

Tick Toxinology: Isolation and Characterisation of
the Toxin from the Australian Paralysis Tick,
Ixodes holocyclus.

By

Michael J. Thurn

Thesis submitted in September, 1994, in fulfilment of the degree of
Doctor of Philosophy
in the Faculty of Applied Sciences,
University of Technology, Sydney.

Acknowledgements

The last four years have been at times rewarding and extremely frustrating. This thesis would not have been possible if not for the help and assistance from the following people.

First and foremost I would like to thank my supervisor and mentor, Kevin Broady. He has been instrumental to this project and his patience knows no bounds.

My sincere thanks must also go to Robert Raison for allowing me to be part of the Immunobiology Unit and the various members of this unit over the years. In particular, Jeff Hook, Roseanne Dunn, Peter Towns, Nick dos Remedios, Charles Robin, and Sue Kehrer. Thanks for your assistance and companionship it has been deeply appreciated.

To the following establishments and their personnel, thanks for all your input and help: The Kolling Institute of Medical Research (Brian Baldo, Greg Donovan and Geraldine O'Neil); Biotechnology Australia (Yella Lahnstein, Chris Costandi, and Co Yen); Norvet Laboratories (Mal Logan and Graham Kelly); Centre for Drug Design and Development (Paul Alewood and Alun Jones).

Closer to home, I would like to thank the following departments and people for their friendship and assistance: Pathology and Immunology (Bob O'Grady, Chris Sinclair, Jennifer Thorpe and Toni Baragith); Biochemistry Department (Bill Booth); Applied Biology and Environmental Biology (Ken Brown, Ben Pearson, Brett Nudd, Peter Ralph, and Ross Mcpherson); Animal House (Don Ernst, Janeen Skewes).

Gillian Lyons and Catherine Dorey deserve a special mention, as without their love and patience this thesis would definitely not have been possible.

Finally, I thank my family for their support and encouragement, and Emma-Jane Barter for her undivided attention and love, and for teaching me that there is more to life (a lot more) than a PhD.

For my loving mother.....

Table of Contents

Abbreviations.....	ii
List of Figures.....	iv
List of Tables.....	viii
Abstract	x
Chapter 1 Introduction / Literature Review.....	1
Chapter 2 Identification and Partial Purification of Holocyclus Toxin.....	31
Chapter 3 Characterisation of Holocyclus Toxin Binding to Rat Brain Membranes	83
Chapter 4 Final Purification and Structural Analysis of Holocyclus Toxin.....	105
Chapter 5 Molecular Characterisation of Holocyclus Neurotoxins	157
Chapter 6 General Discussion	226
Chapter 7 Bibliography.....	232
Appendix 1.....	257
Appendix 2.....	259

Abbreviations

$[\alpha\text{-}^{32}\text{P}]\text{dATP}$	deoxyadenosine 5'- $[\alpha\text{-}^{32}\text{P}]$ triphosphate
$[\alpha\text{-}^{32}\text{S}]\text{dATP}$	deoxyadenosine 5'- $[\alpha\text{-}^{32}\text{S}]$ thiotriphosphate
aa	amino acid
ADP	adenosine diphosphate
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indopyranoside
bp	basepair
BSA	bovine serum albumin
BWSV	black widow spider venom
%C	percentage of crosslinker in an acrylamide solution
cDNA	complementary DNA
cpm	counts per minute
D	dalton
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DEAE	diethylaminoethyl
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl
DSS	suberic acid bis(N-hydroxy-succinimide ester)
EtOH	ethanol
g	gravity
$[\gamma\text{-}^{32}\text{P}]\text{dATP}$	adenosine 5'- $[\gamma\text{-}^{32}\text{P}]$ triphosphate
HPLC	high pressure liquid chromatography
hr	hour
IEF	isoelectric focusing
IPTG	isopropylthio- β -D-galactopyranoside
Kb	kilobasepairs
kD	kilodalton
λ	bacteriophage lambda
MAb	monoclonal antibody

MeOH	methanol
min	minute
mM	millimolar
mRNA	messenger RNA
M _r	relative molecular weight
MW	molecular wieght
NaN ₃	sodium azide
NBCS	newborn calf serum
NBT	nitro blue tetrazolium
ng	nanogram
OD ₆₀₀	optical density measured at 600nm
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
pI	isoelectric point
pmol	picomole
PMSF	phenyl methyl sulphonyl fluoride
PTD	paralysing tick dose
PVDF	polyvinylidenedifluoride
QS	quackenback strain
RT	room temperature
SEM	standard error of the mean
SDS	sodium dodecyl sulphate
SGE	salivary gland extract
SSC	sodium chloride trisodium citrate
SPD	standard paralysing dose
%T	total acrylamide concentration (w/v)
TBS	tris buffered saline
TBE	tris buffered ethylenediaminetetra-acetic acid
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylenediamine
TFA	trifluoroacetic acid
Tris	tris(hydroxymethyl)aminomethane
U	unit
UV	ultraviolet
WBE	whole body extract
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
2-DE	two-dimensional electrophoresis

List of Figures

Figure 1.1	Conduction of nerve impulses.....	5
Figure 1.2	Distribution of the Australian paralysis tick <i>I. holocyclus</i> , the Tasmanian paralysis tick <i>I. cornuatus</i> , and the Hirst's marsupial tick <i>I. hirsti</i> (Roberts, 1970; Stone, 1987).....	16
Figure 1.3	An electron micrograph (x250 in magnification) showing the powerful mouth parts of <i>I. holocyclus</i>	19
Figure 1.4	A cross-sectional diagrammatic representation of an Ixodid tick shortly after penetrating the host's skin.....	20
Figure 1.5	Relationship between change in the secretory material within salivary cells "a" and "b" of Acinus II, and the toxicity of homogenates of salivary glands from <i>I. holocyclus</i> fed for varying lengths of time.....	23
Figure 1.6	End-plate potentials (EPPs) and miniature end-plate potentials (MEPPs) in a diaphragm from a mouse paralysed by <i>I. holocyclus</i>	27
Figure 1.7	The standard experimental arrangement for recording EPPs.....	27
Figure 1.8	A schematic outline illustrating the various aims of this thesis.	30
Figure 2.1	The essential components for protein purification.	37
Figure 2.2	10-20% Tricine-SDS-PAGE analysis of each toxic fraction obtained during the partial purification of Holocyclus toxin (Silver stain).....	51
Figure 2.3	Elution profile obtained from the chromatography of the 70% ammonium sulphate precipitate on heparin Sepharose.....	54
Figure 2.4	Elution profile obtained from the chromatography of the heparin Sepharose flow through on DEAE Affi-Gel Blue.....	55
Figure 2.5	Outline of the technique for the identification of Holocyclus toxin and the audioradiogram showing the outcome.	60

Figure 2.6	Synaptosomes adsorption patterns obtained for ¹²⁵ I-HT preparation after analysis by Tricine-SDS-PAGE under reducing and non-reducing conditions.....	61
Figure 2.7	The specificity of the interaction between synaptosomes and HT.....	63
Figure 2.8	HPLC gel filtration of HT preparation.....	64
Figure 2.9	Polypeptides identified by the synaptosome adsorption technique from taipan venom.....	66
Figure 2.10	Polypeptides identified by the synaptosome adsorption technique from tiger snake venom.....	67
Figure 2.11	Polypeptides identified by the synaptosome adsorption technique from Black Widow Spider Venom.	69
Figure 2.12	Polypeptides identified by the synaptosome adsorption technique from North African Scorpion.....	72
Figure 2.13	PVDF blot of HT preparation and N-terminal sequence results.....	74
Figure 3.1	Time course for the association of ¹²⁵ I-labelled HT with synaptic membranes.....	95
Figure 3.2	The association of ¹²⁵ I-labelled HT with synaptic membranes over a range of temperatures.	97
Figure 3.3	Characterisation of ¹²⁵ I-labelled HT binding to synaptic membranes.....	98
Figure 3.4	The effects of pretreating synaptic membranes with several venoms on the ability to bind ¹²⁵ I-labelled HT.....	99
Figure 3.5	Cross-linking ¹²⁵ I-labelled HT binding to synaptic membranes.....	101
Figure 4.1	Normal versus microbore reverse-phase HPLC.....	110
Figure 4.2	Elution profile and Tricine-SDS-PAGE analysis of fractions obtained after reverse-phase C4 HPLC chromatography of the toxic fraction obtained from DEAE Affi-Gel Blue chromatography.	127

Figure 4.3	Elution profile obtained from the HIC of the toxic fraction obtained by DEAE Affi-Gel Blue chromatography.....	128
Figure 4.4	Separation of peak 1 (HIC) by Reverse-phase C4 HPLC.....	131
Figure 4.5	Separation of peak 1 (Figure 4.4) by reverse-phase C8 HPLC.....	132
Figure 4.6	Separation of peak 2 (Figure 4.4) by reverse-phase C8 HPLC.....	133
Figure 4.7	Total amino acid analysis.....	135
Figure 4.8	Ionspray mass spectrometric analysis of HT-I.....	138
Figure 4.9	Ionspray mass spectrometric analysis of HT-II.....	139
Figure 4.10	The effect of the column diameter (ID) on the sensitivity of the detection system available for this study.....	141
Figure 4.11	Separation of tryptic peptides by reverse-phase HPLC.....	143
Figure 4.12	Amino acid sequence analysis of tryptic peptides separated by reverse-phase HPLC (refer to figure 4.11).....	144
Figure 4.13	Characterisation of the neurotoxin HT-I and HT-II for the presence of glycoconjugates using a DIG glycan detection kit (Boehringer Mannheim).....	146
Figure 4.14	Western blot analysis of the MAb 9B6 1G6.....	147
Figure 4.15	Dot blot analysis of MAb 9B6 1G6.....	149
Figure 4.16	Purification scheme for HT-I, HT-II, and HT-III. Tricine-SDS-PAGE was conducted under both reducing (R) and non-reducing (NR).....	152
Figure 5.1	Diagrammatic representation of the basic technique adopted for cDNA synthesis and cloning.....	161
Figure 5.2	SDS-PAGE analysis of the translation products.....	196
Figure 5.3	Electrophoretic analysis of adult tick and nymph cDNA.....	198
Figure 5.4	Size fractionation of adult cDNA.....	200
Figure 5.5	PCR amplification of ubiquitin sequences.....	203

Figure 5.6	Immunoscreening.....	204
Figure 5.7	PCR Analysis of the immunopositive clone M920.....	206
Figure 5.8	Partial nucleotide sequence and corresponding in-frame amino acid sequence of cloneM920.....	208
Figure 5.9	Electrophoretic analysis of tick total RNA.....	213
Figure 5.10	Northern hybridisation analysis of total RNA.	215
Figure 5.11	The production of toxin during engorgement.....	217
Figure 5.12	Diagrammatic representation of RACE.	219
Figure 5.13	PAGE analysis of the PCR products produced by RACE.	220
Figure A.1	Measuring the molecular mass of a protein by ionspray mass spectrometry.	258
Figure A.2	Nucleotide sequence obtained for the 209 bp product derived from the amplification of adult tick cDNA library with degenerate primers for ubiquitin.....	259

List of Tables

Table 1.1	Steps in the transmission of electrical impulses at the neuromuscular junction and their inhibitors (adapted from Mebs and Hucho, 1990).....	7
Table 1.2	Neurotoxins in spiders (adapted from Kawai, 1991).....	9
Table 1.3	World distribution of ticks implicated in tick paralysis.....	14
Table 2.1	The comparative potencies of ticks engorged on different hosts.....	47
Table 2.2	Partial purification of Holocyclus toxin.....	50
Table 2.3	Fractionation of the crude tick extract with ammonium sulphate.....	53
Table 2.4	Summary of the additional purification techniques attempted.....	57
Table 2.5	The various venoms used and their presynaptic neurotoxins.....	71
Table 3.1	Estimated acute toxicity in rodents of some of the most toxic toxins (adapted from Habermann, 1992).....	87
Table 4.1	Chemical cleavage of specific peptide bonds.....	114
Table 4.2	Some common commercially available proteases.....	114
Table 4.3	Purification of Holocyclus neurotoxin.....	129
Table 4.4	Amino acid analysis of Holocyclus neurotoxins.....	136
Table 4.5	Comparison of the HT-II peptide amino acid sequence, AXTYPLGSKVGYK with protein databases using the BLAST algorithm.....	154
Table 5.1	Oligonucleotide probes and primers.....	176
Table 5.2	% Incorporation of ³⁵ S-methionine.....	195
Table 5.3	First strand incorporation of [α - ³² P]dATP.....	297

Table 5.4	FASTA sequence homology analysis of the amino acid deduced from clone M920.....	209
Table 6.1	Previous characterisations of the toxin from <i>I. holocyclus</i>	227
Table 6.2	Holocyclus neurotoxins and their characteristics.....	229
Table A.1	FASTA sequence homology analysis of the nucleotide sequence obtained for the 209 bp PCR product.....	260

Abstract

Several species of ticks have been reported to secrete neurotoxins which can be fatal to mammals, including man. Envenomation by the Australian paralysis tick, *I. holocyclus*, is a considerable veterinary problem affecting tens of thousands of domestic and native animals each year. Envenomation is characterised by a progressive ascending flaccid paralysis and is due to a neurotoxin present in the salivary secretions of the tick. Purification of the neurotoxic component has been largely unsuccessful mainly due to the insensitivity of the biological assay, the minute quantities present and the reported labile nature of the neurotoxin. This dissertation describes the identification, final purification, partial characterisation and preliminary structural characteristics of the neurotoxins from *I. holocyclus*.

The neurotoxins were identified as having a molecular weight of 5 kD by SDS-PAGE using an innovative technique involving adsorption to synaptosomes. The identification subsequently enabled the final purification of three closely related neurotoxins. The neurotoxins were purified from extracts of engorged adult female *I. holocyclus* by a series of conventional chromatography techniques (heparin Sepharose, DEAE Affi-gel blue, Alkyl Sepharose) followed by C4 and C8 reverse phase HPLC. Purity was demonstrated by Tricine-SDS-PAGE and biological activity confirmed by injection into neonatal mice. Total amino acid analysis of the three neurotoxins showed that the neurotoxins were essentially identical. This was latter confirmed by a non-neutralising monoclonal antibody which was capable of recognising a common epitope shared by all three neurotoxins. Amino acid sequence of the neurotoxins was restricted to peptides generated by proteolytic digestion with trypsin as direct N-terminal sequencing revealed an amino-terminal blockage. An accurate molecular weight of 5,640 (HT-II) and 5,460 (HT-I) Daltons has been determined by mass spectrometry for two of the neurotoxins. The small size is comparable to neurotoxins isolated from other arachnids (spiders and scorpions). However, comparisons of the peptide amino acid sequences derived from the proteolytic digestion of HT-II with known protein sequences failed to display any significant homologies.

cDNA libraries were produced from poly(A⁺) mRNA isolated from unengorged *I. holocyclus* ticks. The libraries were characterised and found to

be well-represented with tick-derived cDNA sequences. PCR primers and oligonucleotide probes were designed from the partial amino acid sequence obtained for HT-II and used to screen the cDNA libraries. After extensively screening the libraries, no positive clones were obtained. Northern hybridisation analysis of total RNA prepared from unengorged and engorged ticks with the oligonucleotide probe RHT produced a band of the expected size (220 bps), only with total RNA prepared from engorged ticks. This indicated the original cDNA libraries prepared from unengorged ticks were unlikely to contain the gene of interest. PCR based strategies were unable to isolate neurotoxin gene(s) from mRNA prepared from engorged ticks.