The Molecular and Biochemical Characterisation of Venom Proteins from the Box Jellyfish, *Chironex fleckeri*

Thesis submitted by

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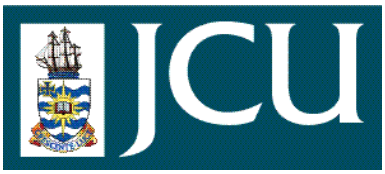

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Declaration on Ethics

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the National Statement on Ethics Conduct in Research Involving Human (1999), the Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997), the James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines (2001), and the James Cook University Statement and Guidelines on Research Practice (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (Approval Number A901).

Diane Brinkman Date

Statement on the Contribution of Others

Scientific Collaborations

I gratefully thank the following people and organisations for providing resources and scientific/technical assistance to me during my candidature:

- Dr Jamie Seymour and Ms Griselda Ávila-Soria (JCU), Reef HQ (Great Barrier Reef Marine Park Authority) and Queensland Surf Life Saving Association for generously providing jellyfish samples
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- CRC Reef Research Grant (2005–2006)

Publications Arising from Thesis

At the time of thesis submission, two manuscripts describing the research findings of Chapters 5 and 6 were already published and an invited review based on Chapters 1 and 8 was submitted. Three additional manuscripts are currently in preparation. Details of each manuscript are provided below.

- **Brinkman, D.**, Burnell, J., 2007. Identification, cloning and sequencing of two major venom proteins from the box jellyfish, *Chironex fleckeri*. *Toxicon* 50, 850-860. (Chapter 5)

- **Brinkman, D.**, Burnell, J., 2008. Partial purification of cytolytic venom proteins from the box jellyfish, *Chironex fleckeri*. *Toxicon* 51, 853-863. (Chapter 6)

- **Brinkman, D.**, Burnell, J., *submitted*. Biochemical and molecular characterisation of cubozoan protein toxins. *Toxicon.* (Chapters 1 and 8)

- Ávila-Soria, G., **Brinkman, D.**, Burnell, J., *in prep*. Molecular aspects of box jellyfish cDNA expression libraries and screening by antibody probes. *FEBS Letters.* (Chapter 3)

- **Brinkman, D**., Burnell, J., *in prep*. Biochemical characterisation of nematocyst-derived venom proteins from the box jellyfish, *Chironex fleckeri*. *Toxicon*. (Chapter 4)

- **Brinkman, D.**, Burnell, J., *in prep*. Expression of box jellyfish (*Chironex fleckeri*) toxins in *Escherichia coli*. *Protein Expression and Purification.* (Chapter 7)

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Abstract

Chironex fleckeri is a dangerous Australasian box jellyfish that inflicts painful, debilitating and potentially life-threatening stings in humans. The venom of *C. fleckeri* contains a variety of bioactive proteins that are cytolytic, cytotoxic, inflammatory or lethal, however, few individual venom proteins have been thoroughly characterised and their mechanism(s) of action remain unclear. Hence, the primary objectives of this thesis were to identify and characterise the major protein components in *C. fleckeri* venom, provide insight into their possible structures, functions and mechanisms of action, and explore the potential to express recombinant venom proteins in bacteria.

Two of the most abundant proteins contained in the nematocysts of *C. fleckeri* were identified in this study as *C. fleckeri* toxin-1 (CfTX-1) and toxin-2 (CfTX-2). The two proteins also represent the first *C. fleckeri* venom proteins to be successfully cloned and sequenced. The molecular masses of CfTX-1 and CfTX-2 (~43 and 45 kDa, respectively) were determined by SDS-PAGE, and both proteins were strongly antigenic to commercially available box jellyfish antivenom (CSL Ltd) and rabbit polyclonal antibodies raised against nematocyst-derived *C. fleckeri* venom. A combination of N-terminal amino acid sequencing, peptide mass fingerprinting, RT-PCR and cDNA library screening was used to isolate and clone cDNA encoding CfTX-1 and -2 (1789 and 1624 bp, respectively). Searches of non-redundant protein databases revealed that the deduced amino acid sequences of mature CfTX-1 and CfTX-2 (436 and 445 residues, respectively) were similar to three lethal, haemolytic box jellyfish toxins: CqTX-A from *Chiropsalmus quadrigatus*, CrTXs from *Carybdea rastoni* and CaTX-A from *Carybdea alata*. The protein and cDNA sequences of the five box jellyfish toxins were not similar to any other sequence in protein or nucleotide databases, supporting a hypothesis that the toxins may have evolved as highly specialised cubozoan toxins. Following a multiple sequence alignment of the five protein sequences, several short, but highly conserved regions of amino acids coincided with a predicted transmembrane spanning region, which could be involved in a pore-forming mechanism of action. Furthermore, remote protein homology predictions for the family of box jellyfish toxins suggested weak structural similarities and, hence, inferred function to pore-forming insecticidal δ-endotoxin proteins.

CfTX-1 and -2 were difficult to separate using electrophoretic or chromatographic methods, however, the two proteins were significantly co-purified from *C. fleckeri* venom using size exclusion chromatography or cation exchange chromatography. The native molecular mass of the co-purified CfTX proteins was 370 kDa, suggesting the formation of oligomeric

quaternary structures. The co-purified CfTX proteins were potently haemolytic to sheep erythrocytes (HU_{50} = 14 ng/mL) and caused the formation of large cleared zones of haemolysis in 5% sheep blood agar, thus confirming predictions of a pore-forming mechanism of action. Due to the significant sequence similarity of CfTX-1 and -2 to CrTX-A, CaTX-A and CqTX-A, the CfTX proteins could also be lethal, painful, inflammatory and dermonecrotic, and as such, may be the primary cause of life-threatening effects in envenomed humans.

During size exclusion and ion exchange chromatography experiments, a second major cytolysin (145 kDa) and a minor cytolysin (70 kDa) were also partially purified from *C. fleckeri* venom. The 145 kDa cytolysin, comprised of two major proteins (~39 and 41 kDa), was twice as haemolytic to sheep erythrocytes (HU_{50} = 7 ng/mL) as co-purified CfTX-1 and -2. Notably, the 39 and 41 kDa proteins were not significantly antigenic to CSL box jellyfish antivenom or rabbit antibodies raised against nematocyst-derived venom and the proteins were not always present in different batches of nematocysts. Due to the relatively high abundance of the 39 and 41 kDa proteins in some batches of *C. fleckeri* nematocysts compared to others, the variable concentrations of the proteins could have a major impact on the potency and variety of biological activities elicited by *C. fleckeri* venom. Furthermore, the 39 and 41 kDa proteins may represent a novel class of cytolytic proteins that are produced by cubozoan jellyfish.

Another strategy used to identify putative *C. fleckeri* venom proteins in this study involved the construction and screening of a *C. fleckeri* tentacle cDNA expression library with antibodies raised against *C. fleckeri* venom. Although no putative venom clones were isolated using this approach, cDNA clones encoding 44 independent non-venom proteins were isolated, sequenced and characterised, thus providing the first preliminary survey of the transcriptome of *C. fleckeri*.

Novel studies were also undertaken to assess whether CfTX-1 and -2 could be heterogeneously expressed in *Escherichia coli* for further functional and structural characterisation and/or for potential use in future antivenom production or other therapeutic applications. Although results of the studies indicated that CfTX-1 and -2 can be expressed in a bacterial host, expression levels were too low (ng protein/g cells range) to permit further characterisation. Furthermore, a large proportion of the expressed CfTX proteins formed insoluble inclusion bodies that required solubilisation and refolding prior to purification as soluble, potentially active recombinant proteins.

The research presented in this thesis, typified by the isolation, identification and characterisation of two major protein toxins in *C. fleckeri* venom, will benefit future research investigating the mechanisms of action of box jellyfish venom proteins in envenomed humans and potentially assist in the development of improved clinical treatments for box jellyfish stings.

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