% formic acid in water (solution A) and peptides were eluted with an increasing gradient of 90% ACN in 0.1% formic acid (solution B) by the following gradient; 3–50% solution B from 0 to 30 min, 50–95% solution B from 30 to 32 min, 95% solution B from 32 to 37 min and 95–3% solution B 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 300 °C. The intact protein spectrum was analyzed in auto MSMS mode from range of 110–3000 *m*/*z* for MS scan and 50–3000 *m*/*z* range for MS/MS scan. The spectrum