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Proteomic characterization and comparison of Malaysian *Bungarus candidus* and *Bungarus fasciatus* venoms

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ABSTRACT

Kraits (*Bungarus spp.*) are highly venomous elapids that are only found in Asia. In the current study, 103 and 86 different proteins were identified from *Bungarus candidus* and *Bungarus fasciatus* venoms, respectively. These proteins were classified into 18 different venom protein families. Both venoms were found to contain a high percentage of three finger toxins, phospholipase A_2 enzymes and Kunitz-type inhibitors. Smaller number of high molecular weight enzymes such as L-amino acid oxidase, hyaluronidases, and acetylcholinesterase were also detected in the venoms. We also detected some unique proteins that were not known to be present in these venoms. The presence of a natriuretic peptide, vespryn, and serine protease families was detected in *B. candidus* venom. We also detected the presence of subunit A and B of β-bungarotoxin and α-bungarotoxin which had not been previously found in *B. fasciatus* venom. Understanding the proteome composition of Malaysian krait species will provide useful information on unique toxins and proteins which are present in the venoms. This knowledge will assist in the management of krait envenoming. In addition, these proteins may have potential use as research tools or as drug-design templates.

Biological significance
This study has revealed the proteome composition of Malaysian *B. candidus* and *B. fasciatus* venoms, two medically important snake species in Asia. Information on the venom proteome of these species will provide useful information for krait bite management and aid in antivenom selection. Venom proteome profiles of these venoms showed that there are significant differences in the venom protein family compositions. Detection of proteins and peptides that have not been documented in these species such as natriuretic peptides, vespryn and serine proteases provides new knowledge on the composition of these venoms. The roles of these new proteins and peptides in krait envenoming are still unknown. Discovery of these proteins and peptides may also be useful for future research tool and therapeutic development.

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1. Introduction

Snake envenoming is considered to be one of the world's neglected diseases by the World Health Organization [1]. The mortality and morbidity caused by snake envenoming are prevalent in rural areas of under developed and developing countries [2,3]. It has been estimated that five million peoples are affected by snakebite around the world with between 25,000 and 125,000 deaths occurring annually [2,3]. *Bungarus candidus* (Malayan krait) and *Bungarus fasciatus* (banded krait) are two highly venomous elapid snakes and are considered to be medically important species in Southeast Asia [1,4]. *B. candidus* is easily recognized by its distinct alternating black and white bands, with black spots in the white bands, while *Bungarus fasciatus* is identified by its alternating thick black and white or yellow bands encircling the body [5,6]. *B. fasciatus* is perhaps the most widely distributed krait species in the world. Its range extends from India to South China and downward to Southeast Asia [5,7]. Unlike *B. fasciatus*, *B. candidus* is only found in certain countries in Southeast Asia such as Peninsular Malaysia, Thailand, Vietnam, Cambodia and Indonesia [5,8].

Venoms from *B. candidus* and *B. fasciatus* contain highly complex mixtures of various bioactive molecules and toxins [7,9,10]. Previous studies showed that *B. candidus* and *B. fasciatus* venoms contain several enzymatic and non-enzymatic components [11,12]. Some of these components exhibited neurotoxicity when tested in-vitro and in-vivo [12–14], an important symptom of envenoming by *Bungarus* spp. [15,16]. Neurotoxins in these venoms are divided into two major types based on their site of action, namely, presynaptic and postsynaptic neurotoxins [7,14,17]. Presynaptic neurotoxins in *B. candidus* and *B. fasciatus* venoms are mainly neurotoxic phospholipase A_2's, whereas the postsynaptic neurotoxins in these venoms are mainly non-enzymatic three finger toxins (3FTx) [7,17–19]. The heterodimeric presynaptic neurotoxin, β-bungarotoxin, is known to be present only in the venom of *Bungarus* species. This neurotoxin is composed of two different subunits, a phospholipase A_2 subunit called subunit A and a subunit homologous to Kunitz-type protease inhibitor called subunit B. Subunit B from *B. multicinctus* is capable of a exerting potassium channel blocking effect independently [20]. β-Bungarotoxin-like proteins have been cloned, isolated and found to be the most lethal toxins in *B. candidus* venom [9]. However β-bungarotoxin has not been isolated from *B. fasciatus* venom and is thought not to exist in the venom [7].

In recent years, new symptoms following krait envenoming in humans have been reported [21–23]. *B. candidus* envenomings in Vietnam were reported to have caused rhadomyolysis and hyponatremia [23]. Similarly, hyponatremia was observed in victims envenomed by *B. multicinctus* [21]. It was postulated that the hyponatremic effect was due to the presence of natriuretic peptides in the venom [23]. The natriuretic peptide gene has been reported in two other *Bungarus* species, i.e. *B. flaviceps* and *B. multicinctus* [24,25]. However, the presence of the peptide has not been detected at peptide or protein level in their venoms. In animal studies, it was found that *B. candidus* venom caused a transient reduction of mean arterial blood pressure and the effects were postulated to resemble calcium channel inhibitor activity [26]. Recent antivenom studies examining neutralization of the neurotoxic effects of Malaysian *B. candidus* and *B. fasciatus* venoms showed that a higher dose than the recommended titer is required to prevent and reverse neurotoxicity [27]. In addition, cross-neutralization was not seen when different monovalent antivenoms were used to prevent in-vitro neurotoxicity [27].

The application of proteomic techniques, development of high resolution mass-spectrometry and the enrichment of protein databases with protein sequences have allowed snake venoms to be profiled in a short time frame [28–30]. In addition, these techniques can be applied in assessing antivenom reactivity towards components in snake venoms [31,32]. A deep knowledge of the composition of snake venoms and reactivity of their components to antivenoms is important to elucidate the likely clinical effects of snake venom, formulating the antivenom and designing management strategies for snake bite treatment [29,31–33]. The composition of *B. fasciatus* venom from different localities has been studied but with limited success in terms of the number of proteins identified [7,34]. Despite a number of proteins having been isolated, characterized, and deposited in the database [9,10,19], no venom proteomic profiling has been conducted for *B. candidus*.

In this work, we have conducted proteome profiling and comparison for venoms from two Malaysian krait species, *Bungarus candidus* and *Bungarus fasciatus*. Some components from *B. candidus* and *B. fasciatus* venoms have been previously identified from crude venom and transcripts from venom gland [7,9,17]. However, many of these toxins are yet to be confirmed at the protein level in the venom. We were also interested to identify low abundance and unique proteins that could play important roles in envenoming and may have the potential to be developed as research tools and/or molecular drug templates. By using the automated *de novo* protein sequencing from tandem mass spectrometry data (MS/MS) and database search using NCBI database, we have identified 103 proteins from *B. candidus* venom and 86 proteins from *B. fasciatus* venom. Our study showed that the venom proteome for Malaysian *B. candidus* and *B. fasciatus* venoms are more diverse and complex than previously reported.

2. Methods and materials

2.1. Venoms

B. fasciatus and *B. candidus* crude venoms were donated by Mr. Zainuddin Ismail, a private snake enthusiast. The snakes used for venom milking originated from the states of Perlis and Kedah, Northwest of Peninsular Malaysia. *B. candidus* and *B. fasciatus* venoms were milked by placing the snake's fangs on a sterile container covered with a parafilm, milkings from 10 adult specimens of the same species were pooled and transported on ice. Venoms were later frozen at −80 °C before being freeze-dried at Sunway campus (Kuala Lumpur). The freeze-dried venoms were later weighed and labeled