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

[α-^{32}P]dATP	deoxyadenosine 5'-[α-^{32}P]triphosphate
[α-^{32}S]dATP	deoxyadenosine 5'-[α-^{32}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
[γ-^{32}P]dATP	adenosine 5'-[γ-^{32}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	polyvinylidinedifluoride
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

List of Tables

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.