THE SEQUENCE, EXPRESSION AND IMMUNOLOGICAL CHARACTERISATION OF THE HT-1 NEUROTOXIN FROM THE AUSTRALIAN PARALYSIS TICK *IXODES HOLOCYCLUS*

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DECLARATION

The experiments presented in this thesis were carried out by myself, except where indicated in the text. None of the material has been presented previously for the purpose of obtaining any other degree.

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ABSTRACT

The paralysis tick of Australia, *Ixodes holocyclus*, causes a severe toxicosis in domestic animals such as dogs and cats, livestock, and in some cases humans. It is characterised by a rapidly ascending flaccid paralysis. The causative agent of the toxicosis is a neurotoxin produced in the tick salivary glands. The current treatment for tick paralysis is in the form of a polyclonal dog antiserum. This antiserum treatment is expensive and effective only in the early stages of paralysis.

This thesis describes the isolation of the cDNA sequence for the neurotoxin HT-1 of *I. holocyclus*. In addition it describes the successful expression of the mature HT-1 toxin as a fusion protein in a bacterial host system and attempts at expression of this recombinant protein in the absence of a fusion partner in a baculovirus system. The antigenic and protective properties of the HT-1 fusion protein raised in mice and dogs is also discussed.

The complete cDNA sequence of the HT-1 neurotoxin was determined using the technique of RACE-PCR. The derived protein sequence of the mature toxin has a calculated molecular weight of 5.9 kDa and contains 8 cysteine residues, suggesting that it can form four disulphide bonds. The HT-1 protein sequence shares similarities with other arachnid neurotoxins. Examples of these similarities are its small size, number and arrangement of cysteine residues and in the length and composition of its signal sequence.

A recombinant form of the HT-1 toxin was produced as a fusion protein in an *E.coli* host system. The fusion protein was shown to be immunogenic by Western blot and ELISA analysis. The antibodies produced against the recombinant fusion protein show partial protection against native toxin in mouse assays. Dogs immunised with the recombinant fusion protein produced specific antibodies to HT-1 as demonstrated in ELISA assay. However, these antibodies were not protective when the dogs were challenged with numerous live ticks.

The results obtained have contributed a novel tick neurotoxin sequence which as a fusion protein produces antibodies that exhibit partial protection in mice against native toxin challenge. These results provide some optimism and basic understanding of what is required for the future development of a recombinant vaccine against paralysis caused by the tick *I. holocyclus*.

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ABBREVIATIONS

3'	three prime
5'	five prime
A	absorbance
Ab	antibody
AcNPV	*Autographa californica* NPV
Ag	antigen
AP	alkaline phosphatase
AVSL	Australian veterinary serum laboratories
Az	sodium azide
Bac	baculovirus
BCIP	5-bromo-4-chloro-3-indopyranoside
bp	base pair
BSA	bovine serum albumin
CAPS	3-[cylcohexylamino]-1-propane-sulfonic-acid
cDNA	complementary DNA
ChTx	charybdotoxin
CIAA	chloroform:isoamyl alcohol
CNBr	cyanogen bromide
Da	Dalton
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
ds	double stranded
DTT	dithiothreitol
ELISA	enzyme linked immuno-absorbent assay
EtBr	ethidium bromide
EtOH	ethanol
Fab	fragment antigen binding
Fwd	forward
HPLC	high pressure liquid chromatography
HT	holocyclus toxin
i.p.	intraperitoneal
IgG	immunoglobulin with γ isotype heavy chain
IMAC	immobilised metal affinity column
IPTG	isopropylthio-β-D-galactopyranoside
kDa	kiloDalton
KLH	keyhole limpet hemocyanin

kV	kilovolts
LB	Luria Bertani
LPS	lipopolysaccaride
mA	milliamps
Mab	monoclonal antibody
MBP	maltose binding protein
MCS	multiple cloning site
µF	micro-Farrad
mRNA	messenger RNA
MWCO	molecular weight cut off
NBT	nitro-blue tetrazolium
NMR	nuclear magnetic resonance
NPV	nucleopolyhedrovirus
OD	optical density
p-NPP	p-nitophenyl phosphate
PAGE	polyacylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
polyA	polyadenosine
PVDF	polyvinylidinedifluoride
RACE	rapid amplification of cDNA ends
RE	restriction endonuclease
Rev	reverse
RNA	ribonucleicacid
RNase	ribonuclease
s.c.	sub-cutaneous
ScFv	single chain fragment variable
SDS	sodium dodecyl sulphate
ss	single stranded
TBE	tris buffered ethylene diamine tetraacetate
TE	tris EDTA
Tris	tris-(hydroxymethyl)-aminomethane
UV	ultraviolet
V	volts
VH	heavy chain variable region
VL	light chain variable region
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER 1

INTRODUCTION

1.1 PREVIEW

Spiders and scorpions rely heavily on their venom for predation and defence. As a consequence, they have evolved potent neurotoxins within the venom that act quickly and specifically on nervous tissue. Ticks, on the other hand, have adapted to a parasitic lifestyle as evidenced by the loss of venom glands and specialised structures (fangs and telsons) for venom delivery. However, a few select species such as *Ixodes holocyclus, Rhipicephalus evertsi evertsi,* and *Argas (Pericargas) walkerae* have been identified with having neurotoxins present in their saliva. The Australian tick *Ixodes holocyclus* presents a serious veterinary problem in Australia due to the action of a neurotoxin secreted by the ticks salivary glands. While a great deal of research has been conducted into the nature of this neurotoxin, relattively little is known about the toxin at the molecular level.

The subject of this thesis was to isolate the gene encoding the neurotoxin responsible for the paralytic symptoms attributed to *Ixodes holocyclus,* and to establish a bacterial expression system to produce large quantities of recombinant toxin in order to assess its toxic and immunological activity.

Comparative analysis of toxins and inhibitory polypeptides belonging to different phyla suggests that these peptides possess certain similarities in one or more aspects such as their mode of action, size, amino acid composition, as well as in conserved structural regions. Extensive physiological studies have been performed on a number of such proteins derived from different biological sources. These include, amongst others, species of arachnids, insects, plants, sea anemones, cone snails and fungi. The homologies found amongst these neurotoxins provide some knowledge of the possible expected molecular structure and pharmacological action of the *I. holocyclus* neurotoxin.

The aim of this chapter is to provide a review of the major studies that have been carried out on the structure and function of medically and agriculturally significant arachnid neurotoxins. Emphasis has been placed, where possible, on research into arachnid neurotoxins in Australia, and on the significance of these investigations for providing effective preventative and treatment strategies against envenomation.

1.2 ARACHNID NEUROTOXINS

Venomous species have been identified in various phyla of the animal kingdom: Cnidarians, Platyhelminths, Echinoderma, Mollusca, Annelida, Arthropoda and Chordata. A variety of toxins have been isolated from animals belonging to these phyla. A large proportion of these are proteins with a small size (less than 100 amino acids) and containing a high density of disulphide bonds (Menez, 1998).

Australia has a collection of the most potent venomous land and sea creatures in the world (Hodgson, 1997). Australia's venomous animals fall into three major groups: snakes, which remain the leading cause of human fatalities; arthropods, which affect more people than snakes although deaths are virtually unknown; and thirdly the vast array of venomous and poisonous fauna present in the Australian marine environment. From a human health viewpoint, the jellyfish and the stinging fish are the most important, followed by sea snakes and the blue-ringed octopus (White, 1998).

Venoms are a complex mixture of substances, mainly proteins. Venoms have been described as the "complete secretion of the specialised venom glands of an animal" (Chahl and Kirk, 1975). Toxins are peptides or proteins having a range of weights, but are generally less than 30 kDa. They target specific receptors usually found on cell membranes. The specificity of toxins can be neurological, cardiovascular, muscular or not differentiated according to the anatomical distribution of the receptors which are recognised (Menez, 1998).

Neurotoxins are defined as substances which impair the functioning of nervous tissue. That is, neurotoxins act directly on neurons or interfere with electrical impulse transmission between the neurones and their target cells (Tu, 1977). Neurotoxins are primarily used to immobilise prey and have been isolated from a number of venoms. They usually produce a rapid paralysis of the envenomated prey by interfering with nerve conduction, pre- or post-synaptically, at the neuromuscular junction (Hodgson, 1997). The pharmacological effect is proportional to the quantity of introduced toxin and to the quantity of receptors to which the neurotoxin binds (Menez, 1998).

In the class Arachnida, which is a member of the largest phylum in the animal kingdom - Arthropoda - three orders have been shown to secrete neurotoxins: the

Scorpionida (scorpions), the Araneida (spiders) and the Acarnia (ticks) (Cupp, 1991). Of the three, the venoms of the scorpions have been most thoroughly studied. The major obstacle to structural studies of toxins is the difficulty in obtaining large quantities of highly pure and conformationally stable proteins (Oren *et al.,* 1998), consequently the toxins from smaller arachnids such as spiders and ticks have been studied to a lesser extent.

1.3 SCORPIONS

Scorpions are found in tropical and temperate regions of the world. There are 1400 species of scorpions identified, belonging to 9 families. Of these the Buthidae family are the only ones which cause clinically significant envenomation and are of medical importance. Only a few genera belonging to the Buthidae are considered to be dangerous. These include the genera *Androctonus*, *Buthus* and *Leiurus* of North Africa and the Middle East, *Centruroides* of Mexico and Southern USA, and *Tityus* of Brazil (Granier *et al.,* 1989, Hutt and Houghton, 1998).

The venom of scorpions is produced by glands in the tail of the organism and envenomation is affected by direct injection of a venom - containing fluid from these glands. There are variations found in the toxicity of scorpion venoms depending on the route of injection and the species of the scorpion. The unique property of scorpion venom is the presence of toxins that tend to act on only one type of animal, namely mammals, insects or crustaceans (Jaravine *et al* ., 1997, Hutt and Houghton, 1998).

Scorpion venoms contain mostly low molecular weight, basic neurotoxins. The polypeptides are 4 - 8 kDa in size, are very resistant to degradation by heat or enzymes, and have been classified according to the targets they recognise (Becerril *et al.*, 1997). There are four main classes of scorpion toxin, which specifically interact with sodium, potassium, chloride and calcium membrane bound ionic channels. The Na^+ channel toxins are polypeptides of 60-70 amino acids with 4 disulphide bridges; the K^+ channel toxins are peptides of 31-39 amino acids comprised of 3 - 4 disulphide bridges; the Cl^- channel toxins are peptides of 36 amino acids and are cross linked by 4 disulphide bridges (Lippens *et al.*,1995). Finally the Ca^{2+} channel toxins are, to date, of unknown structure (Becerril *et al.,* 1995, Becerril *et al* ., 1997).

The Na^+ channel toxins are of the most medical importance. The K^+ channel toxins are commonly found to augment or even act synergistically with the Na^+ channel toxins. In general however, the contribution of the remaining three classes (K^+, Ca^{2+} and Cl^-) to human intoxication is relatively minor, due to the fact that they are less abundant components of the venom or they are present in species of scorpions not dangerous to humans (Calderon-Aranda *et al.,* 1995). Therefore, the best studied toxins are those that recognise the voltage gated Na^+ channel. This group of toxins has been sub-divided into α and β neurotoxins. This classification has been based on the different effects these toxins have on the Na^+ channel activity. The α-toxins bind to site 3 of the Na^+ channel in a voltage dependent manner and inhibit Na^+ current inactivation, whereas β–toxins bind to site 4 independently of membrane potential and affect sodium activation (Becerril *et al*., 1995; Oren *et al.,* 1998).

The α-scorpion class of toxins can be further divided into several subgroups according to their activity on mammals and insects as well as their binding properties. Classical α- toxins are those that are active on mammals and include the 'Old World' (Africa and Eurasia) toxins such as AaH I, AaH II, AaH III (from the scorpion *Androctonus australis hector)* Lqq V (from *Leiurus quinquestriatus quinquestriatus*) and Bot III (from *Buthus occitanus tunetanus*). AaH II is the most active α-toxin and is the most extensively studied scorpion neurotoxin. It serves as a prototype for other anti-mammalian scorpion α-toxins. The α-toxins which are highly active on insects include: LqhαIT (*Leiurus quinquestriatus hebraeus*) Lqq III (*Leiurus q.quinquestriatus*), Bot IT1 (*Buthus occitanus tunetanus*) (Gordon *et al.,* 1998, Nakagawa *et al.,* 1998). There is also a third group of α-toxins termed the α-toxin-like group of which the toxins are active against both mammals and insects. Examples from this group include Bom III and Bom IV (from *Buthus occitanus mardachei*) (Vargas *et al.*, 1987). The β-toxins are present only in the 'New World' (The Americas) scorpions, and include anti-mammalian and anti-insect toxins. Examples of β–toxins include: Cs V (*Centruroides sculpturatus* Ewing); Ts IV (*Tityus serrulatus*) and Cn II (*Centruroides noxius*) (Gordon *et al*., 1998).

Some scorpion toxins however, do not fit the above classifications. For example, it was recently published that a new class of toxin exists, which acts on K^+ channels, and which is 35 amino acids in length but cross-linked by 4 disulphide bonds instead of the 3 commonly found (Delepierre *et al.,*1998). This toxin, named Pil, is from the African scorpion *Pandinus imperator.* The disulphide bridges at position C_{20}-C_{35} are the novel structural characteristics of this toxin. Its position differs from the 4th disulphide bridge in insect toxins or in chlorotoxin (a short chain scorpion toxin active on Cl^- channels from *L. quinquestriatus*) where it is located on the N-terminus (Lippens *et al.,* 1995). Similarly, the Tunisian scorpion, *Scorpio maurus* has a toxin named maurotoxin which is also active on K^+ channels, has 34 amino acids with 4 disulphide bonds, and which has striking homology with the sequence of Pil, although the disulphide bridge pattern differs (Blanc *et al.*, 1997; Rochat *et al.*, 1998).

In addition, Legros and coworkers (1998), have reported long chain scorpion toxins active on K^+ channels which are cross-linked by only 3 disulphide bonds. The Ts TX Kβ toxin from *Tityus serrulatus* and Aa TX Kβ from *Androctonus australis* were the first toxins described with more than 60 amino acids but only 3 disulphide bonds. Therefore these toxins represent an evolutionary link between the long chain toxins active against Na^+ channels and the short chain toxins active against K^+ channels.

Lastly, Blanc *et al* (1997), describe a toxin AaH STR1 (from *A. australis*) as being the first 'Old World' α-toxin having a β-toxin fold. Therefore, the high sequence similarity of AaH STR1 with α-type scorpion toxins places it as a possible phylogenetic link between 'Old World' and 'New World' scorpion toxins.

The Structure of Scorpion Toxins

Primary Structure Studies

Genes encoding neurotoxins from scorpions of the *Androctonus*, *Centruroides*, *Leiurus, Tityus* and *Buthus* genera have been cloned and their nucleotide sequence determined. From studies of these genes, several conclusions can be made about the architecture of scorpion neurotoxins.

Among scorpion neurotoxins, the amino terminus contains an 18-21 amino acid signal peptide. Comparison of signal peptide sequences showed a richness of hydrophobic and or aromatic residues and that valine is consistently present in position 3 and leucine at position 9 (Becerril *et al.*, 1995). The sequences, as presented in Figure 1.1, are in agreement with the rules proposed by von Heijne (1986) for cleavage sites in eukaryotes. Figure 1.1 depicts the significant homology among members of 'Old World' and New World' toxin signal peptides, with a consensus sequence being derived for the various toxins shown.

A comparison of 3' non-coding cDNA sequences (Figure 1.2) indicates several well conserved regions particularly the sequences from nucleotides 35-45 and 70-80. This last region contains the polyadenylation signal (Becerril *et al* ., 1995).

Figure 1.1: Comparison of signal peptide sequences of scorpion neurotoxins.

The signal peptides of representative 'New World' and 'Old World' scorpion neurotoxins are compared. 'New World' toxins include those from group 1 (the *Centruroides noxius* toxins: Cngt II, Cngt III, Cngt IV, Cngt V, and the *Tityus serrulatus* toxin TS VII). The 'Old World' toxins are in groups 2 and 3. These are represented by the *Androctonus australis* toxins AaH I, AaH III, AaH II and LqhαIT from *Leiurus quinquestriatus hebraeus* (Group 2). Group 3 includes the toxins AaH IT, AaH IT1 and AaH IT2 from *A. australis,* together with BjIT2 from *Buthus judaicus*. References for all sequences represented can be found in Becerril *et al.*, 1995. Gaps (-) were introduced where necessary to maximise similarities. The amino acid residues were numbered from -1 to -21. A consensus sequence was derived for each group of signal peptide sequence. A global consensus was deduced from each partial group consensus. For consensus sequences X = variable residue, a = aromatic residue, b = basic residue and h = hydrophobic residue (adapted from Becerril *et al.*, 1995).

		-20					-15					-10					-5				-1
Cngt II	M	N	S	L	L	M	I	T	A	C	L	F	L	I	G	T	-	-	V	W	A
Cngt III	M	N	S	L	L	M	I	T	A	C	L	V	L	F	G	T	-	-	V	W	A
Cngt IV	M	N	S	L	L	I	I	T	A	C	L	V	L	I	G	T	-	-	V	W	A
Cngt V	M	N	S	L	L	M	I	T	A	C	L	A	L	V	G	T	-	-	V	W	A
TsV II	M	K	G	M	I	L	F	I	S	C	L	L	L	I	G	I	-	V	V	E	C
Consensus Group 1	M	N	S	L	L	M	I	T	A	C	L	V	L	I	G	T	-	-	V	W	A
AaH I	M	N	Y	L	V	M	I	S	L	A	L	-	L	L	M	I	G	-	V	E	S
AaH III	M	N	Y	L	V	M	I	S	L	A	L	-	L	L	M	T	G	-	V	E	S
AaH II	M	N	Y	L	V	M	I	S	L	A	L	-	L	F	V	T	G	-	V	E	S
LqhαIT	M	N	Y	L	V	M	I	S	L	A	L	-	L	L	L	L	G	-	V	E	S
Consenesus Group 2	M	N	Y	L	V	M	I	S	L	A	L	-	L	L	M	T	G	-	V	E	S
AaH IT	M	K	F	L	L	L	F	L	V	V	L	P	I	M	-	-	G	-	V	L	G
AaH IT1	M	K	F	L	L	L	F	L	V	V	L	P	I	M	-	-	G	-	V	L	G
AaH IT2	M	K	F	L	L	L	F	L	V	V	L	P	I	M	-	-	G	-	V	L	G
Bj IT2	M	K	L	L	L	L	L	V	I	S	A	S	M	L	L	E	C	L	V	N	A
Consenesus Group 3	M	K	F	L	L	L	F	L	V	V	L	P	I	M	-	-	G	-	V	L	G
Consensus Groups 1-3	M	N	X	L	L	M	I	X	h	X	L	X	L	h	X	X	G	-	V	X	X

Figure 1.2: Alignment of 3'-non-coding sequences of scorpion neurotoxins.

The 3' non-coding regions of the various representative scorpion cDNAs were aligned. Abbreviations for toxin cDNAs represented include: The *Centruroides noxius* toxins: Cngt III, Cngt IV, Cngt II, Cngt V; The *Tityus serrulatus* toxin TS VII; The *Androctonus australis* toxins AaH I, AaHIT, AaH III, AaH II; LqhαIT from *Leiurus quinquestriatus hebraeus* and BjIT2 from *Buthus judaicus*. Refer to Becerril *et al.*, (1995) for references for each sequence presented. Gaps (-) were introduced where necessary to maximise similarities. Numbers on top of sequences are for reference purposes only (adapted from Becerril *et al.*, 1995).

```
                     10            20          30            40
Cngt III   TGGCAACGACTTTTTATTGTCCATAAACAGAAATAT-TGTAACGCTT
Cngt IV    TGGCAACGACTTTTTATTGTCCACCAACAGAAATAG-TGTAACGCTT
Cngt II    TGGCAACGACTTTTTATTGTCCACCAACAGAAATAT-TGTAACGCTT
Cngt V     TGGCAACGACTTTTTATTGTCCACCAACAGAAATAT-TGTAACGCTT
Ts VII                ATT-TGTTTCGCTGAAAATCCTTTACAAATG-AA--CTG
AaH I                 ACC-TGTA--GAGTAAAATCAGAAAGAA-TGTATC-CTA
LqhαIT                ATC-TGTA--GAACATAAACACAAAGAA-TGTATC-CTA
AaHIT            T--TGTA------ATAAT--TAT-GAA--GTAT---T-
AaH III               ACC-TGTA--AAGCAAAA-CACAAAGAAT-GTATC-CTG
AaH II         AT--TATAA-GATG-----------GAAT-GTATC-CTA
BjIT2        GCC--CTAC--TGCAT-GAATATGTTTGTCA-GAAA--AATC---A

             50           60            70           80           90
Cngt III   CTTAATTGCA-GTTAAATG----AAATAAAATGC-T-----AATAGC
Cngt IV    CTTAATTCCAAGT---------------------------------
Cngt II    CTTAATTGCA-GTTAAATG---------------------------
Cngt V     CTTAATTTCAA-TTAAATG----AAATAAAA--TAT-----TATACC
Ts VII     --TAAT---AAGTT---TGGCAAAAATAAAA---AAA----TGTT-C
AaH I      A-AAATAACTGGT----------AAATAAA---CATA----AGTA--
LqhαIT     A-GAATTGATC------------AAATAAA---TAT-----------
AaHIT      --GAATTGATC--TA--------AAATAAATGCACA----TATAT--
AaH III    A-AAATAACTGGT----------AAATAAA---CATA---AGTA---
AaH II     A-GTATC--------AATGTT--AAATAA----TATAATCAAAAA--
BjIT2      A--TAT-ACG-------TG-C-AAAATAAA----GTAAACTTCATGC
```

Analysis of the complete nucleotide sequence of several scorpion neurotoxins, enables a number of significant conclusions to be drawn. Firstly the cDNAs from scorpion neurotoxins of the same species, such as those of AaH (AaH I-III), show well conserved sequences from the 5' non-coding region through to the 3' non-coding region (Bougis *et al.*, 1989). This observation suggests that the AaH I-III neurotoxins are encoded by genes that may have originated from duplications of an ancestral gene with each duplicate evolving independently of one another.

There are also significant similarities observed between cDNAs from different scorpion neurotoxins which have entirely different specificities. Examples of these are the LqhαIT and AaH I-AaH III cDNAs. These toxins share a 95% similarity at the signal peptide sequence, a 72% similarity at the mature peptide sequence and a 60% similarity at the 3' non-coding sequence (Becerril *et al.*, 1995). These are clear indications that the genes specified by these two species are closely related. Despite the noticeable similarity in their cDNAs the two neurotoxins have marked differences in their specificities. AaH II affects mammals (Rochat *et al.*, 1972) while LqhαIT is highly toxic to insects and crustaceans but less toxic to mice (Eitan *et al.*, 1990).

From studies on the *Tityus* (Becerril *et al.*, 1993) and *Androctonus* (Martin-Euclaire *et al.*, 1992) scorpion toxins it appears that the transcription of their genomic regions generates pre-messenger mRNAs of approximately 800 nucleotides containing an intron of approximately 470 nucleotides located within the region encoding the signal peptide. The processing of these pre-mRNAs gives rise to a mature molecule of approximately 370 nucleotides. A comparison of 5' and 3' introns of genes from other arachnids revealed a close similarity with 6 nucleotides located at the 5' end and the last 7 nucleotides of the 3' end of the introns. When these consensus sequences were compared with other eukaryotic intron boundaries it appeared that they conformed to a feature of all eukaryotic introns so far reported (Becerril *et al.*, 1993).

The independent evolution of each duplicate scorpion neurotoxin gene via insertions, deletions and mutations at different levels and sites can be used to explain the variability in amino acid sequence and specificity of action of the neurotoxins. To date, some indicative features of the relationship between structure and specificity of scorpion neurotoxins are emerging.

	10 20 30 40
AaH I	KRDGYIVYPNN-CVYHCVPP-----CDGLCK-KNGGSS-GSCSFL
AaH II	VKDGYIVDDVN-CTYFCGR---NAYCNEECT-KLKGES-GYCQWA
AaH III	VRDGYIVDSKN-CVYHCVPP-----CDGLCK-KNGAKS-GSCGFL
LqhαIT	VRDAYIAKNYN-CVYECFR---DAYCNELCT-KNGASS-GYCQWA
BjIT2	--DGYIRKK-DGCKVSCII-G-NEGCRKECVA--HGGSFGYC-W-
Ts VII	-KEGYLMDH-EGCKLSCFI-RPSGYCGRECGIKKGSS--GYCAWP
Cngt II	-KEGYLVRKSTGCKYGCLLLGKNEGCDKECKAKNQGGSYGYCYAF
Cngt IV	-KDGYLVDV-KGCKAHCYKLGENDYCNRECKMKHRGGSYGYCYGF
AaHIT	KKNGYAVDS-SGKAPECLL---SNYCNNECTKVHYADK-GYCCLL

	50 60 70
AaH I	VPSGLACWCKDLPDNVPIKDT--SRK--CTR
AaH II	SPYGNACYCYKLPDHVRTKG---PGR--CHGR
AaH III	IPSGLACWCVALPDNVPIKDP--SYK--CHSR
LqhαIT	GKYGNACWCYALPDNVPIRV---PGK--CHRK
BjIT2	-TWGLACWCENLPDAVTWK-SS-TNT--CGRKK
Ts VII	-----ACYCYGLPNWVKVWDRA-TNK--CG-KK
Cngt II	-----GCWCEGLPESTFTVPL--PNKS-C-SKK
Cngt IV	-----GCYCEGLSDSTPTWPL--PNKR-CGGK
AaHIT	-----SCYCFGLNDDKKVLEISDTRKSYCDTTIIN

Figure 1.3: Comparison of amino acid sequences of scorpion neurotoxins.

Representative scorpion toxin sequenc from North America, South America and North Africa were aligned. Abbreviations for cDNAs represented include: The *Androctonus australis* toxins AaH III, AaH I, AaH II; AaHIT; LqhαIT from *Leiurus quinquestriatus hebraeus*; BjIT2 from *Buthus judaicus*. The *Tityus serrulatus* toxin TS VII; And the *Centruroides noxius* toxins: Cngt II and Cngt IV. Refer to Becerril *et al.*, (1995) for references for each sequence presented. Gaps (-) were included where necessary to maximise similarities. The numbers on top of the sequence were placed for reference, with the first digit of each numeral being aligned with the corresponding amino acid (adapted from Becerril *et al.*, 1995).

Structural Motifs

The molecular structural motifs of the various scorpion toxin classes appear to follow a similar pattern (Figure 1.4), that is, a well defined and conserved motif of an α-helix of approximately 2.5 turns and a 3-stranded β-sheet arrangement which form a dense core maintained by three or four well-conserved disulphide bridges (Bontems *et al.,* 1991). This structure is known as the α/β scorpion toxin scaffold. The most thoroughly studied scorpion toxin structures belong to those that recognise Na^+ and K^+ channels (Lebreton and Delepierre, 1994). The highly conserved protein scaffold is used to introduce different biological activities on Na^+ and K^+ channels and to bind to distinct receptor sites (Gordon *et al.,* 1998). In addition, the folding pattern confers a very compact structure to the molecule where all residues are exposed to the solvent, except for the cysteine residues which are buried within the peptide (Garcia *et al.*, 1998)

The same fold is adopted both by toxins that act on Cl^- channels (Lippens *et al.*, 1995) and by some proteins synthesised in tissues other than the venom glands, for example, the defensins found in the hemolymph of arthropods (Cornet *et al.*, 1995) and the γ-thions from plants (Bruix *et al.*, 1993). Therefore, different toxic functions can be attributed to this unique, stable and ubiquitous fold. Among the conserved amino acids of the primary structures in pharmacologically diverse scorpion toxins, are the cysteine residues and two peptide stretches with the sequences Cys-X-X-X-Cys (where Cys = cysteine and X = any amino acid) located in an α–helix, and Cys-X-Cys contained in a β-sheet. These segments of primary structure seem to be responsible for the formation of the constant motif underlying the main structural core of the scorpion toxins (Leberton and Delepierre, 1994).

Scorpion toxins possess highly variable amino acid sequences as well as conserved residues. The conserved residues form a large flattened surface rich in aromatic residues which has been referred to as the conserved hydrophobic surface (CHS). It has been suggested that the hydrophobic surface is responsible for the interactions of these polypeptides with their receptors and thus being involved in the toxic site (Gordon *et al.*, 1998). However, the regions composed of highly variable and exposed amino acids coincide with locations of antigenic epitopes and may modulate the scorpions' selectivity to insects or mammals (Bahraoui *et al.*, 1986; Granier *et al.,* 1989). The differences found in the length of the highly solvent exposed loops as well as in the nature of the solvent exposed amino acids are likely

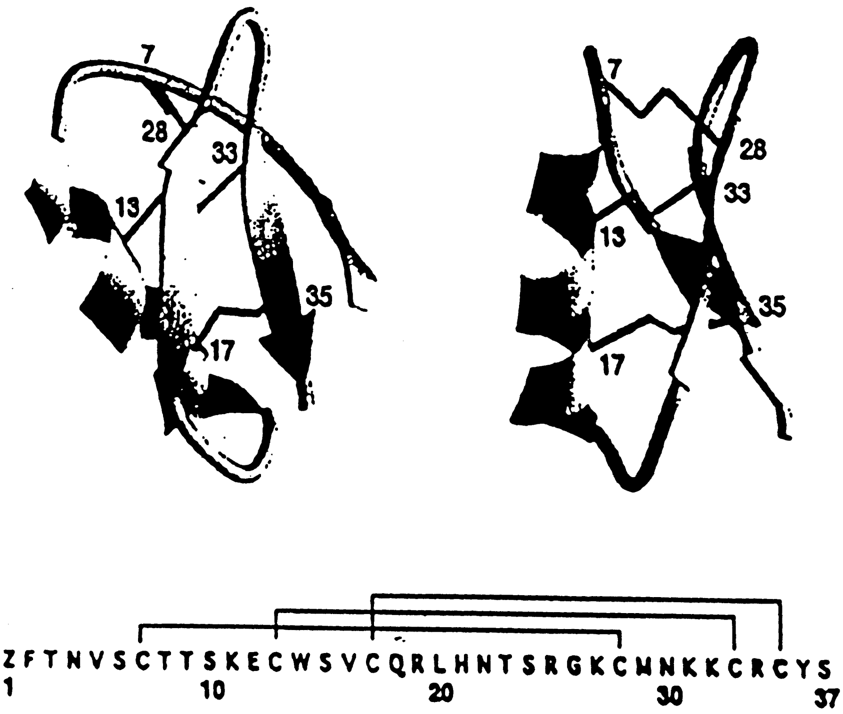


Figure 1.4: The three-dimensional structure and sequence of the scorpion charybdotoxin.

Charybdotoxin is a classic example of a toxin containing the α/β scorpion fold found in all scorpion structures thus far reported. The identifiable structures are the two β-sheets and short α-helix, which are stabilised by three disulphide bonds. The numbers indicate the positions of the 6 cysteines. The sequence is in one letter code (z- pyroglutamic acid) (adapted from Vita *et al.*, 1998).

to provide the molecular basis for the different activities and phylogenetic preferences of scorpion neurotoxins.

One way to gain information about the role of particular residues in the expression of biological properties of a protein is to selectively modify these residues and to determine the consequences with regard to the functions in question. Such studies have been performed on the two most potent scorpion toxins: AaH II and LqhαIT.

Mutational Analysis of Scorpion Toxins

The determination of the three-dimensional structures of toxins displaying similar structures but different pharmacology, or of toxins with different structures but identical pharmacology may be useful in identifying structural domains with functional features. In this regard LqhαIT, the most potent insecticidal scorpion α-toxin, and AaH II, the most potent anti-mammalian α-toxin, are suitable for such studies. The polypeptide backbone of LqhαIT toxin is highly homologous to that of the toxin AaH II. Additionally, the secondary structure is essentially identical in both toxins and the location and type of turns are also very similar (Tugarinov *et al.,* 1997). Therefore, structural differences between the two may serve as a basis for mutational analysis to evaluate the role of such characteristics as the 5-residue turn (residues 8-12) and the C-terminal region residues 2-3, as these are the residues that differ between the toxins (Gordon *et al.,* 1998). The following examples of mutational studies on AaH II and LqhαIT are based on studies performed by Gordon and co-workers (1998).

The solvent-exposed sequence DDV at the beginning of the 5-residue turn in AaH II is conserved in many α-toxins highly active on mammals but is substituted by KNY in LqhαIT. This variation causes changes in the charge of the surface and different side-chain:side-chain interactions stabilising the 5-residue turn, thus leading to differences in the arrangement of the turns relative to the C-termini in the two toxins. Substituting residues KNY in LqhαIT as found in AaH II resulted in the apparent affinity for insect receptor site being decreased 21000-fold and the toxicity almost negated. Tyrosine 10 is unique to the scorpion α-insect toxins that are highly active on insects and may be correlated with this specificity. The substitution of the aromatic tyrosine with a neutral serine in LqhαIT induced a 114-fold decrease in affinity for the receptor, while substitution with tryptophan (a conservative substitution) had only minor effects.

Positively charged residues are found in the C-terminal region in α-toxins such as lysine 58 in AaH II and arginine 58 in LqhαIT. When lysine 58 of AaH II was biotinylated it had a devastating effect in that only 1% of the original toxicity to mice was observed. This could be due to a combination of structural disruption or alteration of the electrostatic potential of the molecule and/or the receptor (Fontecilla-Camps *et al.,* 1988; Zilberberg *et al.,* 1997). Interestingly Bot XI (from the scorpion *Buthus occitanus tunetanus*), unlike most α toxins, does not contain a positively charged residue at position 58. Instead it has a valine (Sampieri *et al.*, 1997). Even though Bot XI has high sequence similarity to AaH II it is 1200 times less toxic to mice and has 120 times lower affinity to the AaH II receptor. This emphasises the importance of lysine 58 for AaH II anti-mammalian activity.

The CHS structure is common to both AaH II and LqhαIT. Modification of the aromatic residues on the CHS had a negligible effect on toxicity to mammals and insects. Thus it is unlikely that the conserved hydrophobic surface is involved in the functional surface of the α-toxins.

Knowledge of the location of such residues as those discussed above allows the ability to draw some structural conclusions with respect to which residues participate in the protein/receptor interaction and which are responsible for the activity of the protein. However additional molecular studies are required before a complete map of all the residues involved in receptor and antigen recognition is elucidated.

Immunotherapy for Scorpion Envenomation

The most common form of treatment for scorpion sting is serotherapy. The antisera used are difficult to prepare reproducibly even under standardised conditions and their neutralising properties may vary from one batch to another (Mousli *et al.*, 1999). Additionally, the efficacy of this treatment is related to the speed with which the anti-serum is administered as the toxic proteins diffuse throughout the body making their subsequent capture by specific antibodies difficult (Zenoulai *et al.,* 1997). However, the selection of antivenom dose and administration protocol remains empirical (Revelo *et al.,* 1996). In Brazil approximately 10 000 human cases of scorpion sting occur and in Mexico more than 200 000 people are affected, with death of at least 1000 cases each year (Calderon-Aranda *et al.*, 1995). It has been demonstrated that a given scorpion antiserum is species specific and therefore cross neutralises only weakly, if at all, a very small number of venoms. This is due to the structural and antigenic polymorphism of scorpion toxins (Bahraoui *et al.*, 1986). Therefore, despite the fact that an effective antiserum exists and is used with some success, these figures indicate that pre-existing high affinity circulating antibodies from a vaccine could neutralise the toxic antigens as soon as they enter the body, immediately after the sting and would therefore protect people at risk from the effects of scorpion venom.

Work involving chemical or physical detoxification of venom for use in protection against the noxious effects of scorpion venoms and toxins has resulted in encouraging and promising results (Possani *et al.*, 1981; Bahraoui *et al* ., 1986). However a practical problem exists in obtaining enough venom from scorpions to prepare the necessary doses for immunisation. Synthetic and genetically engineered peptides offer an alternative to the use of native venom from scorpions (Zenouaki *et al.,* 1997). Synthetic peptide immunogens have made it possible to manipulate the immune response by targeting the host humoral response to the determined regions of the protein to be recognised. The use of synthetic peptides as immunogens has advantages over the use of whole or part of the venom. These advantages are expressed in terms of the complete absence of toxicity of the immunogen as well as in the low cost and reproducible production (Mousli *et al.*, 1999).

The use of monoclonal antibodies (Mab) as immunoprotective agents offer advantages in terms of potency, reproducability and freedom from contaminants, however they are not easy to develop (Evans *et al.,* 1995; Devaux *et al.*, 1997). Treatment of scorpion envenomation requires a rapidly penetrative antivenom which has high affinity for its target neurotoxin, so that the toxin can be readily neutralised and eliminated prior to exerting any further damages upon the host. A single chain Fv (ScFv) fits these requirements and may be able to trap the toxin in the vascular and extravascular compartments before it binds to its target (Mousli *et al.*, 1999). The ScFv is a polypeptide composed of the immunoglobulin heavy chain variable region (VH) covalently linked to the immunoglobulin light chain variable region (VL) by a short flexible peptide of about 25 residues. The binding specificity of the parental antibody is thus retained (Bird *et al.*, 1988; Huston *et al.*, 1988). Numerous ScFv's have been produced for potential therapeutic applications including treatment of cancer and detoxification (Yokota *et al.*, 1992; Denton *et al.*, 1997; Lemeulle *et al.*, 1998). ScFvs are cleared more rapidly from the blood to extravascular spaces than whole IgG or other larger fragments of immunoglobulin molecules (such as Fab and $F(ab')_2$), resulting in better tissue penetration. The small size of the ScFvs (27kDa) permits their rapid diffusion through the body for detoxification and the fast *in vivo* clearance of immunocomplexes via the urine. This minimises the chance of release of bound toxin and the re-emergence of toxicity (Mousli *et al.,* 1999).

The murine Mab 4C1 of the toxin AaH II, can prevent the toxin binding to its receptor as the epitope overlaps or is close to the pharmacologic binding region of the toxin (Bahraoui *et al.*, 1988). A ScFv derived from 4C1 was designed based on the first 8 residues of the N-terminus of AaH II (Mousli *et al.*, 1999). These residues were previously found to be part of a neutralising epitope recognised by the Mab 4C1 (Bahraoui *et al.*, 1988). *In vitro* and *in vivo* experiments indicated that ScFv-4C1 was soluble and retained structural features that defined the parent antibodies specificity, and therefore it had a high capacity to neutralise the pharmacological effects and lethal potency of toxin AaH II (Mousli *et al.*, 1999). Thus this work by Mousli and coworkers (1999), describes the first fully functional ScFv that actively neutralises the toxin produced by the scorpion *A. australis.*

A baculovirus system may prove useful for producing large quantities of ScFv for further pharmacological and neurophysiological studies. By this method the ScFv 4C1 would be produced in a culture system devoid of contaminants toxic to humans such as endotoxins which are released by *E. coli* (Lemeulle *et al.*, 1998). Therefore ScFv-4C1 is a promising step towards the design of new antivenoms with improved properties for immunotherapy.

Frontiers in Scorpion Toxin Research

Baculovirus Expression of Scorpion Toxins as Biopesticides.

Scorpion insect toxins have a high insect toxicity and strict species selectivity and therefore they can be considered as ideal candidates for potential application as biological insecticides in pest control (Xiong *et al* ., 1999). Scorpion insect toxins are selectively active on lepidopterous, dipterous and other insect orders. The insect-selective toxins are sub-divided into two categories: the excitatory and depressant toxins. The depressant toxins, to date, are composed of 61 amino acids and induce a slow progressive onset of flaccid paralysis preceded by a short transient contraction paralysis. The excitatory toxins, to date, are composed of 70 amino acids and induce an immediate reversible fast contraction paralysis upon injection (Moskowitz *et al* ., 1998, Xiong *et al.,* 1999).

Biological insect control agents offer an attractive alternative to chemical pesticides as they do not pose the environmental and health risks associated with chemical residues. However many such agents lack the sufficient potency in the field to be commercially useful (McCutchen *et al.,* 1991). Baculoviruses are pathogens of insects. Among them nucleopolyhedroviruses (NPV) infective to Lepidoptera are used as protein expression vectors and for insect pest control. The insecticidal efficiency of baculovirus has been improved by 30-40% when their pathogenicity is combined with expression of anti-insect toxins (Gershburg *et al*., 1998).

Gershburg and coworkers (1998), demonstrated that when the potent excitatory anti-insect toxin AaIT (from *A. australis*) was genetically incorporated into *Autographa californica* NPV (AcNPV), there was a significant decrease in the time required to kill hosts through oral infection of the AcNPV. In a separate study by Chejanovsky and coworkers (1995), the anti-insect toxin LqhαIT was also expressed in a baculovirus based eukaryotic system. The expressed toxin significantly enhanced the insecticidal efficacy towards lepidopterous larva. Additionally, LqhαIT is a depressant toxin, and therefore has a different binding site on the insect sodium channel from that of excitatory insect toxins (Chejanovsky *et al.*, 1995). Thus it could be a good candidate for engineering double expression vectors together with the excitatory anti-insect toxin which may yield a synergistic interaction between the toxins. In addition these insecticides reduce the risks to non-target insect species with minor predicted effects on the surrounding environment.

Miniprotein design.

Another field of scorpion toxin research which is popular in the current literature is in the area of miniprotein design. The concept of miniprotein design involves small, independently folded motifs acting as templates for the construction of functional polypeptides (Vita *et al.*, 1995). These new constructs may also be useful as highly simplified systems for theoretical and experimental studies of protein structure and folding (Imperiali and Ottesen, 1998). Toxin protein scaffolds are chosen to engineer new miniproteins because they have small, highly permissive scaffolds that have high structural stability and can display a multiplicity of functional sites spread on various regions of their surface available for mutations. An example of such a toxin is charybdotoxin (ChTx) from *Leiurus quinquestriatus hebraeus*. ChTx is a small 37 residue protein with a well known globular structure of the α/β scorpion scaffold, that consists of a triple stranded antiparallel β–sheet on one face, linked to a short α-helix on the opposite face, and stabilised by three disulphide bonds in the interior core (Bontems *et al.*, 1991)

An example of miniprotein design research involved the transfer of a portion of the metal binding site of human carbonic anhydrase onto the α/β scorpion toxin scaffold. To do this ChTx received a total of 9 mutations mostly located on the 2 stranded β-sheet. As the cysteine residues are buried inside the molecule and are conserved in all scorpion toxins, the cysteine pairing was maintained in the engineering of the new sequences and only the solvent exposed residues were mutated (Vita *et al.*, 1995; Drakopoulou *et al.*, 1998).

The introduction of these mutations enabled ChTx to recognise various metals, though with lower affinity when compared with human carbonic anhydrase. As demonstrated by ^{1}H-NMR analysis, the mutations did not affect the fold of ChTx. The conserved cysteine spacings determined the specific native disulphide arrangements, thus explaining the efficient folding properties of both ChTx and its engineered sequences. The chimeric protein did not however, recognise K^+ channels as did the native ChTx. This was expected as a number of functional residues of ChTx were mutated during the transfer operation. Therefore by substituting 9 residues on ChTx the original potassium channel recognition function was transformed into a metal binding activity (Drakopoulou *et al.*, 1998).

This work demonstrated that the toxin structural motif can act as a template for miniprotein engineering and that new activities can be incorporated into it by the transfer of appropriate active sites. Therefore, tolerance for sequence mutations and retained stability following multiple mutations is a unique property of this scaffold. However these results are still preliminary as the transfer activities are still low. Toxin scaffolds have the potential to design minimised proteins expressing new functions. This may be of significance as deriving smaller molecules as therapeutic drugs from these active miniproteins will be easier than starting from the much larger natural proteins (Vita *et al.*,1998)

1.4 SPIDERS

Spiders are one of the many animal groups that use their venom as a tool of aggression in order to paralyse or immobilise their prey. Almost all of the 40 000 spider species are venomous with the exception of the two families, the Liphistiidae and Uloboridae (Malli *et al.*, 1998). Spiders are mainly insectivorous predators, and of the 180 that bite humans only a handful are venomous (Ori and Ikeda, 1998).

Spider neurotoxins can be classified into two groups according to their chemical structure. These are polyamine-like toxins which potentially block neuromuscular transmission mediated by glutamic acid. Examples of spider toxins from this group include the Joro spider toxin (JSTX) from *Nephila clavata* and NSTX from *Nephila maculata* (Kawai, 1991). Secondly there are the cysteine rich neuroactive peptides which specifically interact with and alter the conductance properties of neuronal ion channels (Hagiwara *et al.*, 1991; Figueiredo *et al.*,1995). Examples of spiders from this group include the Australian funnel-web spiders and black widow spiders. As published research on the biochemistry and structure of spider toxins is limited, this review will primarily focus on discussing the research on Australian funnel web spiders, widow spiders and structural homologies among spider neurotoxins and other inhibitory polypeptides.

Australian Funnel-Web Spiders

Australian funnel-web spiders (FWS) (Araneae: Hexathelidae: Atracinae) have a lethal and aggressive nature that contests them as being the worlds most dangerous spiders. The toxins of these spiders have been recently reclassified as atracotoxins (ACTX) because they belong to the subfamily Atracinae. Therefore robustoxin (from *Atrax robustus*) often found in and around Sydney metropolitan households is now known as δ-ACTX-Ar1 and versutoxin (from *Hadronyche versuta*), located in the Blue Mountains west of Sydney, is known as δ-ACTX-Hv1 (Fletcher *et al* ., 1997a; Fletcher *et al*., 1997b; Nicholson *et al.*, 1998). This nomenclature has been used widely for describing disulphide bridged polypeptide toxins from animals such as cone snails (Conus spp.) and the American FWS *Agelenopsis aperta.*

Australian FWS are mainly restricted to the southeastern coast of Australia (Gray, 1988). There have been at least 14 human fatalities, all attributed to the male *Atrax robustus,* since 1927 (Wiener, 1961). The envenomation syndrome is associated with a variety of autonomic and motor symptoms with death resulting from respiratory and circulatory failure (Sutherland, 1990).There have been no reports of fatalities since the introduction of the antivenom in 1980 (Sutherland, 1980) which is produced by the immunisation of rabbits with male *A. robustus* venom (Collins *et al.,* 1995). The venom of *A. robustus* and *H. versuta* produce severe neurotoxic symptoms in primates and newborn mice but not in other vertebrates (Mylecharane *et al* ., 1988; Mylecharane *et al.*, 1989). Non-primates do not exhibit symptoms following *A. robustus* envenomation due to the presence of inactivating IgG in their plasma (Sheumack *et al.*, 1991).

Biochemical Studies on Australian Funnel-Web Spiders

Spider venoms are biochemically very complex mixtures of proteins. Characterisation of the active components and analysis of their physiological functions has been difficult due to the venom being synthesised in relatively small quantities (Kawai, 1991). The first spider toxins to actually have their amino acid sequences deduced were those of the Australian FWS *Atrax robustus* (Sheumack *et al.*, 1985) and *Hadronyche versuta* (Brown *et al.*, 1988).

Both δ-ACTX-Ar1 and δ-ACTX-Hv1 are peptide neurotoxins consisting of a single chain of 42 amino acid residues. They have a number of basic residues and appear to be tightly folded molecules cross-linked by 4 conserved intramolecular disulfide bonds including unblocked C- and N-terminal cysteines. They are 83% homologous with 8 differing residues that are mostly conservative substitutions (Brown *et al.*, 1988). The sequences are unusual in that they contain three consecutive cysteine residues at positions 14-16. (See figure 1.5). The differences may be due to point mutations from an ancestral gene. The toxins show very weak overall homology with any other neurotoxins, but have a cysteine motif similar to other spider toxins (Nicholson *et al* ., 1998).

δ-ACTX-Ar1 and its homologue δ-ACTX-Hv1 have been recently shown to target the voltage gated Na^+ channel. Thus they have been named the δ-atracotoxins (δ-ACTX's). This nomenclature is again consistent with that of other peptide toxins mentioned previously, where the Greek symbol (in this case δ) donates the activity of the toxin for Na^+ channels (Fletcher *et al.,* 1997a). This inactivation of action is similar to polypeptide scorpion α-toxins (Narahashi *et al.,* 1972; Strichartz and Wang., 1986) and sea-anemone toxins which bind to neurotoxin receptor site 3 on the extracellular surface of the sodium channel (Warashina and Fujita, 1983; Wasserstrom *et al.,* 1993). This was confirmed by experiments showing that δ-ACTXs inhibited binding of the mammalian selective α-scorpion toxin AaH II from *Androcotnus australis hector* and the insect selective α-scorpion toxin LqhαIT from *Leiurus quinquestriatus quinquestriatus* to rat brain and cockroach synaptosomes respectively (Little *et al.,* 1998). Therefore δ-ACTXs may be defined as a new class of toxins which interact with site 3 on the Na^+ channel. However they are able to potently interact with insect sodium channels as well as mammalian channels, unlike the scorpion α-toxins. These toxins would therefore be useful in investigations regarding the structural requirements for anti-insect versus anti-mammal activity (Little *et al.,* 1998).

1 10 20
δ-ACTX-Ar1: C A K K R N W C G K **N** E D C C C P M K C **I** Y A
δ-ACTX-Hv1: C A K K R N W C G K **T** E D C C C P M K C **V** Y A

30 40
δ-ACTX-Ar1: W Y N **Q** Q G S C **E** **T** T I **T** **G** L **F** K K C
δ-ACTX-Hv1: W Y N **E** Q G S C **Q** **S** T I **S** **A** L **W** K K C

Figure 1.5: The mature amino acid sequence of the Australian funnel-web spiders δ-ACTX-Ar1 and δ-ACTX-Hv1.

The sequences for δ-ACTX-Ar1 and δ-ACTX-Hv1 were obtained from Sheumack *et al.*, (1995) and Brown *et al.*, (1988) respectively. The 8 substitutions are shown in bold type. Numbering above the sequence is for reference purposes only.

In the sea anemone and scorpion toxins, combinations of charged (especially cationic) and hydrophobic side chains are responsible for binding to receptor site 3 on the channel (Rogers *et al.*, 1996). A study by Fletcher and co-workers (1997) showed that δ-ACTX-Hv1 shares a number of topologically related anionic and cationic residues to that of the α-scorpion toxins and sea anemone toxins. A superimposition of the structure δ-ACTX-Hv1 with those of AaH II and anthopleurin-B (from the sea anemone *Anthopleura xanthogrammica*) showed that three cationic and two anionic residues were superimposed including those suggested to be important for binding. Therefore the Australian FWS toxins represent new toxins which, together with sea anemone and scorpion toxins, can be used in further studies to map the dimensions of the Na^+ channel site.

Interestingly a new Australian FWS toxin was recently isolated from the venom of *H. versuta* (Fletcher *et al.,* 1997b). This toxin named ω-atracotoxin-Hv1, due to its activity on Ca^{2+} channels, was isolated from *H. versuta* venom by screening for anti-*Helicoverpa* activity. *Helicoverpa armigera* is one of the world's most economically devastating agricultural pests and one of the most difficult to control. ω-atracotoxin-HV1 is a 37 residue peptide that contains three disulfide bonds and it is lethal to *H. armigera* larvae, yet harmless to mice. The structure of ω-ACTX-Hv1 shows that it is similar to other vertebrate Ca^{2+} antagonists and falls into the toxic/inhibitory cysteine knot family of polypeptides (discussed on page 26). Therefore this toxin can provide a lead into the design of insect-specific pesticides directed against agricultural pests such as *H. armigera.* This insecticidal potency and apparent specificity of ω-ACTX-HV1 recommends it as a potential template for the design of non-peptidic analogues that may be used in the control of *H. armigera.*

Recently, the solution structures for both δ-ACTX-Ar1 (Pallaghy *et al.*,1997) and δ-ACTX-Hv1 (Fletcher *et al.,* 1997a) have been determined by NMR. Both toxins display a small triple stranded anti-parallel β-sheet and disulphide bond pattern, similar to that of other polypeptides such as ω-conotoxin (Olivera *et al.*, 1997), that fall into the inhibitor cysteine knot family. The three dimensional fold of these toxins is completely different to the previously determined structures for the scorpion α-toxins AaH II and LqhαIT and the sea anemone toxin anthopleune-B despite similar apparent actions on sodium current inactivation. However key amino acids may be shared between the δ-ACTX's and AaH II or LqhαIT.

The work by Fletcher *et al* .(1997a) on the strucutre of δ-ACTX-Hv1 indicates that it has high structural homology with μ-Agatoxin-I (μ-Aga-I), and ω-Agatoxin-IVB (ω-Aga-IVB) which are sodium channel and Purkinje-type voltage gated antagonists respectively, from the North Amercian funnel web spider *Agelenopsis aperta.* Despite their lack of sequence homology the structural homology which consists of a similar cysteine knot determined primarily by their disulfide bond pattern, can be explained as they are all ion channel toxins. μ-Aga-1, like δ-ACTX-Hv1, modifies the kinetics of voltage gated sodium channels. The solution structure of δ-ACTX-Hv1 is also very similar to that of gurmarin, a plant polypeptide. Gurmarin and ACTX-Hv1 share structural and sequence homology which supports the hypothesis that gurmarin elicits its suppressing effect by interacting with an ion channel involved in taste transduction. It is interesting that such structurally similar peptides have evolved (presumably independently) in such unrelated phyla.

Black Widow Spiders: Latrodectism.

The most extensive studies on spider venom have been of the *Latrodectus* genus belonging to the widow spiders. The main widow spider species are the Australian red back spider (*L. hasseltii)*, the North American black widow spider (BWS) (*L. mactans*), the New Zealand katipo (*L. katipo*), the brown widow spider which is found on most continents (*L. geometricus*), the South African knoppie (*L. indistinctus*) and the European *L. tredecimguttatus.* All species produce venom with similar properties and cause a similar and distinctive clinical syndrome termed latrodectism. The most venomous species are the Australian red back and the South African knoppie (Jelinek, 1997).

Australia has the highest per capita rate of latrodectism at approximately 200 bites per annum. The last documented death in Australia occurred in 1955, the year before introduction of antivenom. This antivenom, which remains the current form of treatment, exhibits cross neutralisation amongst certain *Latrodectus* species world-wide (Jelinek *et al*., 1989).

Biochemical Studies on Black Widow Spiders

Black widow spider venom (BWSV) contains a number of high molecular weight proteins that act on vertebrate and invertebrate synapses with the main effect being a massive release of neurotransmitter. The venom contains at least three related latrotoxins, vertebrate specific α-latrotoxin (LTX) and invertebrate specific α and δ-latroinsectotoxins (LIT). All three are potent neurotoxins that trigger neurotransmitter release from pre-synaptic nerve terminals (Ichtchenko *et al.*, 1998).

All latrotoxins are large proteins of approximately 130 kDa and composed of 4 domains: a cleaved signal peptide; a conserved N-terminal domain with invariant cysteine residues; a domain composed of ankyrin-like repeats; and a relatively short C-terminal domain that is less conserved among latrotoxins (Ichtchenko *et al.*, 1998).

The central domains of the BWS toxins are almost entirely composed of a series of 33 imperfect ankyrin-like repeats. Ankyrins constitute a family of proteins that co-ordinate interactions between various integral membrane protein and cytoskeletal elements. Recent studies indicate that a repetitive motif in the ankyrin molecule is responsible for high affinity binding to membrane proteins. Therefore it has been proposed that the structural domains of latrotoxins containing ankyrin-like repeats could also play a role in binding to presynaptic membrane components (Grishin, 1998).

Alignment of the sequence of the 3 latrotoxins indicates that they contain conserved regions extending over the entire protein. δ-LIT has 37% sequence identity with α-LTX and a 38% identity with α-LIT, whereas α-LIT has a 38% identity with α-LTX, 16% of amino acid residues being conserved in the 3 proteins. This indicates that the latrotoxins have most likely evolved from a single ancestral gene. The greatest similarity between all latrotoxins is found in their N-terminal domains. Sequences preceding the N-terminal domain which are removed during maturation, differ greatly among all latrotoxins (Grishin, 1998). The similar domain structure of different latrotoxins and their high degree of sequence identity suggest that the vertebrate specific α-LTX and the invertebrate specific α- and δ-LIT use the same mechanism of action.

In order to determine the mechanism of action of latrotoxin, attempts have been made to produce recombinant forms of α-LTX, allowing for mutational analysis of the toxin. It is known that the venom of α-LTX causes a massive release of neurotransmitters from nerve terminals and endocrine cells in vertebrates. The production of active soluble α-LTX and its homologues in bacteria have proved difficult due to incorrect protein folding (Dulubova *et al.*, 1996). Attempts have also been made to express α-LTX and α-LIT precursors in a baculovirus system, however the proteins synthesised were non-functional and insoluble (Kiyatkin *et al.*, 1995). More recently, Volynski and coworkers (1999) produced active recombinant α-LTX in a baculovirus system that possessed all the properties of natural α-LTX and also determined that the toxin bound specifically to two presynaptic toxin receptors: latrophilin and neurexin Ia.

Homology Studies on Spider Neurotoxins

A variety of toxins are produced which are associated with a small number of protein scaffolds. The inhibitor cysteine knot (ICK) structural motif is an example of one such global fold widely found in an increasing number of small disulfide linked polypeptides from diverse sources and with diverse functions. The ICK motif is characterised by a triple-stranded anti-parallel β-sheet which is stabilised by half-cysteine residues occurring in fixed positions to form a cysteine knot (Figure 1.6). All known examples of this fold satisfy the following consensus determined by the size of the gaps between the half cysteine residues: $CX_{3-7}CX_{3-6}CX_{0-5}CX_{1-4}CX_{4-13}C$, where X can be any amino acid (Pallaghy *et al.*, 1994; Norton and Pallaghy, 1998).

Members of the ICK motif include toxins from species of spiders, cone snails, plants and a fungus, each with varying mechanisms of toxic or inhibitory action as indicated in Figure 1.7. The third and fourth half cysteines of the motif are usually contiguous particularly for spider and cone shell polypeptides. The structural motif is robust and versatile as there is little sequence identity between members belonging to the ICK family. Most of the members contain only three disulfide bridges that comprise the cyteine knot. There are four examples that contain the 4th disulphide bridge, δ-ACTX-Ar1, δ-ACTX-Hv1, ω-agatoxin IVB (ω-Aga-IVB), and μ-agatoxin I (μ-Aga-I) (Norton and Pallaghy, 1998; Fletcher *et al.*, 1997a).

The cysteine knot consists of a ring formed by two disulfide bridges and an interconnecting backbone through which the third disulfide passes. The topology of the motif is βαββ-like, with the α-segment representing coil or turn structures, similar to that of the α/β motif represented by scorpion toxins such as charybdotoxin from *Leiurus quinquestriatus quinquestriatus*) (Bontems *et al.*, 1991) and the insect defensins (Cornet *et al.*, 1995), where the α-segment is an α-helix. Interestingly, the disulfide pairings between the α/β fold and the ICK motif are the same. However, using charybdotoxin as an example, it does not satisfy the motif consensus sequence described above nor does it contain a cysteine knot (Norton and Pallaghy, 1998).

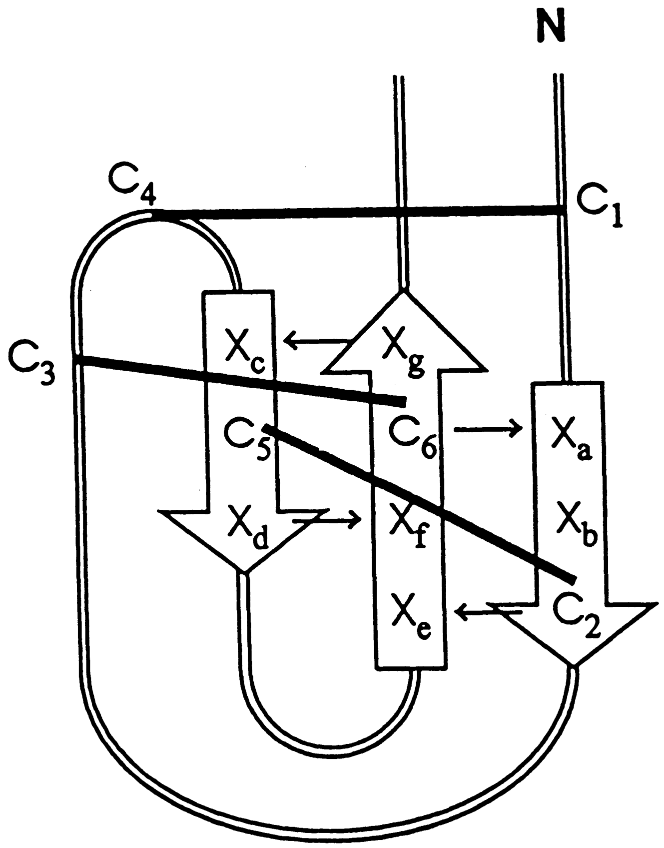


Figure 1.6: Schematic diagram of the inhibitor cysteine knot (ICK) motif.

C represents a half cysteine residue and X represents any other residue. The common hydrogen bonds are indicated by small arrows directed from the donor to the acceptor, and the solid black lines represent cysteine pairing (adapted from Pallaghy *et al.*, 1994).

Figure 1.7: Sequence alignments of members of the ICK motif.

Sequences presented are those that have determined structures available and which have non-redundant sequences or distinct functions. The sites at which the ICK members target (those for which it is known) are listed below. The half cysteine residues of the motif are highlighted in bold. The SWISS-PROT or PIR database accession codes and literature references are given for the amino acid sequences in Norton and Pallaghy (1998).

Target regions of ICK members: robustoxin, Na^+ channel; ω-ACTX-Hv1, Ca^{2+} channel; ω-Aga IVB, Ca^{2+} channel (P-type); μ-Aga I, Na^+ channel; huwentoxin-I, nicotinic acetylcholine recptor; protein 5, K^+ channel; ω-CgTx GVIA, Ca^{2+} channel (N-type); ω-CmTx MVIIC, Ca^{2+} channel (P/Q-type); κ-CpTx PVIIA, K^+ channel; conotoxin GS, Na^+ channel; CMTI-I, trypsin; CPI, carboxypeptidase.

Spiders	robustoxin	**C**AKKR-NW**C**-GKNED**C**-----**C***C*PMK**C**IYAWYNQQ---GS**C**QTTITGLFKK*C*
	ω-ACTX-Hv1	SPT**C**IP-SGQP**C**-PYNEN**C**-----**C**-SOS**C**TFKENENGNTVKR**C**D
	ω-Aga I	EDN**C**IAEDYGK**C**TWGGTK**C**-----**C**RGRP**C**R*C*SMIGTN---*C*E**C**TPRLIMEGLSFA
	μ–Aga I	E**C**VPE-NGH**C**RDWYDE**C**-----**C**EGFY**C**S*C*RQ----PPK*C*I**C**RNNN
	huwentoxin- I	A**C**-KGVFDA**C**TPGKNE**C**-----**C**PNRV**C**SD-------KHKW**C**KWKL
	protein 5	S**C**V-DFQTK**C**KK-DSD**C**-----**C**GKLE**C**SSRW-------KW**C**VYPSPF
Cone Shells	ω-CgTx GVIA	**C**-KSOGSS**C**SOTSYN**C**-----**C**--RS**C**NOYT-------KR**C**Y
	ω-CmTx MVIIC	**C**-KGKGAP**C**RKTMYD**C**-----**C**-SGS**C**GRR--------GK**C**
	κ-CpTx PVIIA	**C**-RIONQK**C**FQHLDD**C**-----**C**-SRK**C**NRF--------NK**C**V
	conotoxin GS	A**C**-SGRGSR**C**OO---Q**C**-----**C**MGLR**C**GRGNP------QK**C**IGAHXDV
Plants	CMTI-I	G**C**-PRILMR**C**KQ-DSD**C**L-AG-**C**---V**C**GPN--------GF**C**G
	CPI	EQHADPI**C**----NKP**C**KTHD-D**C**SGAWF**C**--QA**C**W-------NSART**C**GPYVG
	gurmarin	XQ**C**VKK-DEL**C**IPYYLD**C**-----**C**EPLE**C**KKVNWWD----HL**C**IG
	kalata B1	NGLPV**C**----GET**C**V-GGT-**C**-NTPG**C**---T**C**SW---------PV**C**TR
Fungus	AVR9	**C**----NSS**C**TRA-FD**C**LGQ--**C**-G-R**C**D-------FHKLQ**C**VH

The occurance of very similar cysteine knot motifs in polypeptides from plants and both terrestrial and aquatic animals suggests that this protein fold has been particularly favoured during the course of evolution and may be the most economical way to construct a very stable, small globular protein (Fletcher *et al.,* 1997a).

The studies which have been described in the preceding pages on research into the structure and function of scorpion and spider neurotoxins and of their relationships with other non-related neurotoxins and inhibitory polypeptides provides a useful basis of knowledge for any future work into these and other related arachnid neurotoxins, such as tick neurotoxins, for which published research is limited.

1.5 TICKS

Ticks are parasitic arthropods that prey on vertebrates. Within the arthropods only mosquitoes cause more human and animal diseases than ticks (Grattan-Smith *et al.*, 1997). Ticks belong to the class Arachnida which includes spiders and scorpions, and to the subclass Acari which includes mites. The suborder Ixodida consists of three families of ticks; the Argasidae, Ixodidae and Natalliellidae (Cupp, 1991). Paralysis induced by ticks has been determined to be the result of salivary neurotoxins transferred to the host during feeding. The functional significance of a toxin produced by a parasite is not clear. The reduction of host mobility, exertion of local anaesthesia and the prevention of blood coagulation during feeding are some suggestions for toxin generation. There has also been speculation that paralysis may be a vestigial function conserved when the tick evolved from a predatory lifestyle to a parasitic lifestyle (Stone *et al.,* 1989).

Tick-induced paralysis occurs in different regions of the world. Over 60 of the 820 tick species (belonging to 10 different genera) have been implicated as capable of causing paralysis (Stone, 1987). Most of these are *Ixodid* species (hard ticks) but at least nine species of Argasid ticks (soft ticks) have been found to cause paralysis. Those species which are of major veterinary and human importance are *Dermacentor andersoni* and *Dermacentor variabilis* of North America; *Ixodes rubicundus*, *Rhipicephalus evertsi evertsi*, and *Argas (Pericargas) walkerae* of Africa and *Ixodes holocyclus* of Australia.

The Australian Paralysis Tick, **Ixodes holocyclus**

The Australian paralysis tick, *Ixodes holocyclus,* occurs along the eastern coastal strip of Australia from North Queensland down to the Lakes Entrance in Victoria (Roberts, 1970) (Figure 1.8). Two other Australian ticks of lesser medical importance are *I. cornuatus* and *I. hirsti* found in Victoria and Tasmania (Stone, 1988). The very limited distribution of *I. holocyclus* appears to depend on its great susceptibility to slight variations in environmental conditions, particularly humidity. The highest incidence of tick paralysis coincides with a seasonal abundance of female ticks in spring and early summer (Concidine, 1985). Another factor governing the incidence of the tick is the presence of its natural host, either the long-nosed bandicoot, *Perameles nasuta,* or the Northern brown bandicoot, *Isodon macrourus* (Ross, 1935). It has been suggested that these natural hosts survive heavy infestations as a result of acquired immunity rather than having an intrinsic resistance to the toxin. This is supported by the observations that when a bandicoot carrying high numbers of *I. holocylus* is captured and held in tick-free conditions its ability to resist tick paralysis diminishes over several months [http://www.peg.apc.org/~ullavet/tick.html] and immune animals (including hyperimmune dogs) are seen to have decreased levels of immunity when no longer exposed to tick infestation.

The principle accidental host of *I. holocyclus* is the dog, but the tick regularly attaches to other domestic pets and livestock including cats, cattle and horses. It occasionally affects humans, with 20 fatal cases due to paralysis having been reported (Kaufman, 1989). Therefore *I. holocyclus* has caused more deaths than the funnel web spider *A. robustus* (14 deaths reported) (Wiener, 1961) and the red back spider *L. mactans hasselti,* with 13 deaths reported (Jelinek, 1989). Humans are more commonly affected by allergic reactions to tick bites and several allergens in tick extracts and tick saliva have been described (Stone *et al* , 1983; Dorey and Broady, 1995). Allergic reactions range from mild skin reactions (urticaria and pruritis) to life-threatening anaphylactic reactions. Humans can also be affected by infections which are transmitted by ticks. *I. holocyclus* is a vector for such diseases as Queensland tick typhus for which the causative agent is *Rickettsia australis,* and for Lyme disease caused by *Borrelia burgdorferi* (Russel, 1998).

The impact of *I. holocylcus* on other animals is enormous. It has been estimated that each year tick paralysis affects 20 000 domestic animals in Australia (Stone, 1988). As a measure of the severity of tick toxicosis in non-immune animals, a single adult female *I. holocyclus* tick may be responsible for many cases of tick

Figure 1.8: Distribution of the Australian paralysis tick *Ixodes holocyclus*.

(adapted from Stone and Binnington, 1986).

paralysis and a single tick can cause death of the host (Ilkiw *et al.*, 1987). Infestations by tick nymphs have been reported to produce paralysis but only rarely (Oxer and Ricardo, 1942), while the larvae have not been associated with paralysis, only with causing local irritation (Albiston, 1965). This contrasts the paralysis caused by the tick *A.(P.) walkerae* (the common poultry tick in South Africa) where only the larvae cause paralysis following at least four days of infestation (Viljoen *et al.,* 1990). Larval ticks of this species feed over several days whereas the other stages are fully engorged within one to two hours during which time insufficient toxin is produced to cause paralysis (Viljoen *et al ,* 1990).

Tick attachment and feeding

All ticks are blood-feeding ectoparasites of terrestrial vertebrates (Klompen *et al.*, 1996). The active stages of larva, nymph and adult require blood as a nutritive source, and in the case of adults for sperm or egg production. *I. holocyclus* is a three host tick. During each of the three developmental stages, the tick attaches to and feeds on a new warm-blooded host, (which may be of the same or a different species) until it becomes completely engorged, then drops off and undergoes further development on the ground (Concidine, 1985). On abandoning the host the fully engorged adult female lays 2000-3000 eggs and dies. The male tick does not attach to the host or suck blood, but may be found wandering on the host seeking a mate (Cooper *et al.,* 1976).

Because of the mechanical processes and salivary secretions associated with blood feeding, the tick-host parasitic interaction is complex. Tick salivary glands are the largest organs in the ticks body (Grattan-Smith, 1997). The salivary secretions play a major role in animal disease because of the introduction of toxic compounds that may affect general metabolism or cause flaccid paralysis. Female *I. holocyclus* ticks have relatively long mouthparts that can be thrust deeply into the skin so that the hypostome, armed with backward projecting spines, anchors the feeding tick and causes injury to the surrounding tissues (Cupp, 1991) (Figure 1.9). The male is smaller than the female with shorter mouth parts that are incapable of piercing the skin of mammals. It feeds off the female and not directly from the host (Beckman, 1989).

Unlike other tick species, *I. holocyclus* does not produce a cement which fixes the mouth parts into the skin. The mouthparts are instead inserted directly into the

Figure 1.9: An electron micrograph showing the mouthparts of *Ixodes holocyclus*

The hypostome (**H**) penetrates and is anchored by the teeth to the host's skin during attachment. The palps (**P**) protect the hypostome from being damaged before attachment. |___| = 100uM tissues. In contrast, the North American wood tick, *D. andersoni,* which affects domestic pets and cattle, feeds more superficially and secretes an external cement to secure shallow penetration of the hypostome. This method of feeding has been postulated to result in a much less severe paralysis in contrast to that of *I. holocyclus* and quicker recovery period once the tick has been removed due to the absence of circulating or bound toxins (Stone *et al.,* 1989).

Feeding of the adult tick occurs in two phases. The slow phase takes one to four days during which the body parts of the tick develop to allow for the rapid increase in size of the tick in the final stage of rapid engorgement. This second stage usually lasts approximately 24 hours and is characterised by a 10-fold increase in weight of the tick. This prolonged period of feeding is in striking contrast to other blood-feeding ectoparasites such as mosquitoes (less than 3 minutes); kissing bugs (less than 20 minutes); vampire bats (less than 30 minutes) and leeches (less than 2 hours) (Bowman *et al.*, 1996). This observed volume of engorged ticks underestimates the total volume of blood taken during feeding of *Ixodid* ticks. The meal is concentrated by removal of fluid which is then taken up by the salivary glands and secreted back into the hosts circulation, thus concentrating the nutrient components of the meal (Cupp, 1991; Beckman, 1989). Components of tick saliva suppress the immune and inflammatory response of the host permitting the ticks to remain on the host for an extended period of time (Bowman *et al.*, 1997). The trauma to the host coupled with the introduction of various salivary products may evoke a wide range of responses including local inflammation, oedema and haemorrhage. The destruction of host tissues which develops as a result of tick feeding is due to the presence of active compounds in the salivary secretions. Several of these components have been partially characterised but their function(s) still require further investigation (Bowman *et al.*, 1987).

Toxicoses caused by *I. holocyclus* are characterised initially by a weakness of the hind limbs followed by a rapidly ascending flaccid paralysis. The paresis then increases and extends to the forelimbs and respiratory muscles (Albiston, 1965). Other symptoms include loss of appetite and voice, uncoordination, excessive vomiting, respiratory distress and often death in the absence of timely antitoxin treatment (Stone *et al.,* 1989). The adult female tick does not secrete detectable amounts of toxin until the third day of attachment to the host. This is in contrast to the tick *D. andersoni* where there is a latent period of about 5 days before sufficient toxin can be produced to cause paralysis. The paralysis caused by *D. andersoni* can be avoided if the tick is removed during this latent period (Stone *et al.,* 1979). The onset of paralysis symptoms is usually not seen before the fourth or the beginning of the fifth day for *I. holocyclus*. This period corresponds to the onset of a final rapid stage of engorgement of the tick and has been shown to be accompanied by a marked increase in the size and activity of the salivary glands of the parasite (Stone and Binnington, 1986).

Prevention and treatment of tick paralysis.

Tick paralysis can be prevented by avoidance of tick infested areas, daily searches for attached ticks and application of acaricidal chemicals. Attached ticks need to be removed carefully and there is much debate over the best method of removal and whether the tick should be killed before removal. The problem revolves around the observation that paralysis and allergic reactions can progress following the removal of a tick, presumably due to the secretion or physical injection of more toxin during the removal process (Stone *et al.*, 1989).

Acaricidal preparations can be administered to animals either orally or directly onto the skin (sprays and collars). Cythioate (Proban, Boehringer-Ingelheim) is an orally administered organophosphate that is absorbed from the gut into the bloodstream where it is ingested by the ticks during engorgement causing death of the tick. Fipronil (Frontline, Rhone-Merieux) used as a spray and rinse is reported to kill ticks *in situ* within 24 hours (Searle *et al.*, 1995). Other organophosphates (coumaphos) and synthetic pyrethrins (permethrin) have been used topically and the chemicals carbamate propoxur and amitraz have been employed in impregnated collars (Cupp, 1991).

These acaricides have a number of deficiencies. Firstly, there is the need for regular application and administration of the acaricides. Secondly, the ticks are able to attach to areas (such as the lips, ear canal and anus) which are not covered by topical applications. The oral treatment has the added disadvantage of not being recommended for use in cats and some veterinarians report it as not being completely effective [www.ozemail.com.au/~norbertf/]. Finally there is the concern of using acaricides in young, aged or pregnant animals, and the concern over the presence of acaricidal residues in the flesh of food-producing animals (Albiston, 1965).

Treatment of tick paralysis involves removal or killing of the tick(s) followed by anti-toxin antiserum administration and veterinary support, such as the administration of adrenaline to treat anaphylaxis and vasodilators such as acepromazine to aid respiratory distress (Ilkiw and Turner, 1988). Removal of the offending tick(s) may be sufficient during early infestation (1-2 days) and after mild paralysis has occurred, with no further treatment being required (Albiston, 1965). The ability of dogs to become immune to *I. holocyclus* toxin following gradual or controlled exposure to tick infestation was recognised early (Ross, 1926; Ross, 1935) and hyperimmune dog serum produced for the treatment of tick paralysis (Oxer and Ricardo, 1942) has been marketed ever since. There has been no report of such immunity with toxins from *D. andersoni*, and the hyperimmune serum from *I. holocyclus* does not appear to relieve *D. andersoni* paralysis (Stone *et al.*, 1979).

There are several disadvantages to the current antiserum treatment. It is only effective in the early stages of the disease (Albiston, 1965) while the toxin is thought to be still either circulating or in the tick lesion, that is, before it has bound to its site of action at the neuromuscular junction. Moreover, it is unreliable when a number of ticks are involved (Concidine, 1985). The cost of treatment is high and in many cases the cost of antiserum exceeds the commercial value of farm animals. The efficacy of hyperimmune serum varies between manufacturers and between batches, so precise doses are difficult to recommend. Finally, a large number of animals develop adverse reactions such as anaphylaxis or serum sickness associated with the use of large doses of foreign serum proteins (Stone *et al.*, 1989). The veterinary aspects of tick paralysis are discussed in detail on two internet sites; www. ozemail. com. au/~norbertf/treatment-dogs. htm and www. peg apc. org/~ullavet/tick. html.

Veterinary and agricultural authorities acknowledge that the Australian paralysis tick is a significant commercial problem and is one of the more important causes of morbidity and mortality in companion and domestic animals in eastern Australia. There is general agreement that the development of an anti-tick vaccine would be the most effective alternative to current anti-serum therapy and chemical control because of the advantages of target-species specificity, environmental safety, lack of human health risks, ease of administration and cost. The observation that the *I. holocyclus* toxin secreted during infestation induces a protective antibody response suggests that a vaccination approach is practical. However, despite the efforts of a number of research units over the past thirty years, the tick neurotoxin has until recently defied complete isolation and characterisation.

Biochemical Characterisation of Tick Neurotoxins

The biochemical characterisation of tick paralysis neurotoxins has only been reported for 3 species: *I. holocyclus, R. e. evertsi and A . P. walkerae.* Clinically, these toxins share the common feature of inducing ascending flaccid paralysis. However they are pharmacologically diverse. The neurotoxins from *R. e. evertsi* and *A .P. walkerae* impair the conduction of nerve impulses along peripheral nerve fibres (Gothe and Kunze, 1981; Gothe *et al.*, 1979), whereas preliminary evidence suggests that paralysis induced by *I. holocyclus* results in the inhibition of acetylcholine release from the neuromuscular junction (Cooper and Spence, 1976). The isolation and characterisation of the paralysing toxin for the Australian tick *I. holocyclus* has been studied in depth.

Ross (1926; 1935) demonstrated that mice and dogs injected with extracts of salivary glands from *I. holocyclus* produced characteristic symptoms of tick paralysis. Since then salivary glands have been used almost exclusively as the source of starting material whenever the toxin has been investigated. Stone and co-workers (1983) verified that the neurotoxin was secreted into the host with the saliva in a study where *I. holocyclus* females which had been partially engorged (4 days) on mice were then attached to an artificial membrane and allowed to feed on a culture medium supplemented with foetal bovine serum. The feeding medium was collected, concentrated and shown to produce characteristic paralysis in mice identical to that observed with salivary gland extracts.

Crude salivary gland extracts obtained from female *R .e. evertsi* ticks in the toxic phase cause no overt symptoms when the salivary glands were introduced into mice, chickens or sheep. They did, however, develop typical paralysis when challenged by tick infestation (Viljoen *et al.*, 1986). Similarly, salivary glands from *D. andersoni* female ticks at a similar or later stage of feeding did not cause paralysis when injected into susceptible animals (Stone *et al.*, 1979). Possible explanations for these differing observations between ticks have been proposed. Firstly, the neurotoxin from *I. holocyclus* may concentrate particularly during the rapid feeding phase during the 5th or 6th days (Murnaghan and O'Rourke, 1978), and secondly that there may be continued synthesis of the neurotoxin for *I. holocyclus* in the salivary glands independent of feeding following their removal after 3-5 days of engorgement. Neither of these speculations has been supported by experimental evidence.

A toxic fraction of *I. holocyclus* was first isolated by Kaire (1966). This fraction produced paralysis in dogs consistent with tick toxicosis. In this study homogenates of engorged female *I. holocyclus* ticks were fractionated by chromatography on DEAE-cellulose columns. A toxic fraction with an LD_{50} Of 30-40 mg/kg in dogs was eluted. This fraction represented 2-3% of the total protein applied to the column. A marked immunological similarity to native neurotoxin was demonstrated as both mice and dogs passively immunised with hyperimmune tick serum were protected against the toxic fraction and dogs acquired immunity to the toxic fraction upon immunisation. The toxic component of this fraction was also shown to be resistant to digestion with proteases (pepsin, trypsin and papain). In contrast, the digestion of the neurotoxin from *R. e. evertsi* with pronase did result in a loss of toxicity (Viljoen *et al*, 1986). The *I. holocyclus* fraction was also resistant to heating at 75°C for 15 minutes (detoxification occurred at 100°C), and moderate pH (3-9) changes. Similar fractionation of extracts from the American tick *D. andersoni* failed to produce any characteristic symptoms in susceptible animals (Gregson, 1973).

Numerous attempts have been made to isolate the neurotoxin from *I. holocyclus* (Kaire, 1966; Stone, 1979; Stone *et al.,* 1979; Stone *et al.*, 1983; Stone, 1987; Stone and Aylward, 1987; Stone, 1988; Davey *et al.,* 1989; Thurn and Broady, 1992). Stone and Aylward (1987) claimed to have successfully purified the paralysis toxin and to have determined "an accurate molecular weight and amino acid sequence", although this data was not published. The studies from the above listed publications all concluded that the neurotoxic activity was associated with a high molecular weight (40-80 kDa) protein fraction isolated from whole engorged tick extracts or salivary gland extracts. Similar results were reported in studies of other ticks such as *R. e. evertsi*, and *A. (P.) walkerae* which also induce an ascending flaccid paralysis. However, the toxins may function by different mechanisms.because the *R. e .evertsi* and *A. (P.) walkerae* neurotoxins impair the conduction of nerve impulses along peripheral nerve fibres (Gothe *et al.*, 1979; Gothe *et al.*, 1982). In contrast, preliminary evidence suggests that paralysis induced by *I. holocyclus* results from the inhibition of acetylcholine release from the neuromuscular junction (Cooper and Spence, 1976).

The neurotoxin isolated from the salivary glands of the female spring lamb paralysis tick, *R. e. evertsi*, was the first neurotoxin to be isolated in a pure form as determined by gel filtration analysis. This neurotoxin has a high molecular weight of 68 kDa with an isoelectric point of 6 (Viljoen *et al.*, 1986; Crause *et al.,* 1994). A non-homogeneous protein toxin inducing paralysis has been isolated from the tick *A. (P.) walkerae* (Albiston, 1965). The toxin has a molecular weight of approximately

60 kDa. The amino acid sequences for both these tick toxins are yet to be determined. More recently Crause and co-workers (1994) have reported the production of a monoclonal antibody that is able to recognise and neutralise the paralysing toxin of *R. e. evertsi*, and cross-reacts with the paralysis toxin present in *A. (P.) walkerae*.

Partially purified toxic preparations from *I. holocyclus* when treated with glutaraldehyde were shown to produce a superior antitoxin response in both rabbits and dogs (Stone *et al.,* 1986). The glutaraldehyde treatment enabled hyperimmunity to be established quickly and larger doses could be injected as the neurotoxin was no longer active. This research, while demonstrating that a vaccine could be developed from the native toxin, also indicated the impracticality of using a native source of immunogen for commercialisation due to the limited availability.

Most reports on the physiology and pharmacology of tick paralysis have been based on the tick *D. andersoni.* In this species the neurotoxin activity appears to be due to a disturbance in conductance of nerve fibres and from failure to release but not to synthesise acetylcholine. In contrast the pharmacological investigations of tick paralysis induced by *I. holocyclus* have largely been reported in one study (Cooper and Spence, 1976). This work first reported that the neurotoxin had a peripheral mode of action, acting either at the neuromuscular junction or in the muscle itself. Further experimentation by this group determined the site of action of the neurotoxin. The initial studies showed that muscle contraction was normal at room temperature (between 20-23°C). However, a decline in contraction was seen as the temperature was progressively raised to 37°C at which point there was virtually no response to nerve stimulation. Electophysiological studies confirmed the above responses. These analysis showed that the neurotoxin acted presynaptically by inhibiting the evoked release of acetylcholine in a temperature dependent manner (and that the effect is more pronounced at higher temperatures) at the neuromuscular junction. The reduction in acetylcholine release in tick paralysis is probably a result of the toxin blocking the influx of calcium ions which seems to be essential before evoked release can occur (Cooper and Spence, 1976).

Recent studies (Thurn *et al.,* 1992) described the isolation of three neurotoxins from *I. holocyclus* which bind to rat brain synaptosomes (pinched off nerve terminals) in a temperature-dependent manner. The neurotoxins (holocyclotoxins; HT-1, HT-2 and HT-3) are polypeptides with apparent molecular weights of 5 kDa. The specificity of the toxin was demonstrated through the binding of the 5 kDa polypeptide to synaptosomes. This binding was prevented by addition of excess unlabelled *Holocyclus* neurotoxin preparation and canine tick antivenom. The observed temperature dependence of the neurotoxins binding to synaptosomes coincided with the *in vivo* observation that paralysis is inhibited at low temperatures (Cooper and Spence, 1976).

A result incompatible with the literature was the apparent molecular weight of the native neurotoxin (Stone and Aylward, 1987). In the the study by Thurn *et al.*, (1992), an ionic detergent (SDS) was incorporated into the gel filtration buffer to prevent non-specific interactions and self association. The tight association of the toxin with other protein molecules, such as host serum albumin, has defied purification in the past. As a result of the addition of SDS a molecular weight shift from 40-60 kDa to below 10 kDa was observed. This molecular weight is comparable with arachnid neurotoxins previously isolated from spiders in the range 3-11 kDa, (Kawai, 1991) and scorpions of 5-8 kDa (Rochat *et al.*, 1979).

The neurotoxins were subsequently purified from extracts of engorged adult female ticks by a series of high resolution chromatography techniques. Earlier attempts to purify the neurotoxin were too crude to provide substantial purification. The techniques used in the above study were more advanced and allowed purification of small amounts of protein. The purity of each preparation of *Holocyclus* neurotoxin was confirmed by the presence of a single band after tricine-SDS-PAGE. Under reducing conditions the neurotoxins all migrated at an almost identical molecular weight of approximately 5 kDa. Additional confirmation of the purity of HT-1 and HT-2 was provided by mass spectrographic analysis resulting in single mass peaks (Thurn, 1994).

Total amino acid analysis of the three neurotoxins revealed that they were very similar in amino acid content and that each had a total of approximately 42 amino acids. This number is only an approximation as total amino acid analysis was carried out on low nanogram amounts of toxin and theses analyses do not detect cysteine and tryptophan residues. The structural relatedness of the neurotoxins was confirmed by Western blotting which indicated that all three neurotoxins share a common epitope. Amino acid sequence of the neurotoxins was restricted to peptides generated by proteolytic digestion with trypsin as direct N-terminal sequencing revealed a blockage (Thurn, 1994). A putative partial sequence for HT-1 was deduced from tryptic peptide digests. The sequence consisted of two peptides, 28 amino acids in length, thus representing approximately two thirds of the molecule, as there is an undefined number of residues at the N-terminally blocked end. An interesting feature of the sequence is that there is a C-X-X-X-C motif (Figure 1.10) present in the peptides, which is common to many related toxins such as those of the spider, scorpion and apamin from bees.

Results from the above study have provided the basis for further molecular, biochemical and physiological studies into the characterisation of the neurotoxins for *Ixodes holocyclus*. The shared structural characteristics and immunological similarity identified for the neurotoxins provides the basic evidence for producing a veterinary vaccine that will be capable of protecting an animal against the three neurotoxins.

1.6 AIMS OF THIS THESIS:

The work presented in this thesis satisfied the following objectives.

1. To isolate the gene encoding the HT-1 neurotoxin from *Ixodes holocyclus.*
2. To establish a bacterial expression system in order to recombinantly produce large quantities of mature *I. holocyclus* neurotoxin HT-1 as a fusion protein.
3. To test the HT-1 fusion protein for antigenicity and toxicity in *in vitro* and *in vivo* assays.
4. To assess the activity of the HT-1 fusion protein as an antigen in clinical trials for potential vaccine development against *I. holocyclus* envenomation.

N-terminal Xn -CNAECSTHCDDAGGP-Xn- KCTYQLKGGSYCK-Xn

PEPTIDE A PEPTIDE B

Figure 1.10: Partial putatative HT-1 neurotoxin sequence.

Peptides A and B (underlined) represent individual peptides derived from Edman sequencing. Xn refers to an unknown number of amino acid residues at the blocked N-terminus.

CHAPTER 2

ISOLATION OF THE cDNA SEQUENCE CODING FOR THE HT-1 NEUROTOXIN OF *IXODES HOLOCYCLUS*

2.1: PREVIEW

In recent years there has been significant progress in defining the biochemical characteristics of the tick neurotoxin(s) from *Ixodes holocyclus*. Part of this work involved obtaining partial amino acid sequence data as well as 3' cDNA sequence data for the HT-1 neurotoxin (Thurn, 1994, Masina, 1995). The initial aim of this thesis was to isolate the complete cDNA sequence for the HT-1 neurotoxin of *Ixodes holocyclus* .

This chapter gives a detailed description of the methodology used to derive the first reported tick neurotoxin sequence and of the significance of this discovery with respect to tick vaccine development.

2.2: INTRODUCTION

The Australian paralysis tick *Ixodes holocyclus* is thought to contain three neurotoxins HT-1, HT-2 and HT-3. Previously, only partial peptide sequence data has been obtained for the HT-1 neurotoxin (Thurn, 1994). Due to the minute quantities of the neurotoxins available, the peptide and mass spectrometric data available for HT-1 could not be repeated, resulting in some initial doubt concerning the accuracy of the data.

To confirm existing peptide sequence data for HT-1 and to obtain the complete cDNA sequence for the gene encoding this neurotoxin, molecular biology techniques were employed. The initial degenerate oligonucleotide primers for PCR were designed based on the available amino acid data. This resulted in a partial cDNA sequence from which specific primers were designed for use in 3' and 5' rapid amplification of cDNA ends (RACE)-PCR (Masina, 1995). The RACE-PCR method was used as it allows for cloning of cDNA for which limited sequence data is available (Frohman *et al.*, 1988). The procedure of RACE is described in more detail in Section 2.3.

This chapter describes the elucidation of both 5' and 3' RACE products for HT-1 and the confirmation of these sequences by the discovery of the full length cDNA for the tick neurotoxin HT-1. The results presented highlight the importance of this discovery for further research with the aim of using the HT-1 sequence as a basis for the production of a recombinant vaccine against tick paralysis. This chapter also outlines the homologous regions that the HT-1 sequence shares with known arachnid neurotoxin sequences such as those from scorpions and spiders, and describes those features of HT-1 which make it a unique tick neurotoxin sequence.

2.3: MATERIALS AND METHODS

Oligonucleotide Primers

The oligonucleotides used as primers for PCR were synthesised by Beckman (Brea, CA, USA) at a 40 nmole scale and purified under HPLC grade purification conditions.

The primers S1 and S2 were used as described (Masina, 1995) to amplify 3' and 5' RACE products for HT-1 respectively.

FORWARD PRIMER S1

S1 5' CG GTC CAA TGC AAA AAA TGC AC 3'

S1 = 22 bp sense primer, designed to extend toward the 3' end of the cDNA.

REVERSE PRIMER S2

S2 3' C CAG GTT ACG TTT TTT ACG TGT 5'

S2 = 22 bp antisense primer, designed to extend toward the 5' end of the cDNA

FORWARD PRIMER S3

The primer S3 was designed based on sequence obtained from 5' RACE PCR (Figure 2.5). The S3 primer (21 bp) was designed to prime from the 5' untranslated region of the cDNA toward the 3' end.

S3 5' GCT ATA GCA CGG GTA GCT TAG 3'

Materials

The following reagents were used in this study: *Taq* polymerase, (Perkin-Elmer Corporation, Norwalk CT, USA); PolyATract^R mRNA isolation kit, cDNA synthesis kit, pGEM^R- T vector cloning kit, (Promega Corporation, Madison, WI, USA); Marathon cDNA amplification kit (CLONTECH Laboratories Inc. Palo Alto, CA, USA); SequiTherm cycle sequencing kit (Li-Cor, Lincoln, NB, USA). Other chemical reagents were of analytical reagent grade. Water was obtained from a Modulab analytical laboratory research grade water system, and was sterilised by autoclaving. Water is referred to as MilliQ water.

Methods

Standard recombinant DNA techniques (DNA purification, phenol:chloroform extraction, ethanol precipitation, electrophoresis and alkaline lysis) were carried out as described by Sambrook *et al.* (1989). Digestion of DNA using restriction endonucleases was performed according to the manufacturers instructions (New England Biolabs, Inc., MA, USA). Ligation reactions were performed using the pGEM^R-T vector cloning kit according to the manufacturers specifications.

Polyacrylamide gel electrophoresis (PAGE)

PAGE was used to separate and purify DNA up to 1 kb in size. DNA samples were diluted with a 6x DNA sample buffer (2 µL (0.5M) EDTA, 2 mL sterile glycerol, 6 mL MilliQ water, bromophenol blue to colour) and loaded onto a 10% vertical polyacrylamide gel. Electrophoresis was carried out in a Mini-Protean II (Bio-Rad laboratories, Hercules, CA, USA) apparatus in 1x TBE (45 mM Tris, 1 mM EDTA pH 8.0) running buffer at 90 V for 10 minutes, then at a constant voltage of 150 V. Electrophoresis was terminated when the bromophenol blue tracking dye reached the bottom of the gel. DNA was visualised by staining with 0.5 µg/mL of ethidium bromide in 1x TBE.

5' and 3' RACE

The 5' and 3' terminal sequences of the HT-1 gene were identified using the Marathon cDNA Amplification kit. An overview of Marathon cDNA amplification protocol is shown in Appendix 1. Gene-specific primers based on known sequence information were used for 5’ and 3’ RACE reactions.

First strand cDNA synthesis: First strand cDNA was synthesised from 1µg of engorged tick mRNA by combining 1 µg (1-4 µL) RNA with 1 µL cDNA synthesis primer (10 µM) and sterile MilliQ water to a final volume of 5 µL. The contents were mixed, centrifuged briefly and incubated at 70°C for 2 minutes and placed directly on ice. The following were then added to each tube: 2µL 5x first strand buffer, 1 µL dNTP mix (10 mM), 1 µL reverse transcriptase (200 U/µL) and sterile MilliQ water to a final volume of 10 µL. The contents of the tube were mixed by gentle pipetting, centrifuged briefly and incubated at 42°C for 1 hour. The tube was then placed on ice to terminate first strand cDNA synthesis.

Second strand cDNA synthesis: The following were combined in a 0.5 mL Eppendorf tube: 10 µL of first strand reaction volume, 48.4 µL MilliQ water, 16 µL 5x second strand buffer, 1.6 µL dNTP mix (10 mM) and 4 µL 20x second strand enzyme cocktail (RNase H, *E.coli* DNA polymerase I, and *E.coli* DNA ligase), to a total volume of 80 µL. The contents were mixed by pipetting, briefly centrifuged at 13000 *g* and then incubated at 16°C for 2 hours. Following incubation, 1 µL (5U) of T4 DNA polymerase mix was added and the mixture incubated at 16°C for 30 minutes (to create blunt ends on the ds cDNA). The reaction was terminated by the addition of 4 µL of 0.2 M EDTA. The ds cDNA was then purified by ethanol precipitation. The precipitated DNA was resuspended in 10 µL of sterile MilliQ water and stored at -20°C.

Adaptor Ligation: The following reagents were combined in a 0.5 mL Eppendorf tube: 5 µL double stranded cDNA, 2 µL Marathon cDNA adaptor (10 µM), 2 µL 5x DNA ligation buffer, 1 µL T4 DNA ligase (1U/µL) to a final volume of 10 µL. The contents were mixed by vortexing, spun briefly in a microcentrifuge and then incubated overnight at 16°C. The sample was then heated to 70°C for 5 minutes to inactivate the ligase. A 1/50 dilution of template was used in subsequent PCR amplifications. Undiluted template was stored at -20°C .

5' and 3' RACE PCR: 5 µL of ds cDNA template was used per reaction, together with 10 pmol of adaptor primer 1 (AP1) and 10 pmol of either 5’ or 3’ primers specific for the toxin gene. Each amplification reaction contained a final concentration of: 1x PCR buffer, $MgCl_2$ (0.25 mM), dNTPs (0.2 mM) and 10 pmol of each primer (forward and reverse). The volume was adjusted to 100 µL with sterile MilliQ water.

The following PCR program was used for the amplification using a Hybaid thermocycler: One cycle at 94°C for 1 minute and 50°C for 5 minutes (addition of 2.5 U of *Taq* polymerase), followed by 25 cycles at 94°C for 30 seconds; 55°C for 30 seconds and 68°C for 2 minutes. On completion of thermocycling 5' and 3' RACE products were electrophoresed through a 10% polyacrylamide gel.

Cloning of amplified DNA

Amplified PCR products were electrophoresed through a 10% polyacrylamide gel with 20% of the total reaction volume together with 5 µL of 6x sample buffer loaded into each well. Discrete bands were excised under low wavelength U.V. illumination and pooled in an Eppendorf tube. The gel was cut into small pieces using a sterile scalpel blade and DNA eluted in TE buffer or MilliQ water, at twice the volume of the gel pieces. The DNA was allowed to elute into the buffer overnight by placing it on an orbital mixer at 4°C. The eluted DNA was ethanol precipitated and resuspended in 20 µL of MilliQ. An aliquot of the purified DNA was electrophoresed through a 10% polyacrylamide gel to confirm the presence of eluted DNA.

The purified PCR fragments were cloned into the pGEM^R-T vector according to the manufacturers recommendations. An aliquot of the ligation reaction was transformed into DH5α cells by electroporation as described below. Putative recombinant clones were inoculated into LB broth containing 50 µg/mL ampicillin and plasmid DNA was prepared from the overnight cultures by alkaline lysis. True recombinants were selected by restriction enzyme analysis and were then further purified by an additional phenol:chloroform and ethanol precipitation prior to dideoxy sequencing. Clones were sequenced using the chain termination method of Sanger *et al.* (1977) using reagents and protocols provided by the SequiTherm cycle sequencing kit , with an automated model 4000L Li-Cor DNA sequencer.

Electroporation

Electro-competent *Escherichia coli* (*E.coli*) DH5α bacterial cells were prepared as outlined in the Gene Pulser^R manufacturers instructions (Bio-Rad). Transformation of DNA into *E.coli* was performed by electroporation using a Gene Pulser^R Apparatus (Bio-Rad). DNA to be transformed (typically 1-5 µL of ligation mix) was added to a 50 µL aliquot of thawed DH5α cells and the mix transferred to a cold 0.1 cm gap electroporation cuvette (Bio-Rad). The electroporator parameters were set at 1.8 kV, 200 Ohms, and 25 µF. Following the electroporation pulse, cells were resuspended in 1 mL of 2xYT medium, incubated at 37°C for one hour with agitation, and then plated onto LB ampicillin (50 µg/mL) agar plates pre-spread with 40 µL of 100 mM IPTG and 40 µL of 20 mg/mL X-gal in dimethyl formamide, and then incubated overnight at 37°C.

Computer Analysis

DNA and protein sequence analysis was performed using basic local alignment search tool programs, BLAST and FASTA in the databases SWISS-PROT, GenBank, and SCOP.

2.4: RESULTS

3' RACE Product

A DNA fragment of 300 bp (Figure 2.2, lane 3) was obtained with 3' RACE primers S1 and AP1. Features of this sequence (Figure 2.3) included amino acids corresponding to a partial open reading frame (in single letter code). Excluding the additional (Q/ glutamine) amino acid at the 3' end, there are 3 remaining residues not underlined which differ to the putative peptide sequence data (Figure 2.1) (Thurn 1994). The sequence also contained two stop codons along with a 3' untranslated region. The putative polyadenylation signal was found to be located 16 bp upstream from the poly(A) tail.

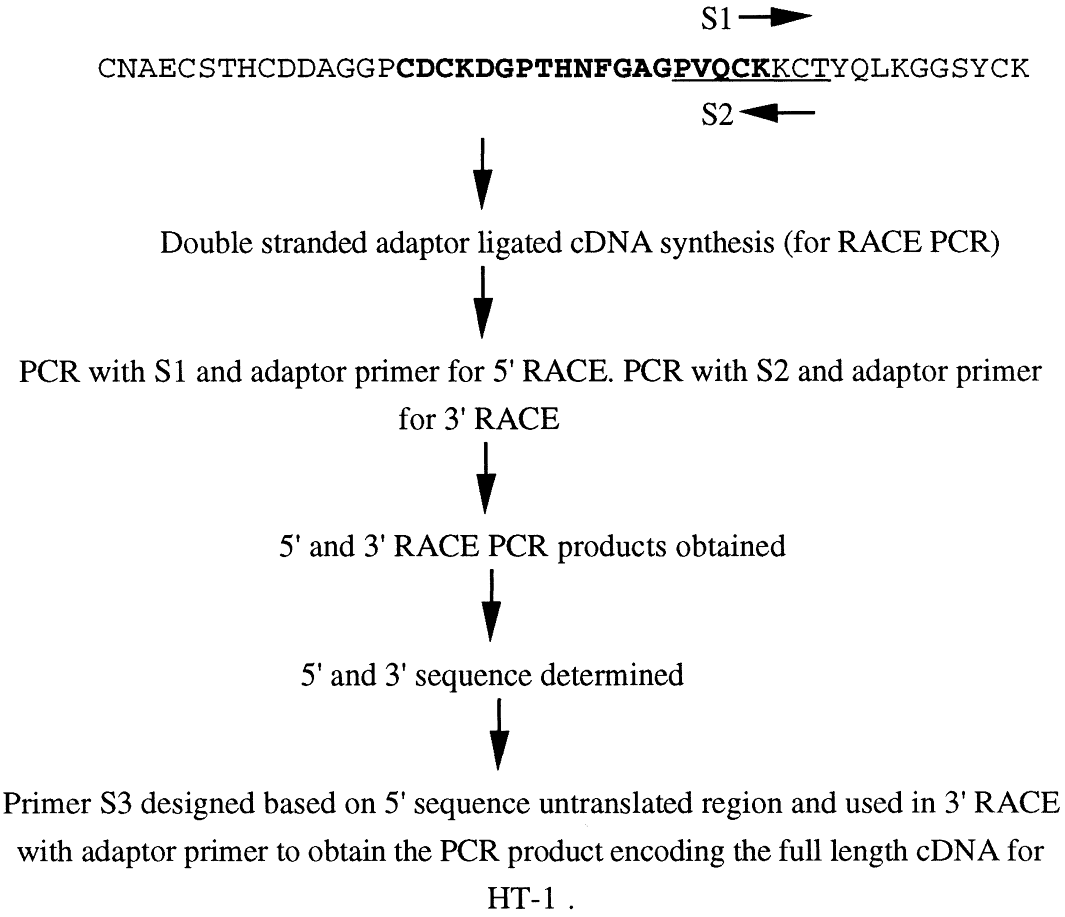
5' RACE Product

Using the 5' RACE method, a DNA fragment of 280 bp (Figure 2.2, lane 2) was obtained with primers S2 and AP1. Several clones of the 5' RACE product were sequenced and were found to be identical. The 5' RACE sequence is underlined in Figure 2.5. The S3 primer used to obtain the complete cDNA for HT-1 was designed based on the untranslated region of the 5' RACE product (Figure 2.5, in italics).

The complete cDNA sequence for HT-1

The complete cDNA sequence of HT-1 was amplified using the S3 primer in conjunction with the AP1 primer. The PCR product for the full length cDNA is shown in Figure 2.4 (lane 2). The PCR product was 450 bp in size. The positive control PCR using ubiquitin primers amplified a product representative of non-tick toxin genes (lane 3). The negative control (lane 4) showed no PCR product.

The PCR product obtained with S3 and adaptor primers was cloned and sequenced several times. Clones of this sequenced product (Figure 2.5), were found to encode a 456 bp cDNA with an open reading frame of 216 bp coding for 72 amino acids starting from the first in frame ATG start codon and ending with TGA and TAA stop codons. The sequence depicted in Figure 2.5 contains a classical signal peptide sequence as described by von Heijne (1986). The predicted signal sequence is 23 residues in length and has a rich hydrophobic region. The mature polypeptide



CNAECSTHCDDAGGP**CDCKDGPTHNFGAGPVQCK**KCTYQLKGGSYCK

Double stranded adaptor ligated cDNA synthesis (for RACE PCR)

PCR with S1 and adaptor primer for 5' RACE. PCR with S2 and adaptor primer for 3' RACE

5' and 3' RACE PCR products obtained

5' and 3' sequence determined

Primer S3 designed based on 5' sequence untranslated region and used in 3' RACE with adaptor primer to obtain the PCR product encoding the full length cDNA for HT-1 .

Figure 2.1: Schematic representation of the protocol used to obtain the cDNA sequence encoding the complete gene for HT-1.

Original peptide information obtained by Thurn (1994) is in normal type. Translated protein sequence obtained by Masina (1995) using PCR with degenerate primers, includes the sequence in normal type and new sequence information in bold type. Non-degenerate primers S1 and S2 were designed for use in 3' and 5' RACE.

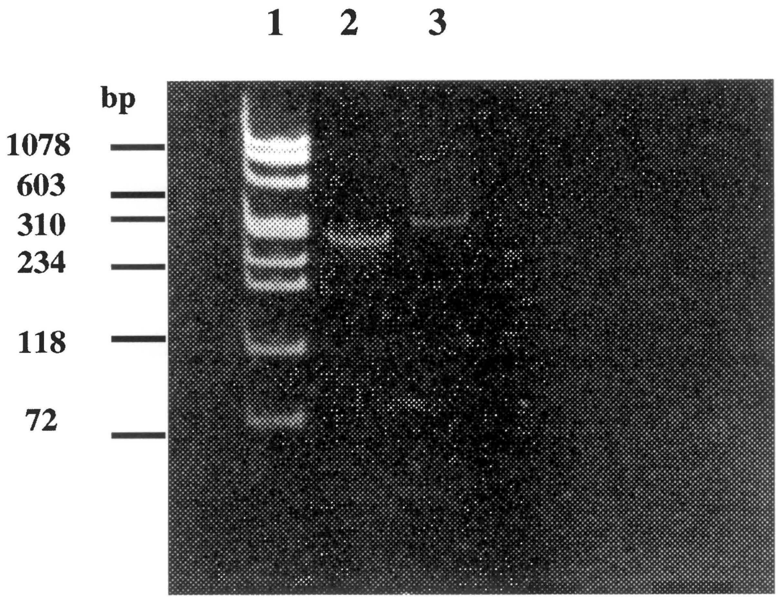


Figure 2.2: The 3' and 5' RACE products of HT-1.

PCR products (10 μL) were electrophoresed on a 10% polyacrylamide gel and visualised by EtBr staining under U.V.

Lane 1: PhiX174/Hae III standards (500 ng, Promega Corporation, Madison, USA)

Lane 2: 5' RACE product, 280 bp. Amplified with S2 and AP1 primers.

Lane 3: 3' RACE product, 300 bp. Amplified with S1 and AP1 primers.

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5′ GTC CAA TGC AAA AAA TGC ACA TAT CAA TTC AAA GGT GAA
    V   Q   C   K   K   C   T   Y   Q   F   K   G   E

GCT TAC TGT AAA CAA TGA TAA CAGTTCCTGCGGAATACGGGAGCAGTCA
 A   Y   C   K   Q   *   *

CCAGAAGAGAGACTAAATGGGGAGGAATATTTCAAGGCACCAAACAGTGTGCGGGT

CATGGGTTTCGATAAAAAGGGACAAAATGGGTAATAAAAATAAGTGTACCAATTAA
(A)n
```

Figure 2.3: 3' RACE sequence (with S1 and AP1 primers).

The 3' RACE sequence was deduced following amplification with S1 and AP1 primers. The residues forming part of the open reading frame have been translated and are represented in single letter code. Amino acids identical to those previously obtained by peptide sequencing (Figure 2.1) are underlined. **Q** = glutamine, and is in the only additional residue found in the 3' open reading frame when compared to original peptide data (Figure 2.1). Stop codons (TGA , TAA) are indicated by (*), followed by the 3' untranslated region including putative polyadenylation signal (underlined) and polyA tail (A_n).

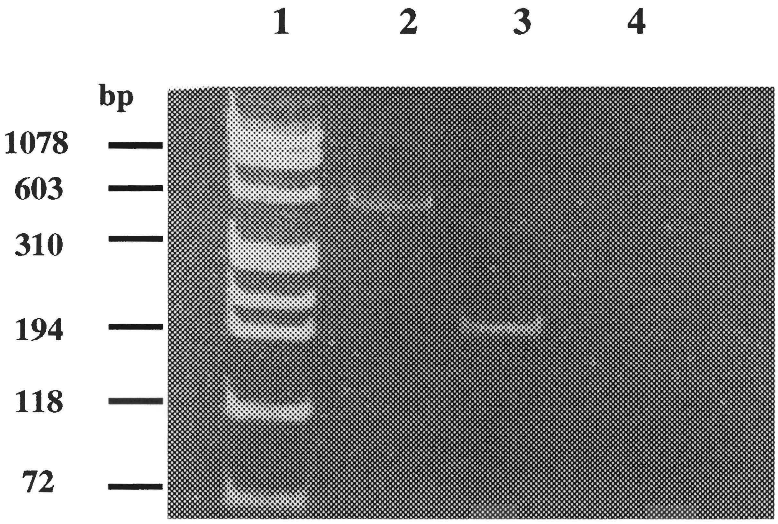

Figure 2.4: PCR product of complete HT-1 cDNA.

PCR products (10 μL) were electrophoresed through a 10% polyacrylamide gel and visualised by EtBr staining under U.V. illumination.

Lane 1: PhiX174/Hae III standards (500 ng, Promega Corporation, Madison, USA)

Lane 2: Complete HT-1 cDNA product of approximately 450 bp amplified with S3 and AP1 primers.

Lane 3: Positive control amplification using ubiquitin forward and reverse primers.

Lane 4: Negative control amplification with primers S3 and AP1 (no template DNA).

S3 Primer

ATTATATTT*GCTATAGCACGGGTAGCTTAG*CGTGATTTGTATAGCC ATG TCC

M S

AAA GTT ACG ACA GTC TTC ATC GGC GCA CTG GTC CTG CTC CTA

K V T T V F I G A L V L L L

CTC ATT GAG AAC GGT TTC TCA TGT ACC AAC CCT GGG AAA AAA

L I E N G F S **C T N P G K K**

AGG TGC AAC GCT AAG TGC TCA ACA CAC TGC GAC TGC AAG GAC

R C N A K C S T H C D C K D

GGA CCC ACT CAC AAT TTC GGG GCT GGG CCG GTC CAG TGC AAA

G P T H N F G A G P V Q C K

AAA TGC ACA TAT CAA TTC AAA GGT GAA GCT TAC TGT AAA CAA

K C T Y Q F K G E A Y C K Q

TGA TAA CAGTTCCTGCGGAATACGGGAGCAGTCACCAGAAGAGAGACTAAATGG

* *

GGAGGAATATTTCAAGGCACCAAACAGTGTGCGGGTCATGGGTTTCGATAAAAAGG

GACAAAATGGGTAATAAAAATAAGTGTACCAATT$(A)_n$

Figure 2.5: The complete HT-1 cDNA and translated protein sequence.

The complete cDNA sequence as derived from amplifications with S3 and adaptor primer, together with the extended 5' region from the original 5' RACE sequence is shown. Both nucleic acid sequence and translated amino acid sequences (in single letter code) are shown. The proposed mature toxin sequence is in bold. Stop codons are indicated by (*). The putative polyadenylation signal is underlined. The nucleotide sequence underlined corresponds to the entire sequence obtained for the 5' RACE product. Sequence corresponding to the S3 primer is in italics.

therefore was deduced to be 49 residues in length with a calculated molecular weight of 5386 Da, and a pI of 9.02. This amino acid sequence is characterised by the presence of 8 cysteine residues, suggesting the presence of 4 disulphide bonds. The full length cDNA confirms previous sequence data obtained for 3' and 5' RACE products. The translated HT-1 peptide sequence has a number of differences from the original putative peptide data shown in Figure 2.1. Figure 2.6 highlights the differences between the translated sequence illustrated in Figure 2.5 and that of the original peptide data as depicted in Figure 2.1. There were 4 substitutions of residues (K, F, E and A, underlined), and 7 residues which are absent (dashed line) in the translated HT-1 sequence obtained by RACE PCR. These differences will be further detailed in the discussion.

Homology analysis of HT-1

The HT-1 peptide and nucleic acid sequences were analysed through searches for homology in the databases SWISSPROT, GenBank and SCOP, using the FASTA and BLAST algorithims. No homology matches could be found when the HT-1 nucleotide sequence was the query sequence. The HT-1 peptide sequence however did give a significant match in the SCOP database using the BLAST algorithm. The similarity was with charybdotoxin, a short chain scorpion neurotoxin where in a stretch of 13 amino acids, 6 residues were identical (Figure 2.7).

Alignment analysis of HT-1

The HT-1 mature peptide sequence (i.e excluding the signal sequence) was subject to alignment analysis using the CLUSTALW programme. The first set of alignments were performed with the HT-1 peptide sequence and a range of known scorpion toxin sequences as depicted in Figure 2.8. The 17 scorpion sequences chosen are representative of long chain α or β-Na^+ channel scorpion neurotoxins, that act specifically on insects or mammals.

The programme GeneDoc (V2.2) was used to group conserved residues within the clustal aligned sequences. The major homology was with 5 cysteine residues which may have some implications relating to the tertiary structural folding of HT-1.

A comparison of the HT-1 mature peptide sequence with spider toxins and other members of the inhibitor cysteine motif family (Figure 1.7) indicates that the

(a) CNAECSTHCDDAGGPCDCKDGPTHNFGAGPVQCKKCTYQLKGGSYCK

(b)...CNAKCSTH-------CDCKDGPTHNFGAGPVQCKKCTYQFKGEAYCKQ...

Figure 2.6: Comparison of peptide sequence data obtained for HT-1.

Sequence (a) (from Masina, 1995), is as shown in Figure 2.1. The sequences underlined correspond to the original peptide information obtained by Thurn (1994). Sequence (b) represents part of the translated HT-1 cDNA sequence obtained with S3 and adaptor primer which corresponds to a region spanning the original peptide sequence information. Residues which differ between the sequences are underlined in sequence (b) and those residues which are absent are indicated by a dashed line (-).

HT-1: 48 HNFGAGPVQCKKC 60

ChTx: 21 HNTSRGKCMNKKC 33

Figure 2.7: Homology between charybdotoxin and HT-1.

A homology search using the SCOP database with the BLAST algorithm for the HT-1 peptide sequence produced a match with the scorpion neurotoxin charybdotoxin (ChTx) from *Leiurus quinquestriatus hebraeus*. The partial sequence for a stretch of 13 residues is shown, the 6 homologous residues are underlined.

cysteine residues of HT-1 essentially fit into the proposed cysteine pattern as described in section 1.4 for members belonging to this family. The proposed consensus sequence is $CX_{3-7}CX_{3-6}CX_{0-5}CX_{1-4}CX_{4-13}C$, for the first 6 cysteine residues of any member. The consensus for the first 6 cysteines of HT-1 is $CX_7CX_3CX_4CX_1C_{14}C$. This suggests that HT-1 may have some structural similarities to members of the inhibitor cysteine knot motif and may itself contain a cysteine knot motif (as depicted Figure 1.6).

Figure 2.8: Amino acid sequence alignment of HT-1 and representative long chain scorpion neurotoxins.

The amino acid sequences were aligned using the ClustalW programme with gaps introduced to maximise homology. Conserved residues are shaded. Scorpion toxins active on insects are indicated in parentheses. Abbreviations: AaH, *Androctonus australis* Hector; AaH I ; AaH II; AaH III; AaH IT1 (insect neurotoxin 1); AaH IT4 (insect neurotoxin 4). BmK, *Buthus martensii* Karch; BmK IT (insect neurotoxin); BmK M1. Lqh, *Leiurus quinquestriatus hebraeus*; Lqh IT2 (depressant insect neurotoxin). Lqq, *Leiurus quinquestriatus quinquestriatus*; Lqq III. Bj, *Buthotus judaicus*; Bj IT2 (insect neurotoxin). Cn, *Centruroides noxius*; Cn II. CsE, *C. sculpturatus* Ewing; Cs I. Css, *C. suffusus suffusus*; Css II. Bot, *Buthus occitanus tunetanus*; Bot I; Bot IT1. Ts, *Tityus serrulatus*; Ts IV ; Ts VII. References for each of these listed toxins can be found in the review by Gordon *et al.* (1998).

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                    10        20        30        40        50
CnII      --KEGYLBDKNTGCKYECLKLGDNDYCLRECKQQYGKGAGGYCY-----AFACWCTHLVE
CssII     --KEGYLVSKSTGCKYECLKLGDNDYCLRECKQQYGKSSGGYCY-----AFACWCTHLYE
CsI       --KDGYLVEK-TGCKKTCYKLGENDFCNRECKWKHIGGSYGYCY-----GFGCYCEGLPD
LqhIT2    ---DGYIRKR-DGCKLSCL-FG-NEGCNKECKSYG--GSYGYCWTW---GLACWCEGLPD
BjIT2     ---DGYIRKK-DGCKVSCI-IG-NEGCRKECVAHG--GSFGYCWTW---GLACWCENLPD
AaHIT1    -KKNGYAVDS-SGKAPECL---LSNYCNNECTKVH-YADKGYCC-----LLSCYCFGLND
BmKIT     -KKNGYAVDS-SGKVSECL---LNNYCNNICTKVY-YATSGYCC-----LLSCYCFGLDD
AaHI      -KRDGYIVYP-NNCVYHCVPP-----CDGLCKKNG--GSSGSCSFLVPSGLACWCKDLPD
AaHIII    -VRDGYIVDS-KNCVYHCVPP-----CDGLCKKNG--AKSGSCGFLIPSGLACWCVALPD
BmkM1     -VRDAYIAKP-HNCVYECARNE---YCNDLCTKNG--AKS■YCQWVGKYGNGCWCIELPD
BotI      -GRDAYIAQP-ENCVYECAQNS---■CNDLCTKNG--ATSGYCQWLGKYGNACWCKDLPD
LqqIII    -VRDAYIAKN-YNCVYECFRDS---YCNDLCTKNG--ASSGYCQWAGKYGNACWCYALPD
BotIT1    -VRDAYIAQN-YNCVYFCMKDD---YCNDLCTKNG--ASSGYCQWAGKYGNACWCYALPD
AaHII     -VKDGYIVDD-VNCTYFCGRNA---YCNEECTKLK--GESGYCQWASPYGNACYCYKLPD
TsIV      -KKDGYPVEY-DNCAYICWNYD-NAYCDKLCKDKK--ADSGYCYWVH---ILCYCYGLPD
TsVII     --KEGYLMDH-EGCKLSCFIRP-SGYCGRECGIKK--GSSGYCAWP-----ACYCYGLPN
AaHIT4    --EHGYLLNKYTGCKVWCVIN--NEECGYLCNKRR-GGYYGYCYFWK---LACYCQGARK
HT-1      CTNPGKKRCN-AKCSTHCD-------CKDGPTHNF--G-AGPVQ----------CKKCTY
              .            *        *          .  *             *

          60        70
CnII      QAIVWPLPNKR---CS-----
CssII     QAVVWPLPNKT---CN-----
CsI       STQTWPLPNK----CT-----
LqhIT2    -EKTWKSETNT---CG-----
BjIT2     -AVTWKSSTNT---■G-----
AaHIT1    DKKVLEISDTRKSYCDTTIIN
BmKIT     DKAVLKIKDATKSYCDVQIIG
AaHI      ---NVPIKDTS-RKCT-----
AaHIII    ---NVPIKDPS-YKCHS----
BmkM1     ---NVPIRVP--GKCHR----
BotI      ---NVPIRIP--GK■HF----
LqqIII    ---NVPIRVP--GKCH-----
BotIT1    ---NVPIRIP--GKCHS----
AaHII     ---HVRTKGP--GRCH-----
TsIV      ---SEPTKTN--GKCKS----
TsVII     WVKVWDRATN---KC------
AaHIT4    -SELWNYKTN---KCDL----
HT-1      ---QFKGEAY----CKQ----
                        *
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2.5: DISCUSSION

Determination of the cDNA sequence for HT-1

Ticks and tick paralysis have attracted the attention of many investigators over the years primarily because they constitute a hazard to the life and health of domestic and companion animals as well as humans. In Australia the tick *Ixodes holocyclus* is the leading cause of tick paralysis. The development of efficient and effective protection against the tick paralysis neurotoxin(s) would be of great veterinary and clinical significance.

This chapter described the isolation of the complete cDNA sequence encoding the HT-1 neurotoxin of *Ixodes holocyclus*. The RACE technique was chosen to determine the sequence. This technique allows very short amino acid sequences to be sufficient starting information for the PCR amplification of a cDNA encoding a full length protein. Therefore alternative classical methods for obtaining the complete cDNA, such as the construction of a cDNA library were not necessary, especially as they do not always yield the full length cDNA sequence (Frohman, 1988).

In this study, RACE was performed on cDNA prepared from mRNA isolated from total RNA obtained from engorged ticks. The primers S1 and S2 (Section 2.3) were used to amplify 5' and 3' RACE products, respectively. Several clones of the individual 5' and 3' RACE products were sequenced. However when the RACE sequences were translated they were not completely identical to the original peptide sequence information The complete cDNA sequence was obtained using the S3 and AP1 primers. The sequence of the individual RACE products were in agreement with the full length gene (Figure 2.5). Therefore, the differences observed between the DNA sequence analysis and that obtained by peptide sequencing (Thurn, 1994) are most likely to be real.

The differences in peptide sequence translated from DNA sequence and that determined by Edman sequencing for HT-1 are illustrated in Figure 2.6. Over the stretch of amino acids representing partial sequence for the whole gene {Figure 2.6 (b)}, there are 4 residues (K, F, E, A) which differ from those in the corresponding positions in Figure 2.6 (a) (E, L, G, S). The codons for these amino acid changes are different, so an error in interpreting DNA sequence data would be highly unlikely. Additionally there are 7 amino acids absent in sequence 2.6 (b) that are present in sequence 2.6 (a). One of these missing residues is a cysteine. The presence of an additional cysteine would mean there would be an odd number of cysteine residues for the entire sequence (from 8 to 9), which is rare in small cysteine rich polypeptides (Gordon, 1998).

The variation between sequence information may be the result of the expression of two or more distinct genes coding for isoforms of the HT-1 neurotoxin. The phenomenon of polymorphism was identified in the AaH and Lqq excitatory insect toxins of scorpions (Xiong *et al.*, 1999). The expression of isoforms for HT-1 may explain the existence of the HT-2 and or 3 neurotoxins originally described (Thurn, 1994). That is, the HT-1 cDNA sequence identified in this study may correspond to any one of the three neurotoxins identified by Thurn (1994). However, according to Thurn (1994), the amino acid composition for HT-1, HT-2 and HT-3 are essentially identical, with the major differences between them being observed with HT-3. However, the amount of protein used for this total amino acid analyses was in "low nanogram amounts" (Thurn, 1994). Thus the amount of sample used was below that required for accurate and reliable results (100 ng minimum) using the procedure (Pico-Tag) available at that time (Bidlingmeyer *et al.*, 1984). Therefore, the degree of accuracy obtained was sufficient for an estimate on the compositional analysis of the neurotoxins but not accurate enough for determining the exact amino acid composition. Peptide sequencing is a difficult technique (Kalapothakis *et al.*, 1998) and errors are not rare in the interpretation of results. Furthermore, the sequence of a peptide can only be established if it is corroborated with a second independent procedure (Bodansky, 1993). In addition, the analysis performed was unable to detect cysteine and tryptophan residues. Therefore, there may actually be greater differences in amino acid composition between HT-1, HT-2 and HT-3 than was originally proposed.

Attempts at direct N-terminal sequencing of HT-1 were unsuccessful. It was suggested that this may be due to an N-terminal blockage (Thurn, 1994). One of the most common problems associated with protein sequencing is N-terminal blockage, and known deblocking methods are not always efficient (Han and Martinage, 1993). Therefore, internal protein sequence analysis is important and is usually determined via chemical or enzymatic cleavage. Digestion with trypsin is the most popular method as it generally gives the most fragments and the resulting small peptides are easily eluted from the membrane (Nokihara, 1998). Using this strategy of trypsin digestion under urea denaturing conditions, partial peptide cleavage was obtained for HT-1. These peptides were sequenced (using amounts below the normal level of detection) and were found to overlap. From these overlapping sequences two peptides labelled A and B in Figure 2.6 (c) were derived. The C-terminus of peptide A (residues T to P) did not form part of an overlapping region of peptides and it's C-terminal (P) has an unnatural cleavage site for trypsin. Therefore, this particular region of peptide data is likely to be incorrect. This is interesting considering that it is within this region of sequence that the translated cDNA sequence for HT-1 (obtained in this chapter), is least homologous as it is missing 7 residues (Figure 2.6 a.). Therefore considering that lower than recommended amounts of protein were used for Edman sequencing and that the derived peptides were based on partial tryptic digestion, it is highly likely that there were errors in interpreting the amino acid derivatives.

Discrepancies between cDNA sequences and peptide sequence have been observed by several groups studying scorpion neurotoxins (Diniz, *et al.*, 1993; Vazquez *et al.*, 1995; Kalapothakis *et al.*, 1998; Xiong *et al.*, 1999). For example, Vazquez and coworkers (1995), working with cDNAs encoding Na^+ channel specific toxins from scorpions noticed some small discrepancies between cDNA deduced sequences and published primary sequences that turned out to be peptide sequencing mistakes. The strategy of repeating peptide sequencing may need to be attempted with *Ixodes holocyclus*. However although several problems still remain in primary structure determination, these are being resolved with the aid of novel ionisation techniques in mass spectrometry (Nokihara, 1998). Presently, sequencing of sub-picomole amounts of proteins using commercial instrumentation permitting up to 40 amino acids from the N-terminus to be determined is possible (Nokihara, 1998). The re-sequencing of purified tick neurotoxin(s) to confirm the sequence and number of neurotoxins present in *Ixodes holocyclus* would be justifiable.

Features of the HT-1 sequence

Multiple sequence analysis of several clones of the 3' RACE, 5' RACE and complete gene products for HT-1 indicated that the cDNA sequence for HT-1 is correct. The following section provides an analysis of the features of the RACE products obtained for the HT-1 neurotoxin.

The 3' RACE product was obtained by PCR with primers S1 and AP1. The 3' sequence was shown to contain characteristic features of 3' end eukaryotic mRNA. This included stop codons, putative polyadenylation signal sequence and poly A tail (Wahle and Keller, 1992). The 5' RACE product was obtained by PCR with primers S2 and AP1. This sequence was shown to overlap with the sequence obtained for the 3' RACE product (Figure 2.3) by 7 residues (V, Q, C, K, K, C, T). Thus, instead of using the untranslated region of the 5' RACE sequence to design the S3 sense primer, an alternative strategy could have been adopted to obtain the complete cDNA sequence. This would have involved 'fusing' the 5' and 3' RACE products so that they would anneal and extend from their overlapping region. Using the AP1 primer and cDNA synthesis primer, a full length cDNA could be amplified by using the fused product as a template. This method (Appendix 1) involves several steps and requires caution especially during the fusion step where unproductive annealing events can result in PCR products where fusion has not occurred.

Therefore, the method of designing the S3 primer based on the 5' RACE untranslated sequence was a more efficient and reliable method for obtaining the full length cDNA as it only required another PCR step using existing double stranded cDNA template. However this method may not have been successful on very long cDNA, though most long distance high fidelity PCR enzyme mixes available today claim to be able to extend cDNAs up to 10 kb in size.

The sequence depicted in Figure 2.5 corresponded to a full-length HT-1 cDNA clone. Excluding the predicted signal peptide it had a calculated molecular mass of 5386 Da and a pI of 9.02. This mass was within the range of other arachnid neurotoxins which have a molecular weight of 4-8 kDa (Becerril *et al.*, 1997). The HT-1 sequence contains 456 nucleotides excluding the polyA rich tail, with an open reading frame of 216 bp. The deduced amino acid sequence is given in single letter code. At the 5' end potential initiation codons are observed. These include Kozac's proposed A(G)CC AUG sequence which is a consensus sequence for eukaryotic initiation sites (Kozac, 1984).

The full length cDNA of HT-1 contains a classic signal sequence as described by von Heijne (1986), suggesting it is a secreted protein, as would be expected for a toxin secreted from tick salivary glands. The signal sequence was predicted by the PSORT database in which the most C-terminal position of a possible signal sequence is calculated (Figure 2.5). Commonly, eukaryotic leader sequences are 16-26 amino acids long (Perlman and Halvorsen, 1983). The predicted signal sequence for HT-1 is 23 residues in length. There are three structurally and possibly functionally distinct regions of a classical signal sequence. These include: the basic N-terminal region; a central hydrophobic region, and a more polar C-terminal region. These three features are present in the HT-1 predicted signal peptide, as well as in other arachnid leader peptides (Becerril *et al.*, 1995).

The low level of overall sequence homology between HT-1 and other toxins suggests that it is a novel arachnid neurotoxin (Figure 2.8). Homology is seen, however, in the number and distribution of cysteine residues (5 out of 8 cysteines). The presence of 8 cysteines in such a small polypeptide suggest that it would be tightly folded as is the case for other disulphide rich small arachnid neurotoxins. This tightly folded property would also support earlier work where the purified toxin was resistant to cleavage with trypsin unless denaturing conditions with urea were incorporated to relax its disulphide backbone (Thurn, 1994).

The disulphide pairings for HT-1 are yet to be elucidated. However there are several features of the positioning of these cysteine residues in the primary sequence which may suggest that it has a similar tertiary structure to that of the closely related scorpion and or spider neurotoxins.

It is well documented that scorpion venoms contain low mass, cysteine rich neuroactive peptides that affect ion channels of excitable tissues (Gurevitz and Zilberberg, 1994). Despite the differences in their primary sequences and specificities, the scorpion toxins share a similar three dimensional conformation. This is evidenced by the comparative analysis of the cysteine pairing in scorpion neurotoxins (Figure 1.3). This analysis revealed a consensus pattern consisting of one pair of half-cysteines spaced by a tripeptide sequence (C-X-X-X-C) disulphide bonded to a second pair of half-cysteines with one amino acid in between (C-X-C). This observation and detection of similar structures in other peptides exhibiting biological activities unrelated to those observed by the scorpion neurotoxins (endothelins from mammals, Yanagisawa *et al.*, 1988; sarafotoxins of reptiles, Takahashi *et al.*, 1985; and apamin from honey bee venom, Pease *et al.*, 1988) suggest that this structural motif, that is, a cysteine stabilised α-helical motif, is a common structural feature involved in membrane recognition by these peptides (refer to Section 1.3 for review). The same structural backbone motif is seen in all scorpion neurotoxins irrespective of their size, sequence and function and has been confirmed in a number of these toxins through crystal structure studies.

HT-1 also contains C-X-C and C-X-X-X-C cysteine motifs that are present in other arachnid toxins. Figure 2.8 illustrates that the C-X-X-X-C motif of HT-1 is homologous with those of the other scorpion sequences analysed. In scorpion toxins this C-X-X-X-C motif is consistent with its location in the α-helix region of the scorpion toxin (Lebreton and Delepierre, 1994). Therefore it can be predicted that this may also be true for the HT-1 neurotoxin. Similarly, the HT-1 sequence also contained the C-X-C motif. However it was found only three residues downstream from the C-X-X-X-C sequence, whereas for the scorpion neurotoxins it was found further downstream at residues 53-55 (Figure 2.8). Adjacent to the C-X-C motif in the scorpion neurotoxins, HT-1 contains a C-X-X-C motif which may stabilise a β-sheet structure as does the C-X-C motif for scorpions (Lebreton and Delepierre, 1994).

The only significant homology revealed by a search of the available databases with the amino acid sequence of HT-1 was with charybdotoxin and this is shown in Figure 2.7. Charybdotoxin is a 37 amino acid protein found in the Israeli *Leiurus quinquestriatus* scorpion venom. It is a powerful inhibitor of K^+ channels. The three-dimensional structure of charybdotoxin presents the same α/β structural motif possessed by the longer chain scorpion toxins acting on Na^+ channels (refer to Section 1.3) (Drakopoulou *et al.*, 1998). Interestingly, there is little sequence homology between these short chain and long chain scorpion toxins. However, as mentioned previously, the well conserved structural motif is observed among scorpion toxins to develop different biological activities (K^+, Na^+ etc. channel specificity) but also by different animals as a defence mechanism (such as insect defensins and plant γ-thionins). This emphasises the great functional diversity of this fold and it would not be surprising for HT-1 to also possess a similar (or part of this) conserved structure.

The cysteine knot motif structure as described in detail in Section 1.4 and illustrated in Figure 1.7 is a motif found commonly among spider neurotoxins and other inhibitory polypeptides. The consensus sequence of $CX_{3\text{-}7}CX_{3\text{-}6}CX_{0\text{-}5}CX_{1\text{-}4}CX_{4\text{-}13}C$ is proposed based on the distances between the first 6 cysteine resides (Pallaghy *et al.*, 1994; Norton and Pallaghy, 1998). The HT-1 mature sequence has the consensus sequence $CX_7CX_3CX_4CX_1C_{14}C$ for the first 6 cysteines. Therefore, the HT-1 sequence conforms to the consensus sequence described for the inhibitor cysteine knot members, with the exception of the gap size between the 5th and 6th half cysteine residues which would need to be extended by 1 to include the 14 residue gap present for HT-1. Despite this, several amino acid sequences have been reported recently that either satisfy or closely resemble the consensus sequence (Norton and Pallaghy, 1998). Some of these require an expansion of gap size if they were to adopt the motif structure proposed. For example, the μ*O*-conotoxins MrVIA and MrVIB from the cone shell *Conus marmoreus* are sodium channel and L-type calcium channel inhibitors (Fainzilber *et al.*, 1995; McIntosh *et al.*, 1995). If they adopt the motif structure, an expansion of the gap size would be required for the second-third cysteine from X3-6 to X3-9. Similarly SNX-325 from the spider *Segestria florentina* probably inhibits the N-type calcium channels

(Newcomb *et al.*, 1995) and would require a major revision of the last gap size from X4-13 to X4-20. Therefore the HT-1 sequence cannot be disregarded as one that may also belong to the inhibitory cysteine knot family.

Significance of HT-1 for future work

HT-1 is the first reported complete cDNA sequence for a tick neurotoxin. This is very significant for the future analysis of tick neurotoxins as it will be the bench mark for all other toxins derived from Australian ticks and ticks around the world. For example the determination of the HT-1 sequence has enabled collaborations with groups in South Africa working on the tick *Rhipicephalus e. evertsi* (Prof. Albert Neitz, personal communication) and Canada with the tick *Dermacentor andersoni* (Dr. Tim Lysyk, personal communication). The long term aim of future research and collaborations is to potentially develop a vaccine against tick toxicoses that can be used on a variety of hosts to control tick envenomation on a global scale.

In the more immediate future there are a number of studies which can be performed with the HT-1 derived sequence. These include monoclonal antibody production, genomic sequence analysis, toxic and antigenic site mapping, tertiary structure predictions and electrophysiological analysis. The remaining chapters of this thesis describe the expression of HT-1 as a fusion protein and analysis of the recombinant HT-1 fusion protein in immunological and biological assays.

CHAPTER 3

THE EXPRESSION OF A RECOMBINANT HT-1 FUSION PROTEIN

3.1: PREVIEW

This chapter describes the expression of the mature HT-1 neurotoxin sequence as a fusion protein in a bacterial system. It illustrates the optimisation of the expression conditions to obtain the maximal yield of expressed protein, together with the problems associated with purification of the fusion protein and how these problems were overcome by the inclusion of a histidine tag. It also illustrates the antigenic nature of the fusion protein as demonstrated by Western blot experiments.

In addition, the expression of HT-1 without a large fusion partner in a baculovirus expression system was attempted. Data and discussion from these experiments is presented.

3.2: INTRODUCTION

Choosing a suitable expression system

Purification of HT-1 from its native source (engorged ticks) is time consuming, costly, and yields only a small amount of protein. To overcome these problems a recombinant expression system was developed to produce large quantities of genetically manipulable HT-1 in *E.coli* .

A number of factors require consideration prior to choosing an expression strategy that is suitable for a particular protein. These include prokaryotic or eukaryotic systems with secreted or intracellular expression; determining protein function requirements including post translational modifications; assessing the requirement for a fusion strategy, and if the system will be simple, cost effective to use and yield high amounts of the recombinant product.

Expression in bacteria

E. coli is the most frequently used prokaryotic expression system for the production of heterologous proteins. An efficient *E. coli* expression system should include a strong, regulated promoter such as those from the *lac* and tryptophan (*trp*) operons or phage promoters such as T7. The highly inducible and repressible *tac* promoter is an example of a hybrid combining different portions of *lac* and *trp* promoters that has been used to develop improved expression vector systems (Sawers and Jarsch, 1996). A transcription terminator should also be included in the expression vector so that it can minimise background transcription, enhance mRNA stability and prevent possible inhibition of its promoter function. Additionally, a plasmid with an elevated copy number is preferred as the basis of a high level expression system in order to increase fermenter productivity (Qui, 1998).

The major advantages of using *E. coli* as an expression system are the ease of subcloning into expression vectors, and the cost effectiveness of growth, selection, and large scale production (Chen, 1994). Additionally, *E. coli* grow at a very fast rate in comparison to mammalian cells, giving the opportunity to purify, analyse and use the expressed protein in a much shorter time (Lemeulle *et al.*, 1998). Bacterial expression systems are also well studied and a large number of efficient bacterial promoters and vectors are available (Vlak and Keus, 1990).

The major disadvantage of a bacterial expression system is the lack of modification of eukaryotic proteins. For example, post-translational modifications such as glycosylation and phosphorylation which occur in some authentic native proteins do not occur in bacterial systems (Hockney, 1994). In addition, the highly expressed proteins are often encapsulated in inclusion bodies which consist of insoluble and incorrectly folded recombinant protein. The formation of inclusion bodies has the advantage that the protein becomes protected from proteolysis in most cases, and large amounts of gene product are usually obtained. However the major disadvantage is that the expressed protein is no longer in its native state and requires to be dissolved and renatured to obtain a functional protein (Schein and Noteborn, 1988). There is also concern over the use of *E.coli* as a production system because of the existence of endotoxins (LPS) in the cell wall as they are pyrogenic and therefore must be removed to yield a safe product (Marston, 1986).

Gene Fusion Systems

The expression of a protein fused to another protein, protein fragment or short polypeptide 'tag' sequence is known as a gene fusion (Hockney, 1994). Bacterial gene fusion systems have been used to facilitate protein purification, to overcome protease degradation problems and to allow secretion of gene products through the cytoplasmic membrane to the periplasm. The choice of a gene fusion system depends on the properties and final use of the gene product to be expressed. Examples of gene fusion systems that have been used to facilitate the purification of soluble recombinant proteins are listed in Table 3.1. Widely used fusion protein and affinity tag systems include the staphylococcal protein A, streptococcal protein G, glutathione-S-transferase from *Schistosoma japonicum*, maltose binding protein, ubiquitin and the FLAG and His_6 tags (Hanning and Makrides, 1998).

Secretion into the periplasm has a number of advantages, including separation from cytoplasmic proteins, particularly proteases (Marston, 1986). This is important considering that the periplasm of *E. coli* contains only approximately 4% of cellular proteins, and therefore a large degree of purification can be achieved by secretion of the recombinant gene product followed by selective release of the periplasmic content. In addition, the production of a soluble fusion protein is enhanced in the oxidative environment outside the cytoplasm where disulphide bond formation may proceed and the protein may fold into a product with full biological activity. Furthermore, if a recombinant protein is toxic to the cell, the secretion approach might offer the only expression alternative (Uhlen and Moks, 1990).

Some examples of gene fusion systems include having a recombinant gene placed downstream of a suitable signal sequence which, when processed during transport to the periplasm, can produce recombinant protein with a native N-terminus. Alternatively the gene can be expressed as a product fused to itself. However, this strategy usually results in the formation of insoluble aggregates. Two of the most common fusion strategies are C-terminal or N-terminal fusions. An advantage with C-terminal fusion where the recombinant product is positioned at the C-terminal side of the fusion partner, is that the promoter and the translation initiation signals are all integrated in the 5' end of the fusion partner and are thus unchanged by different fusions at the 3' end. The N-terminal fusion has the disadvantage that a product specific transcriptional and translational start must be engineered in the 5' end of the recombinant gene (Uhlen and Moks, 1990; Scopes, 1994).

Mammalian expression systems

Mammalian expression systems are theoretically most suitable for expressing mammalian proteins (Scopes, 1994). The signals for synthesis, processing and secretion of eukaryotic proteins are effectively recognised by the mammalian cells (Verma *et al.*, 1998). The disadvantages are that mammalian cells are not very well understood in terms of promoter activity, polyadenylation sites and signal sequence specificity. Moreover, with mammalian expression it is usually difficult to obtain a large scale preparation (>1 mg of purified protein) due to tedious purification procedures (Chen, 1994). The unpredictability of these systems, as compared to yeast or bacterial systems, coupled with the increased expense required for media and growth equipment of mammalian cells precludes their routine use (Vlak and Keus, 1990).

Expression in yeast

The main advantages of yeast over other expression systems are related to the fact that it is both a microorganism and a eukaryote. Unlike *E. coli,* yeast provide advanced protein folding pathways for heterologous proteins and when yeast signal sequences are used they can secrete correctly folded and processed proteins. For example, glycosylation will usually occur although it is not the same glycosylation as found on proteins expressed in mammalian cells (Kukuruzinska *et al.*, 1987). Unlike mammalian systems, yeast can be rapidly grown on simple growth medium. For the expression of clinically and industrially important proteins, yeast is an attractive option as industrial scale fermentation technology is widely used (Verma *et al.*, 1998). The disadvantages are that their genetics and life cycles are more complex than the bacterial system, only a limited number of promoter and vectors are available and the yield of foreign recombinant protein is often low (Vlak and Kaus, 1990).

Expression in insect cells

Recombinant baculovirus expression is now a mature technology and is widely used to express high-level eukaryotic heterologous proteins. Baculovirus vectors have several unique and advantageous characteristics. They contain a polyhedrin gene which is suitable for the insertion of foreign genes because it is non-essential for viral replication, it is a late gene with a strong promoter and can be easily detected by light microscopy. (Maeda, 1989). These systems frequently produce high yields of proteins due to the strength of the viral promoters. Moreover, insect cells carry out most of the post-translational processing events that occur in mammalian cells. Consequently, the eukaryotic proteins produced by a baculovirus expression system are usually similar to the authentic protein in their biological activity, structure and antigenicity. In addition the proteins are produced with much higher yields and a significantly lower cost than could be obtained by using a mammalian expression system (Kitts and Posse, 1993)

Furthermore, baculoviruses have a highly restricted host range which makes them safer to use in human clinical studies than mammalian expression systems (Verma *et al.*, 1998). As far as it is known, insect cells and baculovirus are devoid of pathogenic or toxic compounds for humans. Moreover, insect cells can be grown in serum free media, that is without mammalian contaminants, leading to easy and safe purification (Lemeulle *et al.*, 1998). However the techniques to generate and select a recombinant baculovirus can be experimentally difficult, expensive and time consuming (Chen, 1994).

The expression strategy for HT-1

The initial strategy chosen for the expression of HT-1 was in an *E.coli* host system as a maltose binding fusion protein (MBP). The particular expression package chosen was supplied by New England Biolabs, Inc. (MA, USA) (NEB). With this system the MBP fusion protein could be exported to the periplasm of *E.coli* and purified using an affinity amylose resin. In addition, the fusion protein could be detected using an anti-MBP antibody. The pMAL^{TM} vectors used with this expression system also contain the sequence for the factor Xa protease recognition site for potential cleavage of the fusion protein, thus removing all vector derived residues from HT-1.

The recombinant HT-1 toxin expressed and secreted by the *E. coli* cells was characterised by immunoblotting, immunoassay, toxicity and protection assays. In an attempt to express HT-1 in the absence of a fusion partner, HT-1 was cloned into a baculovirus transfer vector and subsequently used to infect insect cells. Results and discussion from the use of these two methods of expression are presented in this chapter.

Gene Product	Origin	Molecular Weight (kDa)	Secretion	Ligand
β-galactosid.	*E. coli*	116	-	TPEG, APTG
Protein A	*S. aureus*	31	+	IgG
CAT	*E. coli*	24	+	Chloramph.
Poly (Arg)	Synthetic	1-3	-	Ion-exchange
Streptavidin	Streptomyces	13	+	Biotin
Poly (glu)	Synthetic	1-2	-	Ion-exchange
Z	Synthetic	7	+	IgG
PhoS	*E. coli*	36	+	Hydroylapatite
Cysteine	Synthetic	<1	+	Thiol
Protein G	Streptococci	28	+	Albumin
MBP	*E. coli*	40	+	Starch
GST	*E. coli*	26	-	Glutathionone
Flag peptide	Synthetic	2-5	+	Specific IgG
Poly (His)	Synthetic	1-7	+	$Ni^{2+}Cu^{2+}Zn^{2+}$

TABLE 3.1: Gene fusion systems used to facilitate protein purification.

The molecular weight of the most common fusion partner is indicated, as well as the secretion of the fusion protein has been demonstrated (+). Abbreviations: CAT, Chloramphenicol acetyltransferase; Z, IgG-binding fragment based on staphylococcal protein A; PhoS, phosphate-binding protein; MBP, maltose-binding protein; GST; glutathione *S*-transferase; TPEG, (*p*-aminophenyl-*β*–D-thiogalactosidase); APTG, *p*-aminophenyl-*β*--D-thiogalactoside (adapted from Uhlen and Moks, 1990).

3.3: MATERIALS AND METHODS

BACTERIAL EXPRESSION OF THE MBP/HT-1 FUSION PROTEIN

DNA Manipulations

DNA techniques for purification, electrophoresis, ligation, cloning, electroporation and sequencing, were carried out as described in Section 2.3 unless otherwise stated. All materials and chemicals were of analytical or research grade.

Primer design for expression in the pMAL-p2 expression vector

FORWARD PRIMER TEPF (21 bp)

5' GAG AAC GGT TTC TCA TGT ACC 3'

REVERSE PRIMER TEPR (38 bp)

5' GC ATA **GGA TCC** TTA TCA TTG TTT ACA GTA AGC TTC ACC 3'
***Bam*H I** HT-1 sequence

REVERSE PRIMER HT-1HISR
(* = stop codons).

5' CAC **GGA TCC** TTA TCA **ATG GTG ATG GTG ATG GTG**
***Bam*H I** * * 6 x His tag

CCT TCC CTC GAT TTG TTT ACA GTA AGC TTC ACC 3'
factor Xa HT-1 sequence

PCR Amplifications

PCR amplifications were carried out using *Taq* polymerase (Perkin Elmer Corporation, Norwalk, CT, USA), according to the manufacturers protocol. A typical 100 µL reaction contained 1X *Taq* buffer, 200 µM each dNTP, 2.5 mM $MgCl_2$, 50 pmol each primer, 0.1 ng template and 2.5 units of *Taq* polymerase. The DNA was denatured at 95°C for 5 minutes, followed by a 5 minute annealing step at 50°C, during which time the *Taq* was added. The PCR was then carried out for 35 cycles of 72°C for 1 minute, 94°C for 30 seconds and 55°C for 30 seconds, in a Hybaid Omnigene (Ashford, U.K.) thermal reactor.

Creating a blunt ended fragment with Klenow

Klenow (large fragment of DNA Polymerase I) was used to create a blunt ended fragment, for purposes of ligating into the *Xmn* I site of the $pMAL^{Tm}$-p2 vector. The DNA was required at a concentration of 50 µg/mL. The reaction contained 1 unit Klenow/ µg of DNA, dNTP's to a final concentration of 33 µM and 1X Klenow buffer. The mixture was incubated at 25 °C for 15 minutes and EDTA to a final concentration of 10 mM was added. The enzyme was then inactivated by heating the reaction to 75°C for 10 minutes. The Klenow treated PCR product was then purified by phenol:chloroform extraction and ethanol precipitation, and prepared for ligation into the $pMAL^{Tm}$-p2 vector by restriction digest with *Bam*H I.

Preparation of the $pMAL^{TM}$-p2 vector for Cloning

The pMAL-p2 vector was digested with the *Bam*H I and *Xmn* I restriction enzymes according to the manufacturers protocol and then purified by phenol: chloroform extraction and ethanol precipitation.

Expression protocol

Expression of the MBP/HT-1 fusion protein was based on a procedure described by Eisele *et al.* (1992). A 5 mL overnight culture of the pMAL/HT-1 fusion protein in Luria broth was inoculated into 500 mL of superbroth containing 10 g tryptone, 5 g yeast extract, 2.5 g NaCl, 1.25 g K_2HPO_4, 1 g $MgSO_4$, 0.1 µg/mL biotin, 0.4% glucose, and 1.5 mL of trace elements solution containing (for a 100 mL stock solution); $FeCl_2$ 1.6 g, $ZnCl_2$ 0.2 g, $CoCl_2$ 0.2 g, Na_2MoO_4 0.2 g, $CaCl_2$ 0.1 g, $CuCl_2$ 0.1 g, H_3BO_4 0.05 g, 8M HCl 10 mL, and 50 µg/mL ampicillin.

The culture was incubated at 37 °C in an orbital incubator to an OD_{600} of 0.5 or 1-1.5, then incubated in an ice water bath (to arrest growth) for 15 minutes. The cells were then pelleted by centrifugation at 15 000 *g* for 20 minutes at 4°C and resuspended in 1 L of fresh superbroth containing no glucose. Following a 1 hour recovery step at 37°C, expression was induced by adding IPTG to a final concentration of between 0.01-0.5 mM, and the temperature adjusted to either 25, 30 or 37°C. Expression of the HT-1 fusion protein was then allowed to proceed for a period of 4-18 hours.

The expressed fusion protein was recovered from the culture supernatant, periplasm or whole cells. The supernatant was obtained by pelleting the cells at 15 000 *g* by centrifugation for 15 minutes at 4°C. Periplasmic proteins were obtained by an osmotic shock procedure. Pelleted cells were resuspended in a solution containing 20% sucrose, 0.3 M Tris-Cl pH 8.0 and 1 mM EDTA (8 mL/0.1 g of cells). The resuspended cells were incubated at room temperature with occasional agitation for 10 minutes, and then pelleted at 15 000 *g* at room temperature. The cells were then resuspended in ice cold 5 mM $MgSO_4$ and incubated in an ice-cold water bath for 10 minutes with occasional agitation. The cells were then pelleted as described above at 4°C and the supernatant was the osmotic shock fluid containing the periplasmic fraction.

A whole cell extraction method (including cytoplasmic and periplasmic proteins) to obtain both soluble and insoluble fractions was carried out as described in the $pFLAG^R$ expression system instruction manual (IBI, New Haven, CT, USA). Briefly, following induction, the expressed whole culture (50 mL) was centrifuged as described above and the pellet resuspended in 5 mL extraction buffer A (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 250 µg/mL lysozyme and 50 µg/mL NaN_3). The cells were incubated at room temperature for approximately 5 minutes until lysis was complete. The extraction buffer B (0.5 mL) (1.5 M NaCl, 100 mM $CaCl_2$, 100 mM $MgCl_2$ and 20 µg/mL DNAse I) was then added and incubated at room temperature until the cells were no longer viscous from lysis. The cells were then pelleted by centrifugation at 1500 *g* for 1 hour. The supernatant containing the soluble whole cell fraction was then collected. The pellet containing the insoluble whole cell fraction was resuspended in 5 mL of extraction buffer A.

Protein electrophoresis

Discontinuous SDS-PAGE was carried out according to Laemmli (1970) to separate proteins using 12 or 15% acrylamide poured gels or on 4-20% pre-cast gels (Gradipore LTD, NSW, Aust.). The protein samples were diluted with a 3X sample buffer (1.8 mL 0.5 M tris; 1.5 mL 20% SDS; 1.5 mL glycerol; 200 μL MilliQ water and bromophenol blue powder to colour). For reducing conditions the 3X sample buffer was supplemented with 1 μL of 1 M DTT and the samples were boiled for 5 minutes prior to electrophoresis to promote cysteine bond dissociation. Electrophoresis was performed in a Mini-Protean II (Bio-Rad laboratories, Hercules, CA, USA) apparatus in Tris/Glycine buffer at an initial voltage of 90 V until the protein reached the separating gel and then at a constant voltage of 150 V. Protein standards of known molecular weight were electrophoresed simultaneously (Gibco BRL, Gaithersberg, M.D, USA).

Protein staining

Coomassie Brilliant Blue

The electrophoresed proteins were visualised by staining with 0.25% Coomassie brilliant Blue in acetic acid/methanol/water for approximately 1 hour, followed by destaining in 90% methanol and 2% acetic acid until a desired background was obtained at which point the gels were transferred to a water-only solution (Salinovich and Montelaro, 1986).

Silver Staining

For more sensitive detection, protein gels were stained with $AgNO_3$ based on the method by Blum and coworkers (1987). Briefly, proteins were fixed in 50% methanol, 8% acetic acid and 0.05% formaldehyde for a minimum of 1 hour. The gels were then washed 3 times for 20 minutes each in 50% ethanol and then pretreated with 0.02 g sodium thiosulfate (per 100 mL) for 1 minute followed by 3 x 20 second washes in water. The $AgNO_3$ solution (0.2 g/100 mL) + 0.07% of 40 % w/w formaldehyde was added for 20 minutes, followed by 2 x 20 second washes in water. The proteins were then developed in 2% pretreatment solution containing 6 g/ 100 mL Na_2CO_3 and 0.05% formaldehyde. The reaction was stopped with the Coomassie destain solution described above and the gels were then placed in water.

Western blotting

Following separation by SDS-PAGE, proteins were transferred to 0.45 or 0.1 µm nitrocellulose sheets (Bio-Rad, Hercules, CA, USA), according to the method described by Towbin *et al.* (1979). The transfer buffer consisted of 0.025 M Tris, 0.192 M glycine and 20% MeOH, and the electro-transfer took place at a constant voltage of 100 V for 1 hour at 4°C. Following protein transfer, the blotting apparatus was disassembled and the nitrocellulose prestained with Ponceaus S (0.5% Ponceau S in 1% acetic acid) for 5-10 minutes, followed by destaining in water for 2- 3 minutes to visualise the transferred proteins (Salinovich and Montelaro, 1986). The membrane was then blocked with a solution containing 3% BSA in PBS/Az (120 mM NaCl 2.7 mM KCl, 8 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 7.2), supplemented with 0.05% Tween 20 and 0.02% NaN_3 final concentration for 1 hour at room temperature. The nitrocellulose was washed 3 x in TBS-Tween-20/Az (150 mM NaCl, 27 mM KCl, 50 mM Tris, ph 7.2, supplemented with 0.05% Tween-20 and 0.02% NaN_3 final concentration) and then incubated with the appropriate primary antibody (in TBS-T/Az) for at least 1 hour at room temperature. The membrane was then washed as previously described and incubated with the appropriate alkaline phosphatase-conjugated secondary antibody for at least 1 hour. Prior to development, the nitrocellulose was washed as before, with an additional wash step in TBS/Az (no Tween) and then developed by the addition of substrate, nitro-blue tetrazolium (NBT) at 0.33 mg/mL and bromo-chloroindolyl phosphate (BCIP) at 0.165 mg/mL (Sigma, St Louis, MO, USA) in alkaline phosphatase (AP) buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5). Development was stopped by addition of MilliQ water.

Preparation of proteins for peptide sequencing

Following electrophoresis proteins were electotransferred onto Immobilon P (PVDF) membrane (Millipore, Bedford, MA, USA) as described by Matsudaira (1987). The transfer buffer consisted of 10 mM CAPS (pH 11.0) and 10% methanol. The electroblotting was carried out as described above for 1 hour at 4°C. After transfer, the membrane was stained for 5-10 minutes with amido black (1 % amido black powder, 40% MeOH, 1% Acetic acid), destained with water, then air dried and stored at 4°C until used for N-terminal peptide sequencing. The N-terminal sequencing was carried out by Dr. Denis Shaw (Australian National University, ACT, Australia).

Affinity purification of fusion proteins

Amylose Resin

The amylose resin (NEB) was used to purify the HT-1 fusion protein by exploiting its specific affinity for maltose. Amylose column preparation was performed as described in the 'Protein Fusion and Purification System' manufacturers instructions (NEB). Briefly, the amylose resin was placed in a 2.5 x 10 cm column and washed with 8 column volumes of column buffer (20 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA). The periplasmic extract, which had been equilibrated in 20 mM Tris-Cl pH 7.5, was then loaded onto the column at a flow rate of 1 mL/min. The column was then washed with 12 column volumes of column buffer. The protein was eluted in a buffer containing the column buffer + 10 mM maltose. Ten to 20 fractions of 3 mL each were collected. The eluted protein was identified by absorbance at 280 nm, followed by SDS-PAGE staining together with Western blot analysis. The column was regenerated by adding 3 column volumes of water, followed by 3 column volumes of 0.1% SDS, 1 column volume of water and finally 3 column volumes of column buffer + NaN_3 (0.02% final concentration), in which the column was stored.

Talon resin

A talon immobilised metal affinity column (IMAC) was used to purify the HT-1 fusion protein with the 6 x His tag. The talon IMAC (CLONTECH Laboratories Inc., Palo Alto, CA, USA) resin was prepared as described in the CLONTECH 'TALON^{TM} Metal Affinity Resin instruction manual, using the small scale batch procedure under native or denaturing (8 M urea) conditions. The bound His tagged-protein was eluted by stripping the column with 100 mM EDTA. Eluted samples were analysed as described for the amylose resin.

Ni-NTA agarose

Ni-NTA IMAC agarose (QIAGEN, Pty Ltd, VIC, Aust.) was used as an alternative strategy to purify the MBP/HT-1/His_6 fusion protein. The fusion protein was successfully purified under denaturing (8 M urea) conditions using the following protocol. Briefly, 1 mL of the 50% Ni-NTA slurry was added to 4 mL of periplasmic lysate (both equilibrated in 8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 8.0) and mixed gently by shaking. The lysate-resin mixture was carefully loaded into an empty support column. The mixture was allowed to pass through the column and the flow through was collected. The column was then washed with 2 x 4 mL volumes of wash buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 6.3), the wash fractions were collected. The fusion protein was then eluted with 4 x

0.5 mL elution buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 M Tris-Cl, pH 4.5). Flow through, wash and eluted fractions were all analysed by SDS-PAGE as previously described.

Concentration of proteins

Prior to protein purification, periplasmic extracts were concentrated in an Amicon ultra filtration stirred cell system (MA, USA) to the required volume using a YM 10 (10 000 MWCO) cellulose triacetate membrane (Sartorius, Gottingen, Germany). Eluted fractions in 8M urea were dialysed against PBS/Az and also concentrated using the above system.

Protein concentration determination

Protein concentration was evaluated spectrophotometrically at 280 nm using an extinction coefficient of 68 000 M^{-1}/cm^{-1} as determined by amino acid compositional analysis. Subsequent to this, when necessary, the protein concentration was confirmed by a 'Micro BCA Protein Assay Reagent Kit' as per manufacturers instructions (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) or maltose binding protein (MBP) as standards.

Cleavage with factor Xa

Factor Xa cleavage was carried as described in the 'Protein Fusion and Purification System' instruction manual (NEB). Factor Xa cleavage was carried out at a w/w ratio of 1% the amount of fusion protein in PBS/Az (for example, 1 mg factor Xa enzyme was used for a reaction containing 100 mg fusion protein). The reaction was incubated at 4°C overnight. Cleaved proteins were visualised by SDS-PAGE and Western blotting as previously described.

BACULOVIRUS EXPRESSION PROTOCOL

The 'BacPAK^{TM} Baculovirus Expression System' (CLONTECH) was used as an alternative strategy to expression in *E. coli* for the HT-1 toxin, without the incorporation of a protein fusion partner, but with a 6 x His tag for purification.

Oligonucleotoide primer design

The first round of PCR involved using primers designed to incorporate part of the mature tick sequence, together with part of a secretion signal from the α-chain of PLA2 snake inhibitors (Sekuloski *et al.*, 1999) and a *Xho* I restriction endonuclease site for cloning (Figure 3.11). This amplification took place with the primers BacHT-1F and BacHT-1R primers. The second round PCR amplification involved using a specific baculovirus primer (ITSS2) which was designed to extend the remainder of the secretion signal by overlapping with the first round PCR sequence, and to include a *BamH* I site for cloning purposes. This second round amplification involved the ITSS2 and BacHT-1R primers.

The entire α-chain PLA2 secretion signal sequence is:

5' ATG AAA TCC CTA CAG ATC ATC TGT CTC CTT TTC GTT TTG
GTA GCC AGA GGA AGC TGT 3'

BacHT-1F (50 bp).

The sequence underlined corresponds to the region of overlap with the ITSS2 primer used in second round amplification. The letters in normal type form part of the α-chain secretion signal. Letters in italics correspond to the 5' end of the HT-1 sequence.

5' CTC CTT TTC GTT TTG GTA GCC AGA GGA AGC TGT *GAG AAC*

GGT TTC TCA TG 3'

BacHT-1R (54 bp)

This reverse primer was designed to incorporate the HT-1 toxin sequence (italics), a methionine site (M) for cleavage with CnBr, a 6 X his tag (underlined), stop codons (*) and a *Xho* I restriction site.

5' ATA **CTC GAG** TTA TCA ATG GTG ATG GTG ATG GTG **CAT** *TTG*
Xho I * * M

TTT ACA GTA AGC TTC 3'

ITSS2 primer (48 bp).

The sequence underlined corresponds to the overlapping sequence of the BacHT-1F primer used in the first round amplification. Therefore the sequence downstream of the *Bam*H I site corresponds to the beginning of the α- chain secretion signal.

5' CAT CGC **GGA TCC** ATG AAA TCC CTA CAG ATC ATC TGT
***Bam* H I**

CTC CTT TTC GTT 3'

Polymerase chain reaction

PCR amplifications were carried out using reagents from the 'Expand' high fidelity PCR kit (Boehringer Mannheim, Basel, SW). A typical 50 µL reaction contained 1X PCR buffer, 25 pmol primer, 40 µM each dNTP, 0.5 units of *Taq* polymerase and 0.1 ng of template. The cycling conditions included an initial denaturation step of 95°C for 30 seconds, followed by 34 cycles of 55°C for 1 minute, 72°C for 45 seconds and 95°C for 30 seconds, with a final 55°C annealing cycle and 2 x 72°C, 2 minute extension cycles. The PCR machine used was a Hybaid 'PCR Express' thermocycler with heated lid (Hybaid LTD., Ashford, U.K.).

DNA manipulations

Gel extraction, purification of DNA, ligation, restriction digest analysis and sequencing were carried out as described in Section 2.3. The amplified insert was prepared for cloning by restriction digest with *Xho* I and *Bam*H I and gel purification. The HT-1 insert was then ligated into the BacPAK 9 vector which had been cut with the same restriction enzymes. Transformation of ligated DNA was via the heat shock method into highly heat competent JM109 bacterial cells (Promega Corporation, Madison, WI, USA;). Typically, a 50 µL aliquot of JM109 cells and 2 µL of ligation mix were placed in a sterile falcon tube on ice for 20 minutes and then heat shocked at 42°C for 47 seconds. The cells were then allowed to recover for 1 hour in 1 mL of SOC media (per litre: 20g tryptone, 5 yeast extract, 0.5 g NaCl, 10 mL 250 mM KCl, pH 7.0, supplemented with sterile 5 mL 1 M MgC12 1 M MgS04, and 20 mL of 1 M glucose) before spreading onto LBA amp plates containing Xgal and IPTG .

Construction of a recombinant viral expression vector

Recombinant virus was generated according to the CLONTECH 'BacPAK Baculovirus Expression System' manufacturers instructions. Briefly, *Spodoptera frugiperda* cells (Sf21) were grown to exponential phase at 27°C in TNM-FH insect media (Sigma) supplemented with 10% insect tested foetal bovine serum (FBS) and gentamycin to a final concentration of 50 µg/mL. The media was decanted and cells were resuspended in fresh media and cell count performed. Approximately 1×10^6 cells were added to a tissue culture dish, placed in a moist plastic storage box and incubated at 27°C for 2 hours. Following the 2 hour incubation the medium was removed from the cells and 2 mL of BacPAK Grace's Basic Medium was added. This wash step was then repeated. The cells were then incubated for 30 minutes until the transfection mix (below) was added.

Plasmid DNA containing the HT-1/His gene (Bac/HT-1/His) was first passed through a 'Chroma-Spin-400™ column as per manufacturers instructions (CLONTECH) for purification. Bac/HT-1/His was then diluted to approximately 100 ng/µL with TE (1 M Tris-Cl, 0.1 M EDTA, pH 8.0) buffer. The transfection mix consisted of 1 µg of Bac/HT-1/His, BacPAK viral DNA (BacPAK 6/Bsu 36 digest, 5 µL) and water to a total volume of 96 µL. The negative control transfection did not contain the viral DNA. To the test transfection 4 µL of Bacfectin (to enhance viral uptake of DNA) was added and incubated at room temperature for 15 minutes. Meanwhile, the medium was removed from the cells and 1.5 mL of fresh Graces Basic Medium was added. The Bacfectin-DNA mixture was then added dropwise to the cells with gentle mixing. This was known as the p0 phase of viral culture and it was incubated overnight at 27°C, then stored at 4°C.

The transfected cells were propagated by adding 200 µL of the p0 phase to exponential Sf21 cells and incubated for 2-3 days at 27°C. This was known as the p1 phase of viral culture. A p2 phase of viral culture was subsequently obtained following the same procedure. The p1 and p2 phase viral cultures were then analysed by PCR and Western blotting for the presence of recombinant virus. Cell supernatants were collected at various intervals post infection and clarified from cellular debris by centrifugation.

Polymerase chain reaction to identify recombinant baculovirus.

The supernatant fractions of p0, p1 and p2 phase virus cultures were screened for the presence of recombinant virus as first described by Malitschek and Schurtl (1991). In this method 10 µL of the supernatant from viral cultures was combined with 90 µL of a buffer containing 50 mM KCl, 10 mM Tris-Cl pH 8.3, 0.1 mg/mL gelatin, 0.45% Triton X, 0.45% Tween 20 and 6 µg proteinase K. This mix was then incubated at 60°C for 1 hour, then at 95°C for 10 minutes to inactivate the proteinase K. Twenty five microlitres of this preparation was used as PCR template. A typical 50 µL PCR amplification contained 15 pmol of each Bac 1 (fwd) and Bac 2 (rev) primers (CLONTECH) (which amplified a flanking region of approximately 100 bp corresponding to the BacPAK 9 vector), 1 µL 10 mM dNTP mix, 2.5 µL 10X Sigma *Taq* polymerase buffer, 1 unit Taq polymerase and 25 µL of template. PCR cycling conditions were as described previously in this baculovirus expression section.

Western blot analysis of viral cultures for recombinant baculovirus.

Western blotting was used to analyse the p0, p1 and p2 virus culture whole cell (obtained by resuspending the cell pellet following centrifugation in 1 X sample buffer) and supernatant fractions for recombinant HT-1/His. The Western blotting technique used was as described previously. 4-20% pre-cast Tris/glycine gels (Gradipore) were used for protein separation. The proteins were blotted onto a 0.1 µM nitrocellulose membrane and probed with relevant antibodies.

3.4: RESULTS

Expression of the MBP/HT-1 fusion protein in bacteria

Amplification of the HT-1 insert

The oligonucleotide primers TEPF and TEPR were used to amplify the desired tick HT-1 sequence (Figure 3.1) using the cloned plasmid DNA of the full length HT-1 sequence (obtained by RACE, Section 2.4) as template DNA (Figure 3.2). The PCR-amplified product of the HT-1 gene used for cloning into the pMAL^{TM}-p2 expression vector is illustrated in Figure 3.3. Serial dilutions of the template were made from 1/100 to 1/100 000 (lanes 2-7 respectively). A PCR product of 180 bp was amplified as expected (lanes 2-7). Lane 3 also shows a faint low molecular weight primer dimer band. Lane 8 corresponds to a negative control amplification with a low molecular weight primer dimer band also present. Lane 9 was a positive control amplification using ubiquitin forward and reverse primers with RACE PCR cDNA as template. A multiple banding pattern was obtained as expected.

The PCR amplified HT-1 insert was cloned into the pGEM^{R}-T vector (Promega Corporation, MA, USA) and sequenced as described in Section 2.3. The insert was then prepared for ligation into the pMAL^{TM}-p2 expression vector by treatment with Klenow enzyme to create a blunt-ended fragment and by restriction endonuclease digest with *Bam*H I. It was then ligated into the pMAL^{TM}-p2 vector which had been prepared with *Bam*H I and *Xmn I* restriction enzymes. Sequencing again confirmed that the insert was correct and in-frame for expression (data not shown).

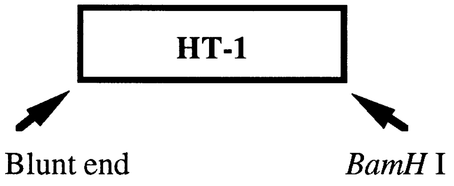
Expression of the MBP/HT-1 fusion protein

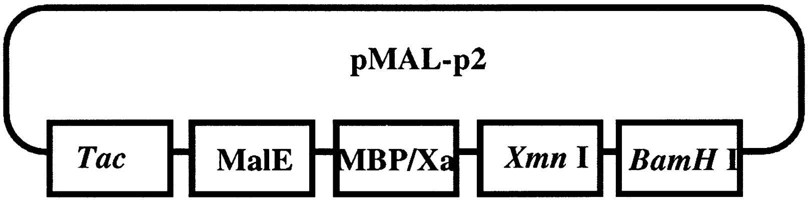
A Western blot analysis of the expression products is shown in Figure 3.4. The expression conditions from which these products were harvested were induction with 0.3 mM IPTG for 4 hours at 37°C. The protein bands were detected with an anti-MBP antibody. A major band at approximately 50 kDa corresponds to the fusion protein (MBP is approximately 42 kDa and the HT-1 insert is approximately 6 kDa). The protein samples were electrophoresed non-reduced and therefore there were some higher molecular weight aggregates that migrated corresponding to a molecular weight of 100 and 200 kDa suggesting that some of the expressed protein may be insoluble. The lower molecular weight bands were demonstrated to correspond to breakdown products of MBP at approximately 40 kDa through

Figure 3.1: Schematic diagram of the cloning and expression strategy for the HT-1 fusion protein in the pMAL-p2 vector.

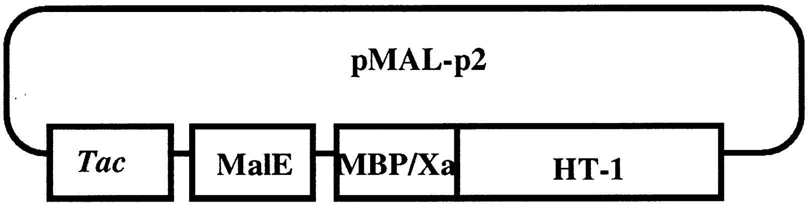
The HT-1 sequence to be cloned into pMAL-p2 was amplified with the primers TEPF and TEPR from a plasmid containing the complete cDNA sequence for HT-1 (Figure 3.2). The amplified product was treated with Klenow to create a blunt end and *BamH* I to create a cohesive end for ligation into the *Xmn* I and *BamH* I sites of the pMAL-p2 vector. The MBP/HT-1 fusion protein was then expressed in *E.coli* TB1 cells. The expression conditions were optimised and the periplasmic fraction used to purify the fusion protein over an amylose affinity resin.

HT-1 fragment amplified by PCR using TEPF and TEPR primers. Treat amplified fragment with Klenow and digest with *BamH* I





Ligate fragment into digested *Xmn* I and *BamH* I sites of the pMAL-p2 expression vector



Express the fusion protein vector in *E.coli* TB1 cells

Optimise expression conditions

Extract periplasmic fraction and purify using amylose affinity column

5'
ATG TCC AAA GTT ACG ACA GTC TTC ATC GGC GCA CTG GTC CTG
M S K V T T V F I G A L V L

CTC CTA CTC ATT *GAG AAC GGT TTC TCA TGT ACC* AAC CCT GGG
L L L I *E N G F S* **C T N P G**

AAA AAA AGG TGC AAC GCT AAG TGC TCA ACA CAC TGC GAC TGC
K K R C N A K C S T H C D C

AAG GAC GGA CCC ACT CAC AAT TTC GGG GCT GGG CCG GTC CAG
K D G P T H N F G A G P V Q

TGC AAA AAA TGC ACA TAT CAA TTC AAA GGT GAA GCT TAC TGT
C K K C T Y Q F K G E A Y C

AAA CAA TGA TAA GGA TCC TAT GC 3
K Q * *

Figure 3.2: The mature toxin sequence for HT-1 and HT-1 expression sequence.

The mature HT-1 sequence is depicted in single letter code. The HT-1 toxin sequence predicted following signal peptide removal is in bold type. The sequence chosen for expression commences with the italics type residue (E) and ends with the bold face type stop codons (*). The primer TEPF is underlined and in italics, the primer TEPR is underlined (adapted from Figure 2.5).

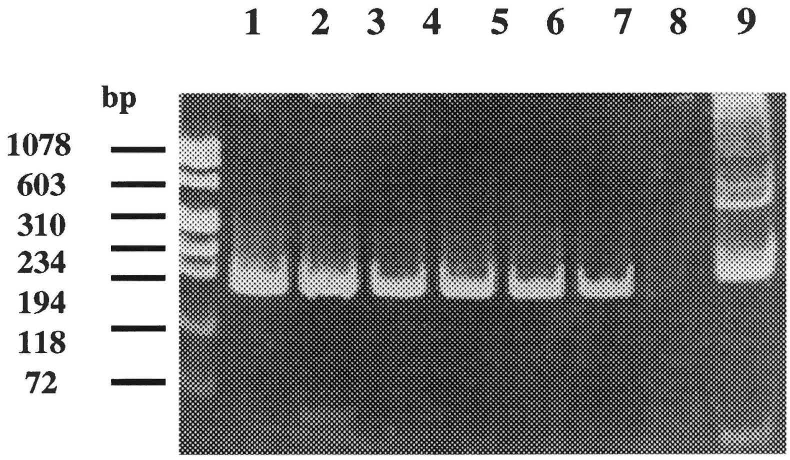


Figure 3.3: PCR amplification of the HT-1 gene that was cloned into the pMAL-p2 expression vector.

PCR products (10 μL) were electrophoresed through a 10% polyacrylamide gel in the presence of DNA loading buffer.

Lane 1:	PhiX/Hae III DNA standards 500 ng (Promega)
Lanes 2-7:	Serial dilutions of cloned HT-1 template from 1/100 to 1/100 000 respectively. A PCR product of approx. 180 bp was amplified for each reaction as expected. A lower molecular weight primer dimer band is visible in lane 3.
Lane 8:	Negative control amplification with no template DNA. No amplified product is visible as expected.
Lane 9:	Positive control amplification using ubiquitin forward and reverse primers with adaptor ligated double stranded tick cDNA as template. A multiple banding pattern was amplified as expected.

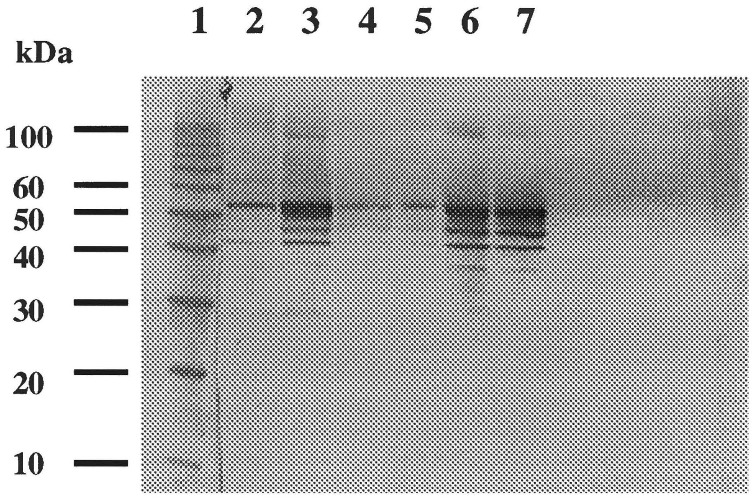


Figure 3.4: Western blot of MBP/HT-1 expression products detected with anti-MBP antibody.

The expressed products were electrophoresed under non-reducing conditions through 12% SDS-polyacrylamide gels and blotted onto nitrocellulose. The nitrocellulose was probed with a 1/10 000 dilution of anti-MBP antibody followed by a 1/20 000 dilution of alkaline phosphatase conjugated anti-rabbit IgG. The fusion protein is the major band visible at ~50 kDa in all lanes of the blot. Bands at ~200 kDa correspond to higher molecular weight aggregates. Lower molecular weight products represent MBP at 42 kDa and MBP or *E.coli* breakdown products at 40 and 35 kDa respectively.

Lane 1: 10 µL 10 kDa protein ladder (Gibco BRL) stained with Ponceau S
Lane 2: 20 µL of uninduced whole culture.
Lane 3: 10 µL of induced whole culture.
Lane 4: 20 µL of post induction whole culture supernatant
Lane 5: 20 µL of sucrose medium prior to osmotic shock.
Lane 6: 20 µL of expressed periplasmic protein following osmotic shock with ice cold 5 mM MgSO4.
Lane 7: 10 µL of periplasmic protein as described for lane 6.

protein sequence analysis and non-specific bacterial proteins cross-reacting with the anti-MBP (data not shown).

Figure 3.4 shows that the majority of the recombinant protein is being exported to the periplasmic fraction (lane 6) and very little fusion protein is found in the post induction whole culture supernatant (lane 4, this supernatant is collected post induction when the cells have been pelleted) or in supernatant following treatment of cells with sucrose (lane 5) prior to periplasmic osmotic shock. The whole culture fraction post induction (lane 3) contains a significant amount of fusion protein as expected (this is a fraction collected immediately after the expression period is completed prior to the cells being pelleted). A faint band corresponding to the fusion protein in the pre-induction (lane 2) whole culture is also visible and this may be due to some cellular leakage prior to induction.

Optimisation of expression of HT-1

To obtain the maximum amount of expression product with minimal amount of impurities and also to assess whether or not most of the protein was being expressed in the soluble periplasmic fraction, a series of expression experiments were performed to identify the optimum conditions for expression of the MBP/HT-1 fusion protein. The variable conditions included, IPTG induction concentration, temperature of induction period and length of induction period. The optimal conditions were found to be induction with 0.3 mM IPTG for 4 hours at 37°C.

IPTG concentration

The pMAL vectors have a *tac* promoter which is inducible by IPTG. The recommended induction concentration of IPTG was 0.3 mM (Protein fusion and purification system, NEB). The IPTG concentration was varied from a range of 0.01 mM to 0.5 mM for the optimisation experiments. All other variables were kept constant, the induction temperature was maintained at 37 °C and length of induction was for a period of 4 hours. The expressed proteins were extracted from the periplasmic fraction and detected by Western blotting as depicted in Figure 3.5 A and B.

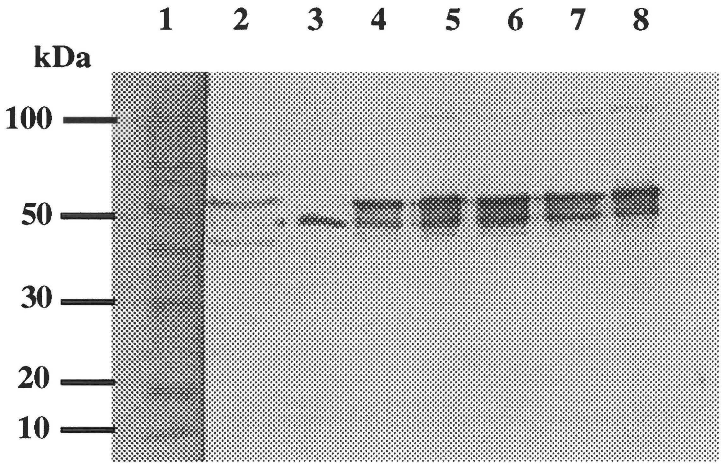
Figure 3. 5A shows the protein samples under non-reducing conditions and Figure 3.5 B under reducing conditions. The major difference between blots A and B in Figure 3.5 is that blot B shows the presence of higher molecular weight aggregates at approximately 200 kDa, which was expected as the samples were

Figure 3.5: Western blot of the periplasmic fraction of the MBP/HT-1 fusion protein under varying concentrations of IPTG.

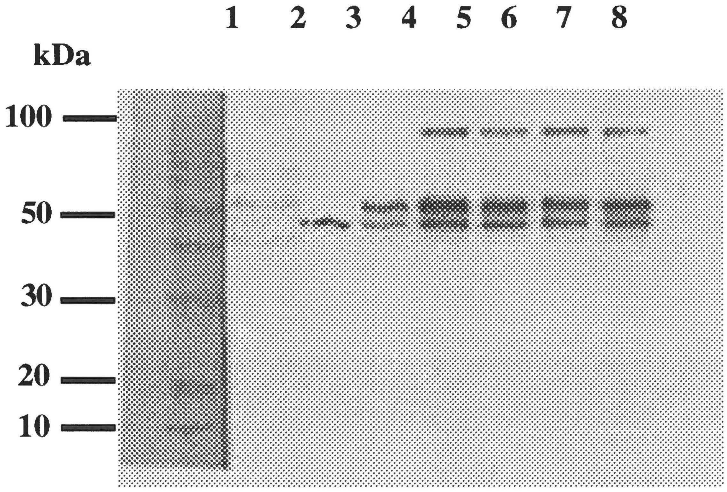
The samples were electrophoresed under reduced (blot A) and nonreduced (blot B) conditions through 12% SDS-polyacrylamide gels and blotted onto nitrocellulose. The nitrocellulose was probed with a 1/10 000 dilution of anti-MBP antibody followed by a 1/20 000 dilution of alkaline phosphatase conjugated anti-rabbit IgG. The fusion protein is the band visible at ~50 kDa (lanes 4-8) with the lower molecular weight band corresponding to MBP. Higher molecular weight aggregates at ~200kDa can be seen in lanes 5-8 in blot B

Lane 1: 10 µL 10 kDa protein ladder (Gibco BRL) stained with Ponceau S
Lane 2: 20 µL uninduced whole culture
Lane 3: MBP standard (80 ng) (NEB)
Lane 4: 5 µL expressed periplasm induced with 0.01 mM
Lane 5: 5 µL expressed periplasm induced with 0.05 mM
Lane 6: 5 µL expressed periplasm induced with 0.1 mM
Lane 7: 5 µL expressed periplasm induced with 0.3 mM
Lane 8: 5 µL expressed periplasm induced with 0.5 mM

A.



B.



electrophoresed non-reduced, while in blot A these higher molecular weight bands are barely visible due to the samples being electrophoresed under reducing conditions.

The difference in expression at the varying IPTG levels is most obvious at a very low concentration of 0.01 mM IPTG (lane 4) where there is less fusion protein present at 50 kDa compared to the concentrations in lanes 5-8. Figure 3.5B illustrates that the 0.3 mM concentration of IPTG yielded a marginally greater amount of fusion protein when compared with the other concentrations between 0.05 and 0.5 mM.

Temperature and period of induction

The MBP/HT-1 fusion protein was expressed under varying temperatures and lengths of induction period. These parameters were examined as it has been reported that lower growth temperatures and shorter periods of induction leads to the formation of fewer insoluble aggregates (Schein and Noteborn, 1998). The IPTG concentration was kept constant at 0.3 mM. Periplasmic fractions were visualised by Western blotting under reduced and non-reduced conditions as illustrated in Figure 3.6A and B. It is clear from these results that overnight induction (~18 hours, lanes 7-9) did not yield more expressed fusion protein than did induction for 4 hours (lanes 4-6). Supernatant fractions were also taken after 4 hours and overnight induction and at the various temperatures during these experiments, and it was found the overnight culture supernatants had more fusion protein than the 4 hour samples (result not shown). This indicated that prolonged induction results in leakage of the fusion protein from the cells into the supernatant fraction. This may explain why a reduction in the amount of periplasmic protein in the overnight samples is evident (Figure 3.6 A and B). Therefore, the optimal length of induction is for a shorter period such as 4 hours. The optimal temperature for induction with a 4 hour incubation appears to be at 37°C where there is the most amount of fusion protein present (lane 6).

Purification of HT-1/MBP using amylose column

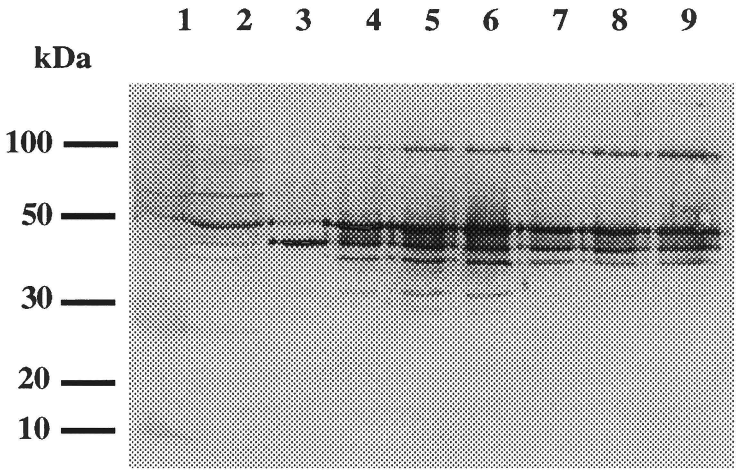
Purification of the HT-1/MBP fusion protein using the amylose affinity resin was not successful. The fusion protein was always found to elute in the flow through or wash fractions even under the most stringent conditions. Therefore an alternative strategy for purification was devised in which a 6 x His tag was incorporated into the C-terminal end of the MBP/HT-1 construct as depicted in Figure 3.7B.

Figure 3.6: Western blot of the periplasmic fraction of the MBP/HT-1 fusion protein at varying temperatures and induction period.

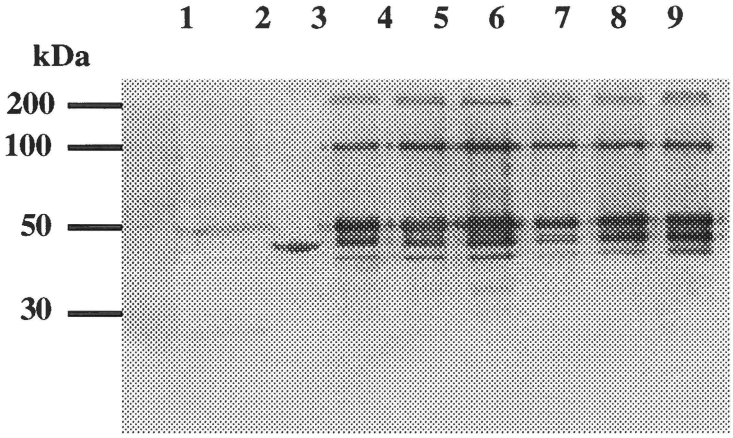
The samples were electrophoresed under reduced (blot A) and nonreduced (blot B) conditions through 12% SDS-polyacrylamide gels and blotted onto nitrocellulose. The nitrocellulose was probed with a 1/10 000 dilution of anti-MBP antibody followed by a 1/20 000 dilution of alkaline phosphatase conjugated anti-rabbit IgG. The fusion protein is the band visible at ~50 kDa (lanes 4-9) with the lower molecular weight band corresponding to MBP. Higher molecular weight aggregates at ~ 100 and 200 kDa can be seen in lanes 4-9.

Lane 1: 10 µL 10 kDa protein ladder (Gibco BRL) stained with Ponceau S
Lane 2: 20 µL uninduced whole culture
Lane 3: MBP standard (80 ng) (NEB)
Lane 4: 5 µL expressed periplasmic fraction at 25°C and 4 hour induction
Lane 5: 5 µL expressed periplasmic fraction at 30°C and 4 hour induction
Lane 6: 5 µL expressed periplasmic fraction at 37°C and 4 hour induction
Lane 7: 5 µL expressed periplasmic fraction at 25°C and 18 hour induction
Lane 8: 5 µL expressed periplasmic fraction at 30°C and 18 hour induction
Lane 9: 5 µL expressed periplasmic fraction at 37°C and 18 hour induction

A



B



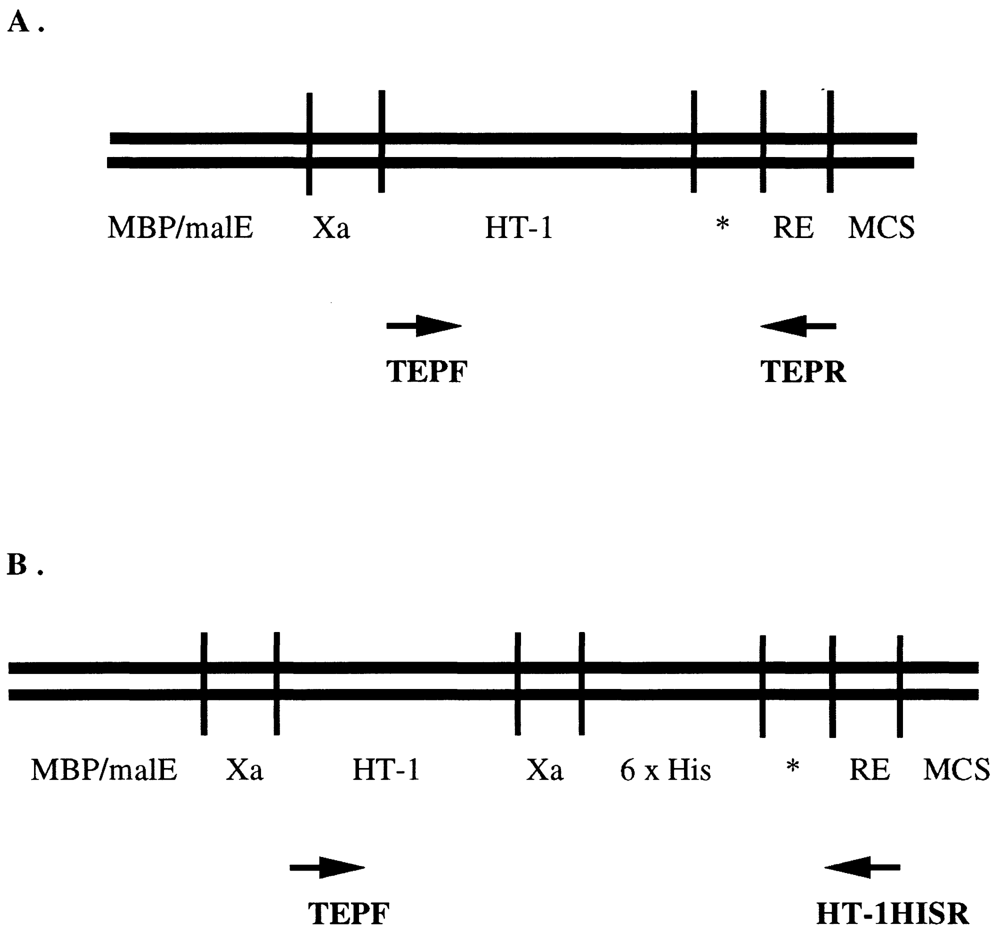


FIGURE 3.7. Schematic representation of HT-1 sequence cloning into the pMAL-p2 vector.

The double stranded lines correspond to linear plasmid DNA. The vertical bars distinguish different regions of sequence as indicated. Figure A, region of sequence amplified with primers TEPF and TEPR, Figure B, region of sequence amplified with primers TEPF and HT-1HISR. Abbreviations: MBP/malE, the maltose binding protein of pMAL-p2 containing the malE secretion signal sequence; Xa, factor Xa cleavage site; HT-1, HT-1 expression sequence; *, stop codons; RE, restriction endonuclease site; MCS, multiple cloning site of the pMAL-p2 vector; 6 x His, Histidine tag; TEPF, forward primer; TEPR, reverse primer; HT-1HISR, reverse primer.

Expression of the MBP/HT-1/His fusion protein

The 6 x His tag was incorporated into the existing MBP/HT-1 fusion protein construct via PCR using the primers TEPF and HT-HISR as described in Section 3.3. This new fusion protein construct not only incorporated a His tag but also an additional factor Xa cleavage site so that the 6 x His tag could also be cleaved from HT-1. The new fusion protein was then cloned into the pMAL^{TM}-p2 vector as previously described and the insert sequence was confirmed by restriction digest analysis and DNA sequencing. Expression optimisation experiments were carried out as for the MBP/HT-1 construct and the optimal expression conditions were found to be the same. An additional investigation was performed with the MBP/HT-1/His construct to ensure that most of the protein being produced was in a soluble form.

Expression of a soluble fusion protein

To ensure that most of the expressed fusion protein was being exported to the periplasm of *E. coli* as a soluble product, experiments were performed as described in Section 3.3 to assess the expression levels in the whole culture insoluble and soluble cellular extract, and to compare these results with periplasmic-extracted fusion protein. The results from these experiments are shown in Figure 3.8 and Figures 3.9. Figure 3.8 depicts a silver stained gel of the different soluble and insoluble fractions collected. The fusion protein is visible at 50 kDa and is typified in lane 5 where a sample of standard periplasmic extract was electrophoresed. The 50 kDa protein is visible in the soluble whole cell extract (lane 6) but not in the insoluble whole cell extract (lane 7). It is also absent from the insoluble whole culture cellular extracts, and the whole culture supernatant, thus indicating that the majority of the fusion protein is localised to the soluble periplasmic fraction of *E.coli*. These observations are supported by the Western blots in Figure 3.9A and B, where the soluble supernatant extract contains the greatest amount of detectable His tagged protein, under reducing and non-reducing conditions (lane 7).

Purification of the MBP/HT-1/His protein

Successful purification of the His tag fusion protein was achieved using the Talon resin (CLONTECH). However, elution of the fusion protein was only possible by stripping the Talon (metal) with EDTA. Though this yielded highly pure fusion

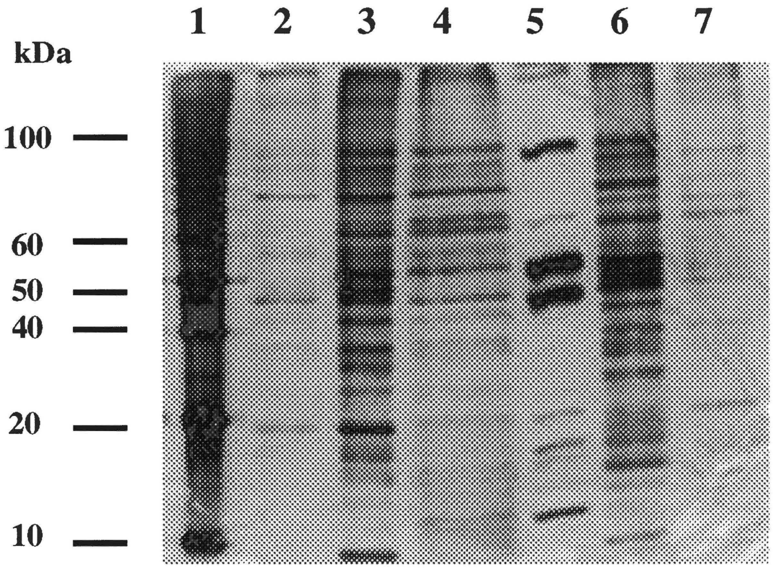


Figure 3.8: Silver stain of insoluble and soluble expression products of the MBP/HT-1 fusion protein.

The samples were electrophoresed through a 12% SDS-polyacrylamide gel under non-reducing conditions. The fusion protein is visible at ~50 kDa.

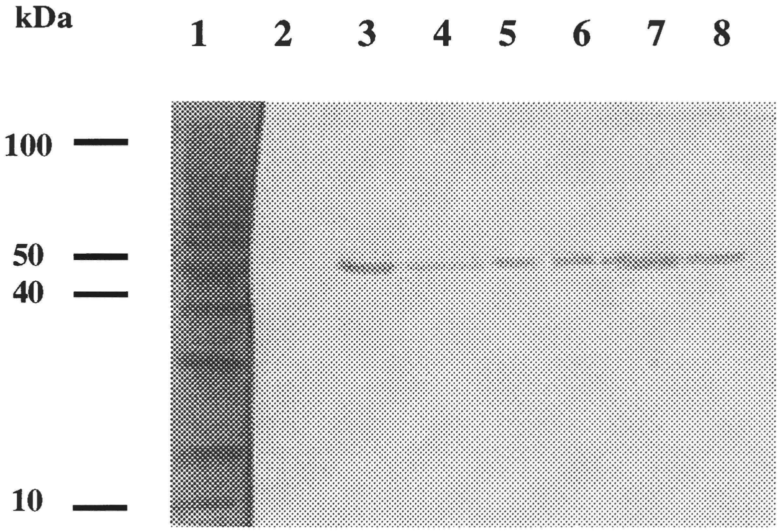
Lane 1: 1 µL 10 kDa protein ladder (Gibco BRL)
Lane 2: 1.5 µL uninduced whole culture
Lane 3: 1 µL induced whole culture
Lane 4: 20 µL post induction whole culture supernatant
Lane 5: 10 µL periplasmic extract
Lane 6: 1.5 µL soluble whole cell extract
Lane 7: 1.5 µL insoluble whole cell extract

Figure 3.9: Western blot of insoluble and soluble expression products of the MBP/HT-1/His fusion protein.

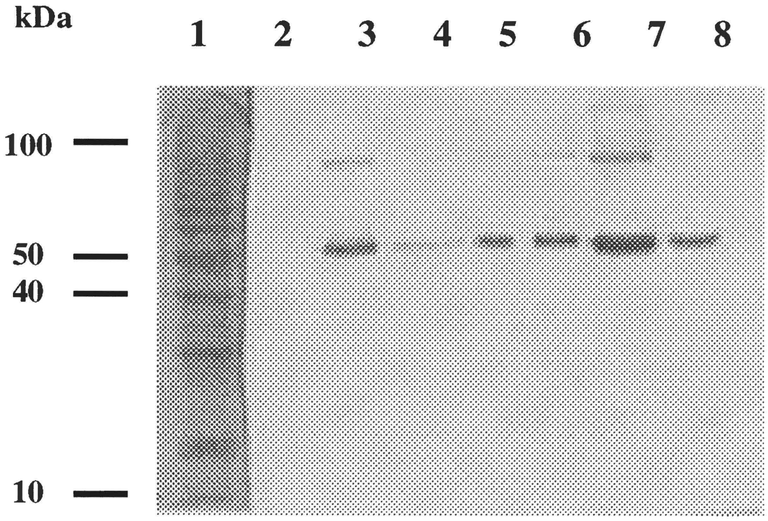
The samples were electrophoresed under reduced (blot A) and nonreduced (blot B) conditions through 12% SDS-polyacrylamdie gels and blotted onto nitocellulose. The nitrocellulose was probed with a 1/10 000 dilution of anti-His antibody followed by a 1/20 000 dilution of alkaline phosphatase conjugated anti-mouse IgG. The fusion protein is the band visible at ~ 50 kDa (lanes 3-8). Higher molecular weight aggregates at ~100 and 200 kDa visible in blot B.

Lane 1: 10 µL 10 kDa protein ladder (Gibco BRL) stained with Ponceau S
Lane 2: 20 µL uninduced whole culture
Lane 3: 10 µL induced whole culture
Lane 4: 20 µL post induction supernatant
Lane 5: 10 µL sucrose medium prior to osmotic shock
Lane 6: 5 µL periplasmic extract
Lane 7: 5 µL soluble whole cell extract
Lane 8: 5 µL insoluble whole cell extract

A.



B.



protein, it was not a very economical way of purifying the protein as a new batch of resin had to be prepared for each subsequent purification. Therefore scale up experiments were not feasible. Results from the Talon purification method are not shown.

An alternative approach to purifying His tag proteins was investigated using Ni-NTA (IMAC) affinity chromatography. Under denaturing conditions of 8 M urea the MBP/HT-1/His fusion protein was eluted in a highly pure form (Figure 3.10) with minimal amounts of unbound protein being collected in the flow through and wash fractions. This purified protein present in 8 M urea was dialysed into a physiological buffer such as PBS/Az. The protein was then suitable for subsequent manipulations such as factor Xa cleavage and immunological and biological assays. In addition, the Ni-NTA agarose could be easily be regenerated and reused several times.

Yield of protein

The yield of expressed periplasmic fusion protein was estimated following purification with the Ni-NTA column. The yield was assessed by spectrophotometric measurements at 280 nm. These estimates were then confirmed by BCA protein assay in which the concentration of the fusion protein was compared to MBP and BSA standards. From these assays it was estimated that the yield of the purified fusion protein was between 5-10 mg/L of expression culture.

Factor Xa cleavage

The tetrapeptide sequence of Ile-Glu-Gly-Arg, which is recognised by the protease blood coagulation factor Xa, was positioned immediately upstream from the HT-1 sequence and Histidine tag so as to cleave the MBP portion of the fusion protein. An additional factor Xa site was positioned between the His tag and HT-1 so as to cleave the His tag. Therefore, following cleavage HT-1 would potentially be left in a native form. Following 24 hours digestion several protein bands were detected by Coomassie staining and Western blot analysis with an anti-His antibody (result not shown). Low molecular weight bands which were visible (<10 kDa) were protein sequenced but were below the levels of detection for accurate data to be obtained. Size exclusion chromatography (Superdex 75, Amershram Pharmacia Biotech., Uppsala, Sweden) was used in an attempt to separate the protein bands. Peak

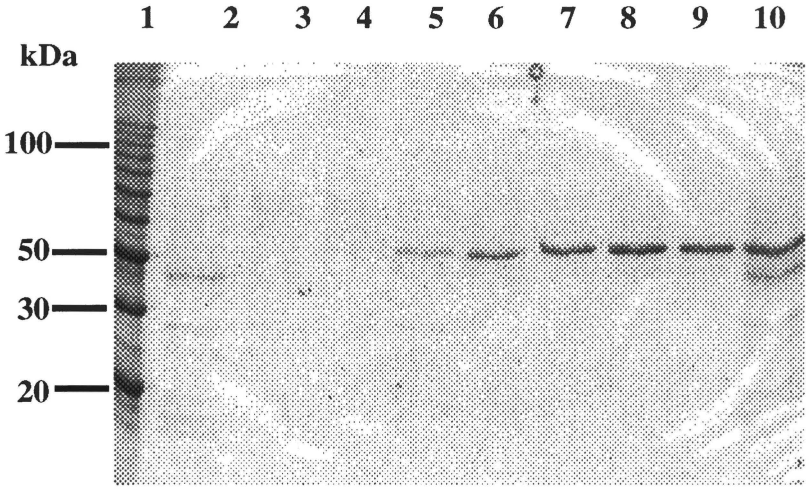


Figure 3.10: Coomassie stained gel of purified MBP/HT-1/His fusion protein using Ni-NTA resin.

The samples were electrophoresed through a 12% SDS-polyacryalmide gel under reducing conditions. The fusion protein is visible at ~50kDa.

Lane 1:	10 µL 10 kDa protein ladder (Gibco BRL)
Lane 2:	20 µL column flow through
Lane 3:	20 µL column wash 1
Lane 4:	20 µL column wash 2
Lanes 5-9:	20 µL elutions 1-5
Lane 10:	20 µL unpurified periplasmic fraction

fractions were collected and analysed by gel electrophoresis, but none were found to contain molecular weight products of less than 10 kDa as is expected for the HT-1 toxin (Matthew Padula, personal communication).

Expression of HT-1 in baculovirus

The expression of HT-1 in baculovirus was trialed in an attempt to express the HT-1 toxin without a fusion partner such as MBP but retaining the His tag for purification. In addition a methionine site was introduced for cleavage of the His tag from HT-1 with CNBr. The baculovirus method would also eliminate the suspected endotoxin contamination of recombinant proteins associated with *E.coli* expression which can be problematic in clinical trials. In addition, baculovirus expression has the potential to yield more of the soluble toxin (Kitts and Posse, 1993).

Amplification of the desired HT-1 fragment to be cloned into the BacPAK vector involved a two step procedure (Figure 3.11) whereby a complete signal sequence from elapid snake α-chain toxin (Sekuloski *et al.*, 1999) would be incorporated into the final product. This signal sequence would export the recombinant HT-1 protein from the insect cells into the cellular supernatant. This signal sequence had been used successfully in the laboratory for several other proteins. The final amplification product, obtained using ITSS2 and BacHT-1R primers is depicted in Figure 3.12. A PCR product of 260 bp was obtained in reactions 1-3 as expected. The positive control amplification using Met forward and reverse primers (primers which are irrelevant to baculovirus insert amplification but which span regions of the template DNA) gave a slightly smaller product as expected and the negative control reaction in lane 5 did not yield a product as there was no template in the reaction mix.

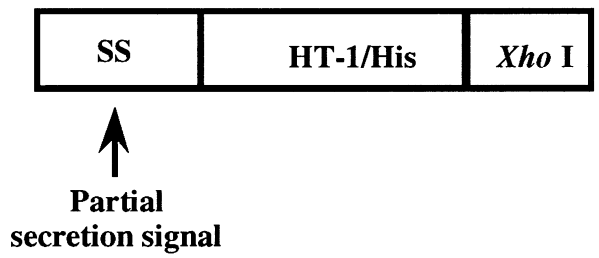
The amplified HT-1 toxin insert was then digested with the *Xho* I and *Bam*H I enzymes and ligated into the BacPAK 9 transfer vector which had been predigested with the same enzymes. The sequence of the cloned insert was confirmed, following which the Bac/HT-1/His DNA was transfected to form recombinant virus as described in Section 3.3. Following transfection the p1 and p2 phase cultures were analysed for the presence of recombinant virus via PCR (Figure 3.13) and Western blotting to detect expressed HT-1/His protein.

Recombinant virus was amplified with primers that flank the MCS of BacPAK 9 (Bac 1 and Bac 2) following extracellular lysis of virus. A product corresponding to

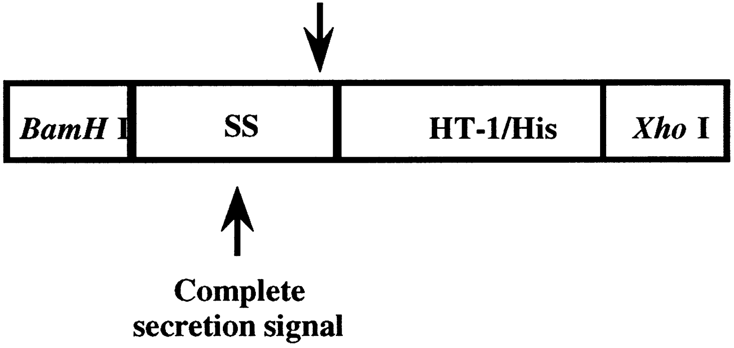
Figure 3.11: Schematic diagram of the cloning of HT-1/His into the BacPAK 9 vector and expression in a baculovirus system.

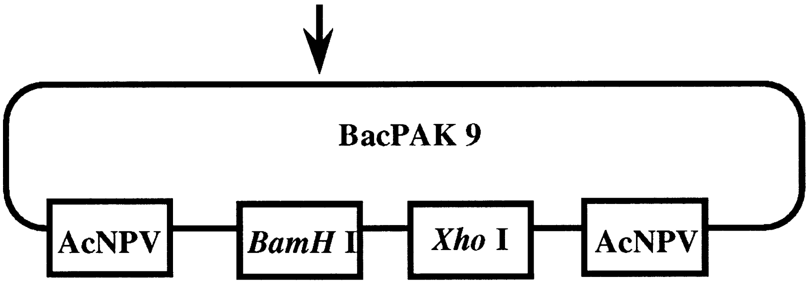
The BacHT-1F and BacHT-1R primers were used to amplify the HT-1/His sequence together with a *Xho* I site and part of the snake α-chain secretion signal (ss). In a second round PCR the primers ITSS2 and BacHT-1R were used to complete the sequence for cloning into the BacPAK 9 vector. The ITSS2 primer completed the sequence for the secretion signal and introduced a *BamH* I site. The amplified product was then ligated into the *BamH* I and *Xho* I sites of BacPAK 9. The Bac/HT-1/His DNA was then transfected into baculovirus and added to insect cells for expression.

First round PCR with BacHT-1F and BacHT-1R primers to amplify HT-1/His sequence, *Xho* I restriction site and part of the snake α-chain secretion signal.

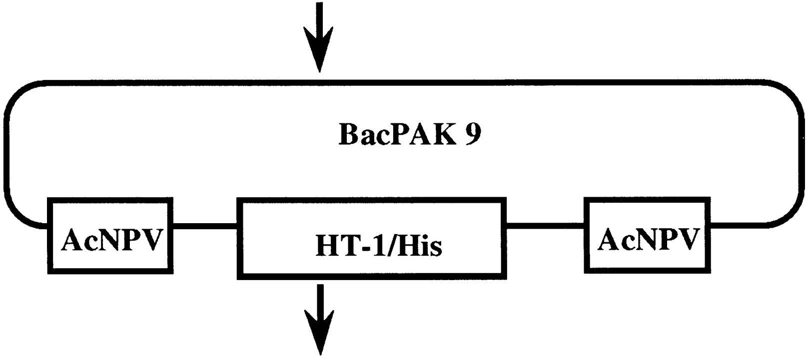


Second round PCR with ITSS2 and BacHT-1R to amplify remainder of secretion signal and *BamH* I site.





Ligation of amplified fragment into the *BamH* I and *Xho* I sites of BacPAK 9



Purify Bac/HT-1/His DNA on chromaspin column and transfect into baculovirus

Add transfection to Sf21 insect cells. Propagate cells/amplify recombinant virus and characterise gene expression

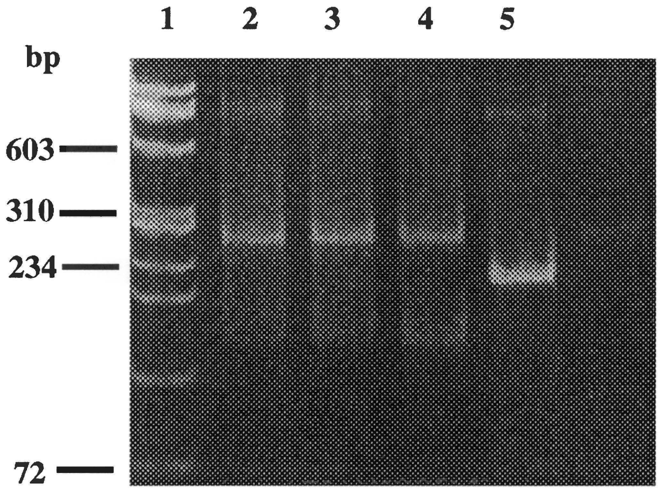


Figure 3.12: PCR amplification of the HT-1 gene with primers ITSS2 and BacHT-1R for Baculovirus cloning

PCR products (10 µL) were electrophoresed through a 10% polyacrylamide gel in the presence of DNA loading buffer.

Lane 1:	PhiX/Hae III DNA standards 500 ng (Promega)
Lane 2-4:	Serial dilutions of template DNA, 1/100, 1/1000 and 1/10 000 respectively. A PCR product of 260 bp was amplified as expected.
Lane 5:	Positive control PCR with primers Met Fwd and Met Rev. A product of 220 bp was amplified as expected.

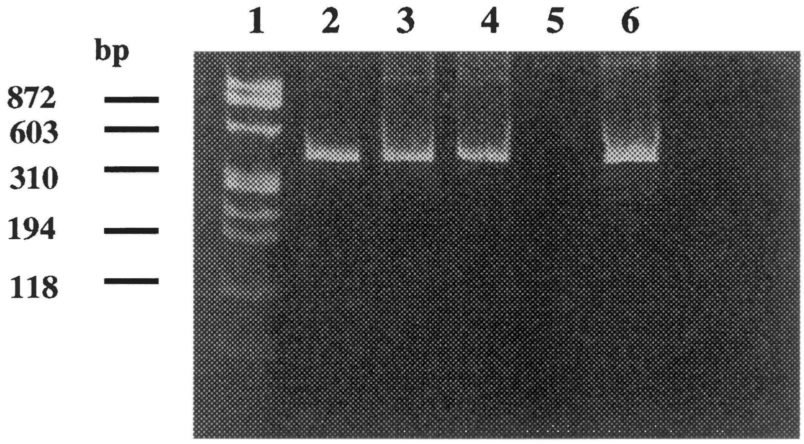


Figure 3.13: PCR amplification with Bac 1 and Bac 2 primers to screen for recombinant HT-1/His virus.

PCR products (10 µL) were electrophoresed through a 10% polyacrylamide gel in the presence of DNA loading buffer. A product of 400 bp was obtained as expected.

Lane 1: PhiX/Hae III DNA standards 500 ng (Promega)
Lane 2: P1 phase supernatant fraction
Lane 3: P2 phase supernatant fraction
Lane 4: P2 phase cellular fraction
Lane 5: Negative control PCR with primers Bac 1 and Bac 2.
Lane 6: Positive control PCR with primers Bac 1 and Bac 2 with cloned HT-1 plasmid DNA

the expected size of the insert and an additional (approximately) 100 bp flanking region of BacPAK 9 was amplified for the p1 and p2 supernatant and cellular fractions (lanes 2-5). This amplified product indicates that the Bac/HT-1/His DNA had infected the viral particles. The Western blot result however indicate that the insect cells infected with virus containing HT-1/His did not appear to be secreting the recombinant HT-1/His as the p1 and p2 phase supernatant fractions were not being detected when probed with the anti-His antibody (result not shown).

3.5: DISCUSSION

This chapter described the expression and purification of the *Ixodes holocyclus* HT-1 neurotoxin as a maltose binding fusion protein in an *E. coli* host system. It also details the attempt to express the HT-1 sequence in a baculovirus model in the absence of a fusion partner.

The purification of HT-1 from native engorged female ticks is time consuming, costly, and yields only a small amount of purified protein. Therefore to obtain large amounts of the toxin protein it was over expressed as a cleavable fusion protein (MBP/factor Xa) in *E. coli*. The expression of a protein fused to another protein fragment or short polypeptide tag sequence can have a number of benefits. It can increase the accumulation of the desired protein sequence and hence increase the availability of the starting material for protein purification. It can provide an epitope by which the fusion protein can be readily purified and/or immobilised, and it can provide a function by which the fusion protein can be detected and quantified reliably (Hockney, 1994). A secretion vector strategy using the pMAL^{TM}-p2 vector was chosen as it was thought that the four putative disulphide bonds in HT-1 were more likely to be properly formed when passing through the secretory pathway to the periplasm of *E. coli* rather than in the reducing environment of the cytoplasm. In addition, secreted HT-1 would potentially be more stable due to fewer extracellular proteases and could also be easily purified from the periplasmic media (Park *et al.*, 1991).

Several scorpion neurotoxins have been expressed successfully as fusion proteins. For example, a gene encoding charybdotoxin (ChTx) from the scorpion *Leiurus quinquestriatus* was expressed as a fusion protein in *E. coli*, fused to the gene *9* protein of T7 bacteriophage with a factor Xa cleavage site in the plasmid pSR9 (Park *et al.*, 1991). The fusion protein strategy was adopted due to difficulties in expressing the ChTx gene directly in bacteria. Stable, soluble and high level expression was obtained with the fusion protein. In addition, factor Xa cleavage was able to release ChTx from the fusion protein. The final yield of recombinant ChTx was 1 mg/L (Park *et al.*, 1991).

Some scorpion neurotoxin expression systems have advanced to a stage where the expressed protein can provide the basis for the design of new vaccines with improved properties for immunotherapy. For example, a recent study described how a single chain Fv (ScFv) against the scorpion neurotoxin AaH II was engineered into *E. coli* and efficiently produced in a soluble and active form (Mousli *et al.*, 1999). This ScFv against AaH II could neutralise the pharmacological and biological effects of the toxin. This was interesting as the ScFv, being a small molecule with high affinity to its target toxin, may be able to neutralise the toxin before it binds to its site of action. This research exemplified the long term aims of research into tick vaccine development using a recombinant toxin product. The remainder of this chapter discusses the progress that was made in expression of the HT-1 neurotoxin.

The construction and expression of the MBP/HT-1/His fusion protein

The predicted mature toxin sequence for HT-1 as depicted in Figure 3.2 (and derived from Figure 2.5) indicated that the mature sequence (excluding the signal peptide) commenced with a cysteine residue. As original N-terminal sequencing did not yield the N-terminal residues for HT-1 due to blockage (Thurn, 1994), the predicted signal was the only N-terminal sequence information available at the time. There was some doubt over the first residue being a cysteine as an N-terminal cysteine is not known to be responsible for any form of post translational modification which could cause such a blockage. Therefore, to ensure that the entire toxin sequence would be expressed, the N-terminal residue was extended to commence from the glutamic acid residue (E), 5 residues upstream. Glutamic acid can be acetylated during post translational processing which is a principle cause of N-terminal blockage (Han and Martinage, 1992). These extra 5 residues were thought not to have an effect on the overall folding pattern for HT-1, especially as it contains 8 cysteine residues (with no additional cysteines) which would stabilise the structure. Moreover, this would make the size of the signal sequence 18 residues instead of 23, which is consistent with the length of all known scorpion leader sequences, which are between 18-21 residues (Becerill *et al.*, 1995).

Optimisation of expression conditions

The expression and transport of the MBP/HT-1 fusion protein to the periplasmic space of *E. coli* was successful (Figure 3.4). Lower molecular weight products corresponding to MBP at 42 kDa and truncated products of MBP at 40 kDa were detected with the anti-MBP antibody. It was reported that at high cell densities nutrients can become limiting leading to the misincorporation of amino acids (Santos and Tuite, 1993) or premature termination of polypeptide chain elongation (Balbas and Bolviar, 1990). In addition, truncated expression products may result from proteolytic degradation. The lower molecular weight proteins at approximately

35 kDa were demonstrated as being non-specific *E.coli* proteins that were cross-reacting with the α-MBP antibody.

It was documented that different expression conditions may influence the yield of protein that is recovered from expression cultures (Hanning and Makrides, 1998). Therefore, the expression of the MBP/HT-1 fusion protein was optimised so that a maximum amount of soluble protein was obtained. The parameters which were optimised included the concentration of the inducer IPTG, the growth temperature and the period of induction.

The recommended concentration of IPTG was 0.3 mM. However, maximum yields of soluble protein using the *tac* promoter in bacteria have been reported with concentrations of IPTG of less than 0.05 mM (Kipriyanov *et al.*, 1997). Expression of the MBP/HT-1 fusion protein was therefore carried out at varying concentrations of IPTG from 0.01 mM to 0.5 mM. The different concentrations of IPTG inducer produced similar patterns of fusion protein expression in the periplasm of *E. coli* (Figure 3.5). The lowest concentration of 0.01 mM produced the least amount of fusion protein at 50 kDa, however the differences between the other concentrations up to 0.5 mM were marginal. Therefore, the recommended amount of 0.3 mM IPTG was used as the optimal concentration.

The effect of temperature on inclusion body formation is well known. In general, temperatures below 37°C have been reported to favour soluble periplasmic expression (Skerra and Pluckthun, 1991; Wetzel, 1992; Pluckthun, 1994). With proteins containing disulphide bonds failure to reach the correct conformation is often associated with incorrect disulphide bond formation (Mitralki and King, 1989). In addition, the high level of expression of a protein with a bacterial signal peptide often results in aggregation after transport to the periplasm. This may be caused by a high concentration of the fusion protein in the periplasmic space which may favour the formation of insoluble aggregates over correct folding (Kipriyanov *et al*, 1997). The higher molecular weight aggregates observed in Figures 3.5 and 3.6 are due to erroneous disulphide bond formation as the aggregates were essentially eliminated as evidenced by SDS-PAGE electrophoresis under non-reducing conditions. The optimal yield of fusion protein was observed at 37°C (lane 6, Figure 3.6), with temperatures at 30 and 25°C inducing less fusion protein at 50 kDa.

It has been suggested that prolonged levels of expression, particularly at higher temperatures can cause the outer membrane of *E. coli* to become permeable

(Pluckthun and Skerra, 1989). Overnight expression of the MBP/HT-1 fusion protein did not yield more expressed fusion protein than did induction for 4 hours. (Figure 3.6). Whole cell supernatant fractions were collected in addition to the periplasmic fractions during these experiments. It was found that the overnight culture supernatants contained more fusion protein than the 4 hour supernatant fractions. This leakage into the surrounding medium of *E. coli* would contribute to the reduction in the amount of periplasmic protein visible in the overnight samples. Therefore, the induction period for the MBP/HT-1 fusion protein was 4 hours for all subsequent experiments.

Affinity Purification

The purification of the MBP/HT-1 fusion protein using the amylose resin did not yield any protein, possibly due to incorrect structural conformation of the recombinant protein. Therefore, an alternative construct was cloned into the pMAL-p2 vector whereby a histidine tag was incorporated at the C-terminus of the existing MBP/HT-1 sequence. The histidine tag was chosen because it was small, easy to incorporate into the construct via PCR, and the product could be purified due to its affinity for various metals (Uhlen and Moks, 1990). As a histidine tag contains no natural cleavage site (Table 3.2) an additional factor Xa site was incorporated between the tag and HT-1 (Figure 3.7).

Soluble expression into the periplasm with the MBP/HT-1 His fusion protein was achieved and was found to be optimal under the conditions determined for the original MBP/HT-1 construct. Highly purified MBP/HT-1/His protein was achieved using Ni-NTA agarose under denaturing conditions (Figure 3.10). Although the majority of the expressed fusion protein was shown to be in the periplasm in a soluble form (Figure 3.8 and 3.9) it could only bind to the column under highly denaturing conditions of 8M urea. It was documented in many cases that over time soluble material precipitates in the periplasm and it is therefore necessary to resolubilise the protein. This is usually done, as was for MBP/HT-1/His, using a chaotropic agent such as urea or guanidine (Verma *et al.*, 1998). The yield of fusion protein determined post-purification was found to be 5-10 mg/L. This yield was sufficient for the subsequent manipulations that were required. These included its uses in factor Xa cleavage, as an antigen in ELISA, mouse *in vivo* assays, clinical trial studies and binding studies.

Tag	**Purification**	**Cleavage**
Polyarginine	Cation exchange	Carboxypeptidase B
Polyhistidine	IMAC	Not available
HDHDH (his-Asp)n	IMAC	Renin
Protein A	IgG column	Chemical
GST-transferase	GSH column	Thrombin
Maltose binding	Amylose column	Factor Xa

Table 3.2: Some to the tags used for fusion proteins to simplify purification.

Cleavage is usually by proteolytic enzyme, and specific sites other than those indicated below may be engineered at the fusion site (adapted from Scopes, 1994).

Cleavage of the purified fusion protein was subsequently attempted to obtain the HT-1 recombinant product in the absence of any fusion partners. Partial cleavage with factor Xa was achieved resulting in a multiple banding pattern visible on SDS-PAGE. A band at less than 10 kDa, which may correspond to the HT-1 toxin or HT-1 attached to a His tag, was visible by gel electrophoresis and Western blotting. However, as this band could not be protein sequenced due to a limitation in the amount of detectable protein, its identity could not be confirmed. It is well documented that factor Xa cleavage of proteins can be unreliable. For example, Nagai and Thogerson (1987), report that the cleavage rate of factor Xa often depends on the sequence following the cleavage site, and some fusion proteins are cleaved internally although the sites do not have the tetrapeptide recognition sequence.

Expression of the HT-1/His fusion protein in Baculovirus

A number of problems were encountered with the MBP/HT-1/His construct in the pMAL-p2 vector . These included the inability to cleave the large MBP fusion and His_6 proteins from the HT-1 gene using factor Xa and concern over problems with endotoxin production in *E.coli* . Endotoxin is toxic to vertebrates and therefore would not be safe for vaccine development in dogs (Marston, 1986). Therefore, an alternative expression strategy was investigated. This strategy involved expressing HT-1 with a His tag and a CNBr cleavage site in a baculovirus system.

The baculovirus system from CLONTECH has been successfully used to express a variety of snake phospholipase inhibitors, snake phospholipase A and a ScFv melittin immunotoxin in the laboratory (Andre Choo, personal communication). Expression in baculovirus would provide a product devoid of contaminants toxic to vertebrates and would potentially produce a large amount of soluble protein.

The HT-1/His sequence was amplified fused to an N-terminal secretion signal sequence from PLA2 snake α-chains. This secretion signal had been used with the proteins mentioned above to successfully export them to the baculovirus culture supernatant. The HT-1/His gene viral construct appeared to successfully infect the cells as recombinant virus was able to be detected by PCR (Figure 3.13) in the supernatant and cellular fractions post-infection. However, expressed protein could not be detected by Western blot analysis using an anti-His antibody (result not shown).

Difficulties encountered with expression in baculovirus have been attributed to a number of factors. These include low, inefficient or partial transcription of the gene, inefficient translation of the mRNA, inefficient secretion of the gene or the rapid uptake and metabolism of the peptide due to protein instability (Carbonnel *et al.*, 1988, McCutchen *et al.*, 1991). A detailed study by Matsuura and coworkers (1987) presented data which indicated that the level of expression of a foreign protein is largely related to the representation of the 5' upstream sequence of that gene.

A number of studies have therefore looked at the signal peptide sequence fused N-terminally to the gene of interest. Although it is unclear if any features of signal peptides other than their hydrophobicity are recognised by the eukaryotic cells secretory machinery, it has been observed in other systems that different signal peptides give different levels of secretion (Kaiser and Botsein, 1990). Moreover, the fusion of foreign proteins with a consensus signal peptide has not resulted in an increased amount of protein from insect cells (Devlin *et al.*, 1989). Tessier and coworkers (1991) investigated the effect of the substitution of the signal peptide of the precursor of the plant cysteine protease papain (propapain) with signal peptidase derived from a 21 amino acid melittin signal peptide. They found that the melittin signal peptide secreted over 5 times more papain precursor than the wild-type prepropapain which used the plant signal peptide.

There have been several reports on the expression of scorpion and spider neurotoxins in baculovirus where an improvement in the yield of baculovirus products were obtained by substituting secretion signal sequences. For example, the expression of the anti-insect α-scorpion toxin LqhαIT (from the scorpion *Leiurus quinquestriatus hebraeus*) in baculovirus was successful with its own secretory leader sequence which was properly processed by the insect cells (Chejanovsky *et al.*, 1995). In contrast the AaIT insect selective scorpion toxin from *Androctonus australis* was fused to a signal peptide coding sequence of an insect protein from the silk worm named bombyxin and to the baculovirus protein gp67, both of which produced soluble and functionally expressed protein (McCutchen *et al.*, 1991; Stewart *et al.*, 1991).

The expression of the spider toxin α-LTX (which lacks its own signal sequence) in baculovirus by Ichtchenko and co workers (1998) involved the addition of a signal peptide from the baculovirus glycoprotein gp67 to the N-terminus of the recombinant toxin to enhance its secretion. In addition, they inserted a histidine tag at the N-and C-terminal ends of the toxin to allow efficient purification using Ni agarose. The α-LTX was expressed and purified in a highly active form. In a separate study α-LTX was expressed in baculovirus using a melittin signal peptide and this construct also possessed all the properties of native α-LTX (Volynski *et al.*, 1999).

The expression of HT-1 in baculovirus requires re-examination. A number of factors including protein stability, translational problems and choice of leader sequence have to be reconsidered before expression of HT-1 in baculovirus is repeated. The successful expression of HT-1 as a cleaved protein would benefit future studies for vaccine development. These benefits would include providing recombinant material for site directed alterations which would help elucidate the structural motifs involved in binding and activity of the toxin. In addition, large amounts of recombinant HT-1 can be used for three dimensional structural analysis, monoclonal antibody production, and biological and toxicity assays.

CHAPTER 4

IMMUNOGENICITY AND PROTECTION ASSAYS OF THE RECOMBINANT HT-1 FUSION PROTEIN

4.1: PREVIEW

This chapter describes the assessment of the immunogenic and protective properties of the recombinant HT-1 fusion protein in biochemical and biological assays. The immunisation protocols and challenge experiments were carried out in mouse and dog animal models. The results provide some insight into the requirements for the development of a protective recombinant vaccine against *Ixodes holocyclus* tick paralysis.

Note: The recombinant HT-1 fusion protein antigen refers to the purified MBP/HT-1/His bacterial construct, described in chapter 3.

4.2: INTRODUCTION

The administration of a polyclonal dog antiserum is a recognised therapeutic means to alleviate paralysis symptoms caused by the tick *Ixodes holocyclus*. However, the efficiency of this treatment remains strongly dependent on the rapidity with which the specific antiserum is administered. In contrast, pre-existing circulating antibodies would neutralise the toxic antigens soon after the tick takes its blood meal. Therefore, a vaccine to protect animals and persons at risk from the effects of the *I. holocyclus* paralysis toxins would be desirable to prevent tick paralysis.

A high degree of immunity is obtained when both rabbits and dogs are immunised with a toxoid preparation developed by treating partially purified extracts of *I. holocyclus* tick neurotoxin with gluteraldehyde (Stone and Binnington, 1986; Stone *et al.*, 1986). This toxoid enabled rapid hyperimmunity and large doses were tolerated as the neurotoxin was inactive. However, the commercialisation of such a toxoid was impractical due to the problem of obtaining sufficient toxin from ticks to satisfy the number of doses required for treatment.

Research into scorpion neurotoxins have indicated that there are a number of methods that can be used to obtain non-toxic but protective neurotoxins. Chavez-Olortegui and coworkers (1997) described the isolation of a non-toxic protein from the toxic fraction of *Tityus serrulatus* scorpion venom using chromatography techniques. This non-toxic protein was used to immunise animals and produce polyclonal antibodies which were capable of recognising and neutralising the toxins in the native *T. serrulatus* venom. The problem with this non-toxic protein was again obtaining sufficient material to fulfil the large number of required immunisations.

Neutralising antibodies have also been produced against scorpion toxins with the use of synthetic peptides. For example, a synthetic octapeptide corresponding to the N-terminal extremity of the neurotoxin AaH II from the scorpion *Androctonus australis* was rendered immunogenic by coupling it to a suitable protein carrier [keyhole limpet hemocyanin (KLH)] (Devaux *et al.*, 1996). The anti-peptide anitsera specific for this synthetic peptide were neutralising to native AaH II. The use of synthetic peptides as opposed to whole or part of the venom has advantages as they are non-toxic and have a low cost and reproducible production (Calderon-Aranda, 1995).

Studies based on developing vaccines against toxic components of scorpion venoms have largely focussed on recombinantly produced toxins. For example, the Bot XIV toxin from the scorpion *Buthus occitanus tunetanus* was expressed in *E. coli* as a fusion protein, which when cleaved produced neutralising antibodies against the native toxin (Bouhaouala-Zahar *et al.*, 1996). In addition, a ScFv was engineered from the monoclonal antibody 4C1 against AaH II. This ScFv was effectively expressed in bacteria in a soluble and active form capable of neutralising the pharmacological and biological effects of the AaH II native toxin (Mousli *et al.*, 1999).

Purified intact recombinant fusion proteins (that is, those whose fusion partner has not been cleaved) have been used in the development of vaccines not only for neurotoxins but also against foot and mouth disease virus (FMDV), bovine papilloma virus (BPV) and cholera toxin (Kleid *et al.*, 1981; Jacob *et al.*, 1985; Pilacinski *et al.*, 1986). Fusion proteins have also been used in the development of diagnostic kits for the AIDS retrovirus (Muesinget *al.*, 1985) and to demonstrate biological activity of the Fc portion of IgE (Liu *et al.*, 1984). This chapter describes how the neurotoxin HT-1 from *Ixodes holocyclus,* which was successfully expressed in a bacterial system as a fusion protein (Chapter 3), was assessed for the ability to protect mice and dogs from the lethal effects of the *I. holocyclus* crude tick extract by the production of protective antibodies.

4.3: MATERIALS AND METHODS

MATERIALS

All chemicals and reagents were of analytical research grade unless otherwise stated. Freund's complete and incomplete adjuvants were purchased from Sigma (St Louis, MO, USA). Molecular weight standards used in Western blot analysis were from Gibco BRL (Gaithersberg, M.D, USA). Commercial dog antisera was a gift from Australian Veterinary Serum Laboratories (AVSL). Engorged adult female *I. holocyclus* ticks were kindly donated from veterinary practices from the Northern and North-Western suburbs of Sydney. These ticks were removed from dogs requiring treatment for tick paralysis and were stored frozen at -20°C.

METHODS

SDS-PAGE electrophoresis and Western blotting

SDS-PAGE, Western blotting, protein staining and protein concentration determination were carried out as described in Chapter 3 (Section 3.3).

ELISA

Antigen at the appropriate concentration (50 μL/well) was incubated in a 96-well polyvinyl EIA microtitre plate (Costar, Cambridge MA, USA) in carbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ and 0.02% NaA3) overnight at 37°C. Following 3 washes (200 μL/well) with PBS/Az, the non-specific binding sites were blocked with 3% BSA/PBS-Az for 1 hour at 37°C (200 μL/well). Fifty microlitres of the required dilution of primary antibody was then added to the plate and incubated at 37°C for 1 hour. Bound antibodies were then detected with the required alkaline phosphatase conjugated secondary IgG antibody at 50 μL/well (Sigma, St Louis MO, USA). The bound immune complexes were visualised by the addition of *p*-nitrophenyl phosphate (NPP) substrate (Sigma , St Louis MO, USA) (1 mg/mL) in ELISA substrate buffer (0.1 M glycine, 1 mM MgC12, 1 mM ZnC12, pH 10.4). Plates were developed at 37°C for 10-30 minutes. The reaction was stopped by the addition of 3 M NaOH. Absorbance was determined at 405 nm using an Organon Teknika micro-Elisa reader. Between each incubation step the wells were washed three times with PBS/Az containing 0.05% Tween-20 (200 µL/well).

Pre-Incubation ELISA

Mouse or dog antisera were preincubated for up to 2 hours with an excess amount (7.6 µg/mL) of MBP2 protein standard (New England Biolabs) to absorb binding of MBP in the fusion protein antigen. An irrelevant recombinant Histidine-tagged protein (donation from Andrew Zybenko, QIAGEN, Australia) was preincubated in excess (1.8 µg/mL) to absorb binding to the 6 x His tag of the HT-1 fusion protein antigen. The preincubated antisera mix was then used as the primary antibody in ELISA.

ELISA Antibody Titres

The end point antibody titres were calculated based on the highest antisera dilution which yielded a significant antibody response. A significant response was greater than 0.1 OD units at 405 nm.

Crude Tick Preparation

Engorged ticks were allowed to thaw and then dissected through their midline with a scalpel. The ticks were then homogenised in a 50 mL manual glass homogeniser containing ice-cold MilliQ water (100 µL/tick). The homogenate was then centrifuged at 12 000 *g* for 20 minutes at 4°C. The pellet was resuspended in two volumes of MilliQ water and homogenised as described above. The combined supernatants containing tick neurotoxins were stored at -20°C.

Crude Tick Homogenate Paralysis Assay

The crude tick homogenate was assessed for the ability to cause paralysis symptoms at a range of serial dilutions from 1:2 to 1:128. The assay was carried out in 5 day old neonatal Balb/C mice weighing between 3-5 g. Crude tick homogenate at each dilution was injected s.c using a Hamilton syringe. The mice were monitored for the development of typical paralysis symptoms following an 18 hour incubation with the injected crude tick homogenate. Characteristic tick paralysis symptoms follow those described by Ilkiw *et al.* (1987). The first stage of paralysis is characterised by signs of in-coordination of the hind quarters. This is followed by hind limb and then forelimb paralysis. The final stages involve paralysis of the head and respiratory muscles and lastly death due to respiratory failure.

Recombinant HT-1 Fusion Protein Assay

The purified recombinant HT-1 fusion protein was assayed for toxicity by s.c. injection into 5 day old neonatal Balb/C mice weighing between 3-5 g. The fusion protein at 1.2 mg/mL was assayed for toxicity in duplicate by injection of 100 µL at 1.2 mg/mL, 0.6 mg/mL and 0.24 mg/mL. Mice were observed for the development of paralysis symptoms over an 18 hour period.

Mouse Immunisation Protocol

Adult female Balb/C mice weighing between 20-30 g received via i.p. injection, 200 µL of purified recombinant HT-1 fusion protein at a concentration of 25 µg/200 µL in complete Freund's adjuvant. Secondary and tertiary i.p. booster injections of 12.5 µg/200 µL fusion protein in incomplete Freund's adjuvant were administered at 2 week intervals. Following the immunisation protocol mice were anaesthetised (1 part O_2, 2% halothane, 2 parts N_2O) and bled via cardiac puncture. The blood was allowed to clot overnight at 4°C, recentrifuged and antisera was collected and stored at -20°C.

Mouse Protection Assays

Control groups of mice were injected with normal (non-immune) mouse sera or AVSL dog antiserum. As a positive control for paralysis, mice were immunised with crude tick homogenate at a 1:16 dilution (without sera). Each dilution of immune sera or control sera were pre-incubated with the 1:16 dilution of crude tick homogenate for 30 minutes to 1 hour at room temperature. Therefore, neutralising antibodies in the anitsera bound to toxin *in vitro*. The mixture was then administered to the mice by s.c. injection. The mice were monitored for the development of paralysis symptoms over an 18 hour period. Results were recorded at 4, 8 and 18 hours post injection.

Dog Immunisation and Protection Protocols

All experiments and materials relating to dog immunisations, antisera collection and dog protection assays (aside from HT-1 fusion protein and crude tick homogenate) were devised, performed and supplied by Bahrs Hill research station, Bahrs Scrub, QLD, Australia (Terence Hopkins, Bayer Australia LTD, QLD).

Crude Tick Homogenate

Crude tick homogenate was administered undiluted to the dogs s.c. at 0.25 mL/10 kg and 1.25 mL/10 kg. Dogs were observed for the development of paralysis.

Immunisation and Challenge Assays

Table 4.1 details the immunisation protocol and physiological characteristics of the individual dogs. Three groups of 8 dogs were each immunised with 2 x 1 mL doses of either 10, 100 or 1000 μg/mL of recombinant HT-1 fusion protein by s.c injection in a proprietary glycolipid adjuvant (Bayer adjuvant system - R1005 diluent) at days 0 and 14. Sera was collected from dogs at days 0, 14 and 35. At day 35 the immunised dogs were challenged with 8 live *I. holocyclus* ticks and observed for the development of characteristic paralysis symptoms. The dogs were rescued from severe paralysis and death by the administration of commercial dog antisera.

Antigen dose	***Dog number***	***Total body weight (kg)***	***Sex***	***Breed***
10 µg/mL	7	18.5	F	German Shepherd
	18	23.3	M	Cattle Dog X
	22	32.8	F	Rottweiler X
	23	15.0	F	Staffordshire X
	27	17.2	F	Cattle Dog
	29	24.3	F	Border Collie
	30	22.3	F	Crossbred
	31	12.4	F	Kelpie
100 µg/mL	2	17.6	F	Cattle Dog X
	3	23.4	M	Kelpie X
	9	25.5	F	Staffordshire X
	12	16.7	F	Bull Terrier X
	20	6.7	F	Chihuahua
	25	22.2	M	Border Collie X
	34	22.3	M	Bull Terrior X
	35	27.5	M	Cattle Dog
1000 µg/mL	10	23.1	M	Dalmation X
	39	18.9	F	Border Collie X
	15	15.3	F	Border Collie X
	17	30.8	M	German Shepherd
	26	17.2	F	Border Collie X
	28	25.0	M	German Shepherd
	36	22.2	M	German Shep. X
	38	22.4	M	Border Collie X

Table 4.1: Dog Immunisation Protocol

24 dogs of varying species, sex and weight, were subject to 2 s.c doses (14 days apart), of 10, 100 or 1000 µg/mL of HT-1 fusion protein antigen emulsified in a proprietary Bayer adjuvant system (R1005 diluent).

4.4: RESULTS

MOUSE ASSAYS

Crude Tick Homogenate

Neonatal mice were monitored for the development of symptoms of tick paralysis following s.c. injection with crude tick homogenate (Table 4.2). Symptoms of characteristic progressive tick paralysis were observed with 1:32 to 1:2 dilutions. The 1:2 dilution caused death due to paralysis and respiratory failure following the 18 hour incubation. The dilutions of 1:64 and 1:128 resulted in no signs of paralysis. Therefore the 1: 16 dilution was chosen as the paralysing dose.

Immunoreactivity of the HT-1 Fusion Protein Mouse antisera

Antisera collected from adult mice that had been immunised with the purified fusion protein were assessed for its reactivity against the HT-1 fusion protein and crude tick homogenate antigens in Western blot and ELISA (Figures 4.1 and 4.2). Western blot analysis (Figure 4.1A, lane 2) revealed that mouse immune sera reacted with the HT-1 fusion protein antigen, while sera from non-immune mice did not (lane 4). The immune sera bound to a 50 kDa protein in lane 2 as well as products of lower molecular weight corresponding to MBP at 42 kDa and MBP breakdown products. These lower molecular weight MBP proteins are being detected as antibodies were being raised against the MBP portion of the fusion as well as to HT-1. The detection of the breakdown products suggests that the purified material used as antigen (visible as a single intact band in Figure 3.10) may be unstable over time. Western blot analysis also indicated that the mouse immune sera did not recognise native crude tick material (Figure 4.1 B, lane 2). This was possibly due to the low levels of HT-1 present in the crude tick preparation, or due to the low amounts of antibodies to HT-1 present in the antisera. The mouse antisera was also shown to produce antibodies to the fusion protein antigen as assessed by ELISA. The immune mouse sera bound to the immobilised HT-1 fusion protein antigen at a range of dilutions (Figure 4.2) and had titres of >10 000 for antigen at concentrations of 1.25 μg/mL to 100 μg/mL.

Mouse Protection Experiments

The results presented in Table 4.3 represent collated average results from a series of five protection experiments that were performed with mouse immune sera. Immune

Dilution of Crude Tick Homogenate	*Paralysis Symptoms*
1:2	death
1:4	total paralysis and respiratory problems
1:8	total body paralysis
1:16	front and hind limb paralysis
1:32	hind limb paralysis
1:64	normal
1:128	normal

Table 4.2: Paralysis symptoms observed in neonatal mice following sub-cutaneous administration of crude tick homogenate.

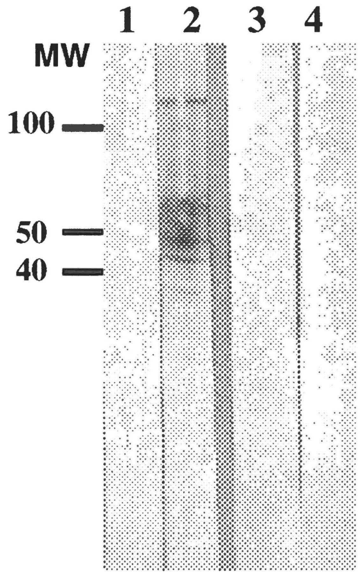
Neonatal mice weighing approximately 5 g were injected in duplicate with 100 μL of a dilution of crude tick homogenate. Mice were observed for the development of typical tick paralysis symptoms following an 18 hour incubation period.

Figure 4.1: Western blot of immune and non-immune mouse sera binding to HT-1 fusion protein and crude tick homogenate.

The HT-1 fusion protein (panel A) or Crude tick homogenate (panel B) were electrophoresed (10 μL and 5 μL/well respectively) nonreduced through a 15% SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose was probed with a 1/100 dilution of anti-mouse sera followed by a 1/10 000 dilution of alkaline phosphatase conjugated anti-mouse IgG. Control lanes were incubated with secondary antibody only to assess non-specific binding. The fusion protein is the band visible at ~50 kDa in lane 2 of panel A. Higher molecular weight bands at ~100 kDa correspond to higher molecular weight aggregates of the fusion protein. Lower molecular weight products represent MBP and its breakdown products as well as non-specific *E.coli* proteins.

Lane 1: secondary antibody control for immune mouse sera.
Lane 2: specific binding of immune mouse sera.
Lane 3: secondary antibody control for non-immune mouse sera.
Lane 4: specific binding of non-immune mouse sera.

A.



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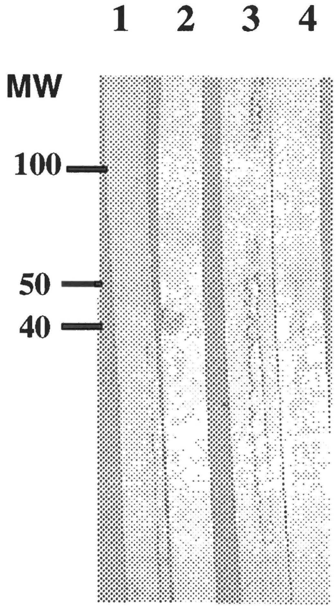

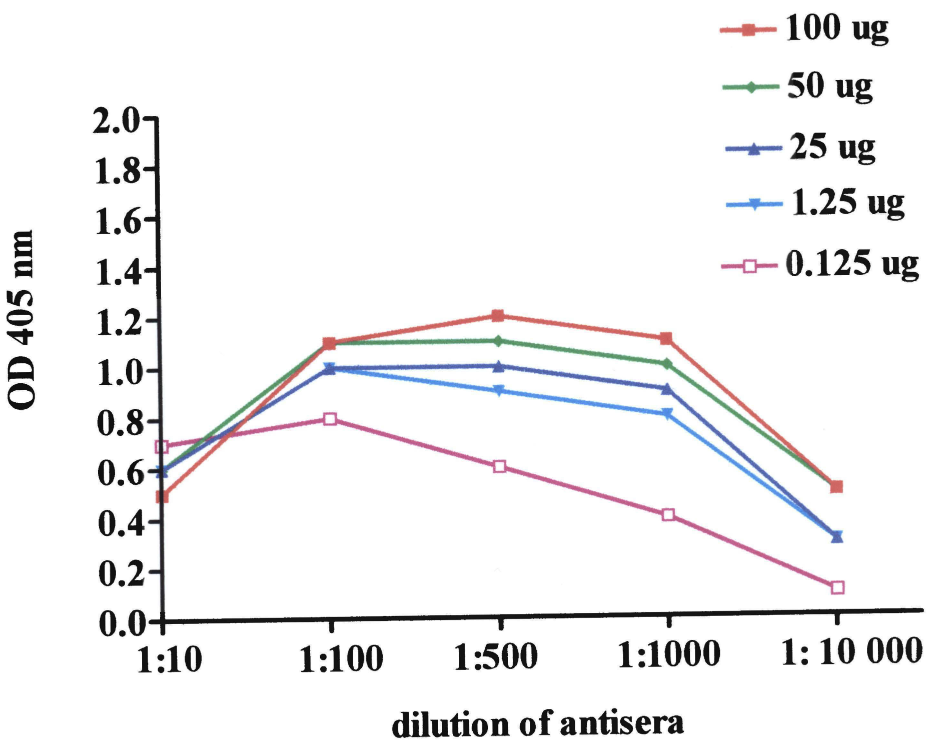

Figure 4.2: Binding of immune mouse sera against varying concentrations of HT-1 fusion protein antigen.

Serial dilutions of HT-1 fusion protein antigen concentrations of between 0.0125 and 100 μg/mL were immobilised to the wells of an EIA plate and subsequently blocked with 3% BSA. Immune mouse sera was added to the wells and serially diluted. The bound immune mouse sera antibodies were detected with alkaline phosphatase conjugated anti-mouse IgG and visualised by the addition of *p*-NPP. The absorbance was measured at 405 nm.

Table 4.3: Protection experiment in neonatal mice.

Neonatal mice each weighing approximately 5 g were administered, in triplicate, s.c with 100 μL of each of the test samples listed. The dilutions refer to the final dilution of sera when combined with a 1:16 final dilution of native tick toxin (crude tick homogenate). Typical paralysis symptoms were observed over an 18 hour period and recorded at 4, 8 and 18 hours post injection.

Degree of paralysis symptoms:

(-) = normal: mice showed no sign of paralysis.
(+) = hind limb paralysis: mice showed paralysis in their hind limbs only.
(++) = complete paralysis: mice showed signs of paralysis in the front and hind limbs.
(+++) = dead: mice were dead due to respiratory failure following complete paralysis.

Serum Tested	*Serum Dilution*	*Crude Tick Homogenate Dilution*	*Paralysis Symptoms Post Immunisation*		
			4 hours	8 hours	18 hours
N/A	N/A	1:16	+	++	+++
non-immune mouse sera	1:2	1:16	+	++	+++
AVSL dog antisera	1:2	1:16	-	-	-
"	1:10	1:16	-	-	-
"	1:50	1:16	-	-	-
"	1:100	1:16	-	-	+
"	1:500	1:16	-	-	+
"	1:1000	1:16	-	-	+
"	1:10 000	1:16	-	-	++
immune mouse sera	1:2	1:16	-	-	+
"	1:10	1:16	-	-	+
"	1:50	1:16	-	-	+
"	1:100	1:16	-	-	++
"	1:500	1:16	-	+	++
"	1:1000	1:16	-	+	++
"	1:10 000	1:16	-	+	+++

mouse sera, control sera (AVSL dog antisera), and non-immune mouse sera were incubated with a paralysing dose (1:16 final dilution) of crude tick homogenate and administered s.c to groups of neonatal mice. The onset of paralysis symptoms was observed and recorded over an 18 hour time period. Mice administered non-immune mouse sera and crude tick homogenate developed hind limb paralysis after 4 hours and were dead following the 18 hour incubation period. Mice injected with the AVSL control antisera were protected from paralysis for the entire 18 hour incubation period up to a 1:50 dilution of antisera. Mice injected with dilutions between 1:100 and 1:1000 developed symptoms of hind limb paralysis at 18 hours, and the 1:10 000 dilution had complete paralysis at 18 hours. The immune mouse sera exhibited some degree of protection. That is, mice appeared normal for 8 hours for the 1:2 to 1:50 dilutions, however at 18 hours these mice were suffering from hind limb paralysis. At 1:100 to 1:1000 dilutions of immune mouse sera complete paralysis symptoms occurred at 18 hours, and at the 1:10 000 dilution death occurred by 18 hours.

DOG ASSAYS

Crude Tick Homogenate

Crude tick homogenate which caused complete paralysis in mice at a 1:32 dilution (100 μg/5 g) was unable to produce any symptoms in dogs when administered (without dilution) at 1.25 mLs/10 kg. This amount equates to a 1:166 dilution which is not effective in the mice. The dose of crude tick homogenate given to the dogs was due to supply limitations and this is the reason why the mouse model was used from the outset.

Dog Protection Experiments

Dogs which were immunised with fusion protein antigen were challenged with live ticks. The results from the challenge of dogs with ticks are presented in Table 4.4. From the 24 dogs challenged with live ticks, only 3 did not develop symptoms of paralysis. Two of the dogs were from the high dose (1000 μg/mL) immunisation group. This suggests that the protective ability of the antisera may relate to the amount of antigen.

To assess the levels of antibodies present in the dog immune sera, ELISAs were performed on antisera collected from the dogs at days 0, 14 and 35. In an attempt to

Group	*Dog Number*	*Paralysis (Yes/No)*
1: 10 μg/mL	7	Yes
	18	No
	22	Yes
	23	Yes
	27	Yes
	29	Yes
	30	Yes
	31	Yes
2: 100 μg/mL	2	Yes
	3	Yes
	9	Yes
	12	Yes
	20	Yes
	25	Yes
	34	Yes
	35	Yes
3: 1000 μg/mL	10	Yes
	39	Yes
	15	No
	17	Yes
	26	No
	28	Yes
	36	Yes
	38	Yes

Table 4.4: Protection experiment in dogs.

24 dogs were administered s.c. with two 1 mL doses of 10, 100 or 1000 μg/mL of recombinant HT-1 fusion protein. Following this they were challenged with 8 live ticks and the development of typical paralysis symptoms was recorded.

eliminate binding of antibodies raised against MBP and the histidine tag, the dog immune sera was preincubated with an excess amount of commercially available MBP and recombinant histidine tagged proteins. There were no proteins available at the time to eliminate binding to factor Xa, however as the factor Xa site is small (only 4 residues) it was not expected to elicit a significant antibody response.

Sera from all 24 dogs were tested in the absorption ELISA and standard ELISA (result not shown). The antibody-binding response of the serum appeared to fall into three groups when analysing immune sera from day 35. That is, there were dogs which had a high antibody response, a medium antibody response and a low or insignificant antibody response. To simplify the presentation of ELISA results, representative dog antisera from each of the high, medium or low responders were plotted. This data was from dogs 36, 35 and 29, and is depicted in Figures 4.3, 4.4 and 4.5, respectively.

In Figure 4.3 it can be seen that the antibody response increases with each immunisation as expected. That is, at day 0 prior to immunisation very little antibodies are produced, at day 14 following the initial immunisation moderate levels of antibodies are produced and at day 35 following the booster injection relatively high levels of antibodies are produced to the whole fusion protein. The dotted lines on the ELISA represent specific binding to HT-1, that is the antibodies to MBP and His have been absorbed in a pre-incubation step. Specific binding to HT-1 is best illustrated in Figure 4.3, where binding to HT-1 is slightly less than that to the whole fusion protein. Figure 4.4 and 4.5 represent dog sera from those injected with medium and low doses of antigen. The results from these ELISAs indicated that lower levels of antibodies are produced to the fusion protein antigen when the antigen dose is only low (10 μg/mL) or moderate (100 μg/mL).

To compare the data from the different dog ELISAs, end-point titres were calculated (Table 4.5). It can be concluded that the antibody titre was greatest from sera collected at day 35. This response was expected considering the nature of a mammalian immune response to repeated doses of antigen (Roitt, 1993). The pre-incubation of the immune sera with MBP and histidine tag proteins successfully absorbed binding to these proteins. This is evident as the titres are lower for these pre-incubation ELISA experiments and therefore provide an indication of antibody levels raised specifically to HT-1. These results also indicate that there is a direct correlation as mentioned above with the dose of HT-1 fusion protein antigen given during the immunisations and the levels of antibody obtained. For example dog 36

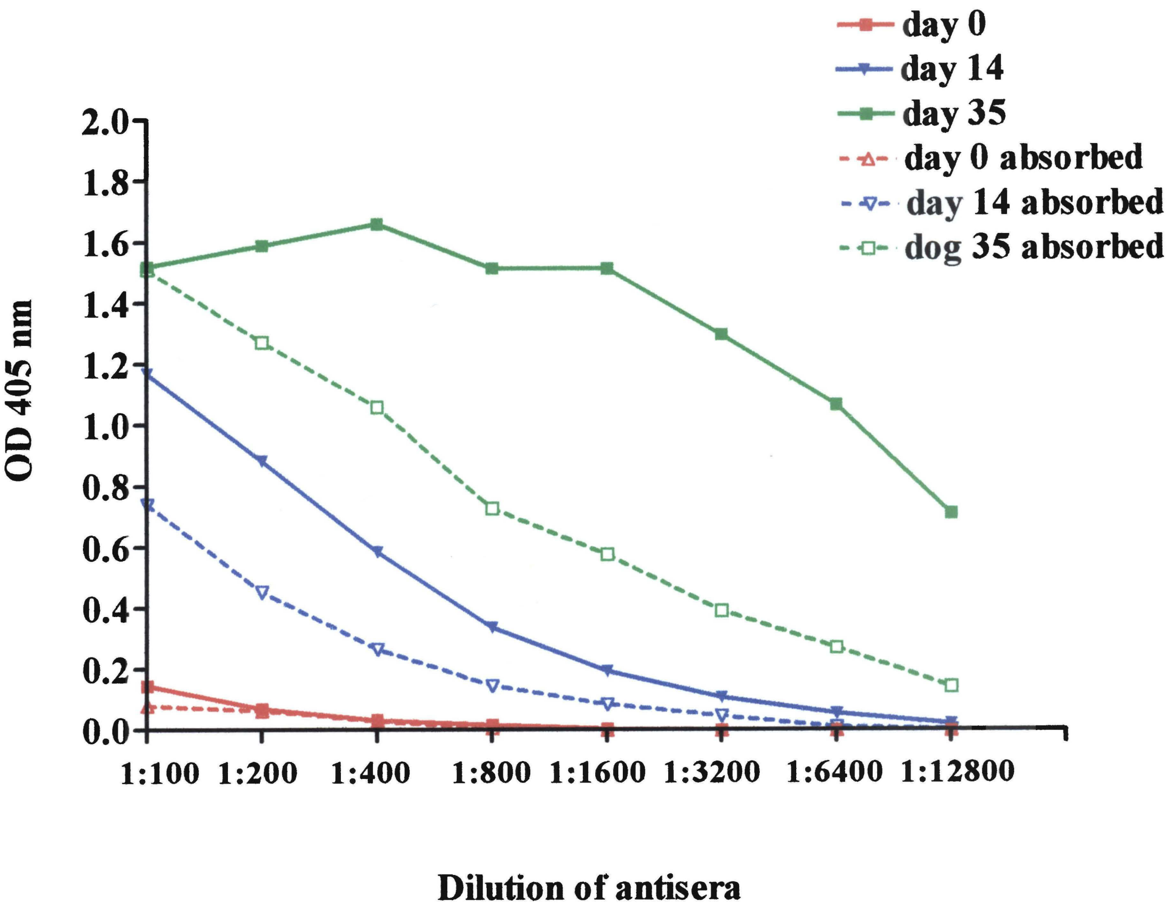

Figure 4.3: Binding response to HT-1 fusion protein - dog 36: example of a high response.

HT-1 fusion protein antigen (25 μg/mL) was immobilised to the wells of an EIA plate which were subsequently blocked with 3% BSA. Dog 36 antisera from days 0, 14 and 35, unabsorbed or absorbed with MBP and His proteins were added to the wells and serially diluted. The bound dog 36 antisera were detected with alkaline phosphatase conjugated anti-dog IgG and visualised by the addition of *p*-NPP. The absorbance was measured at 405 nm.

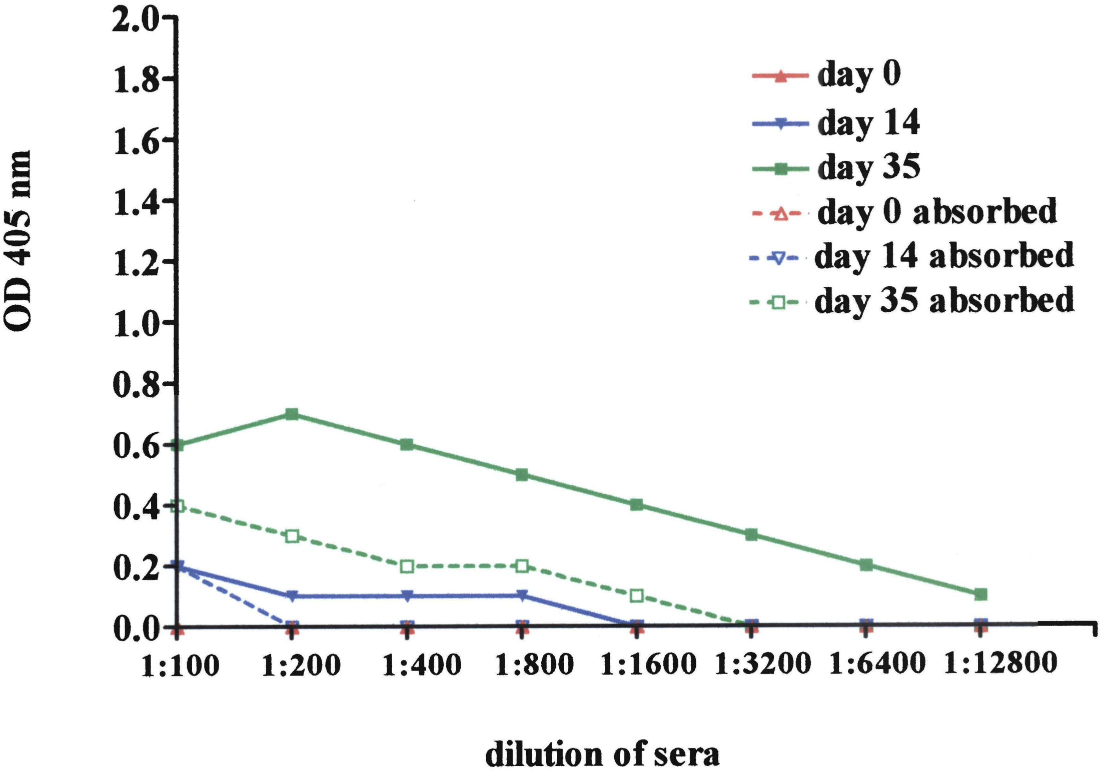

Figure 4.4: Binding response to HT-1 fusion protein - dog 35: example of a medium response.

HT-1 fusion protein antigen (25 µg/mL) was immobilised to the wells of an EIA plate which were subsequently blocked with 3% BSA. Dog 35 antisera from days 0, 14 and 35, unabsorbed or absorbed with MBP and His proteins were added to the wells and serially diluted. The bound dog 35 antisera was detected with alkaline phosphatase conjugated anti-dog IgG and visualised by the addition of *p*-NPP. The absorbance was measured at 405 nm.

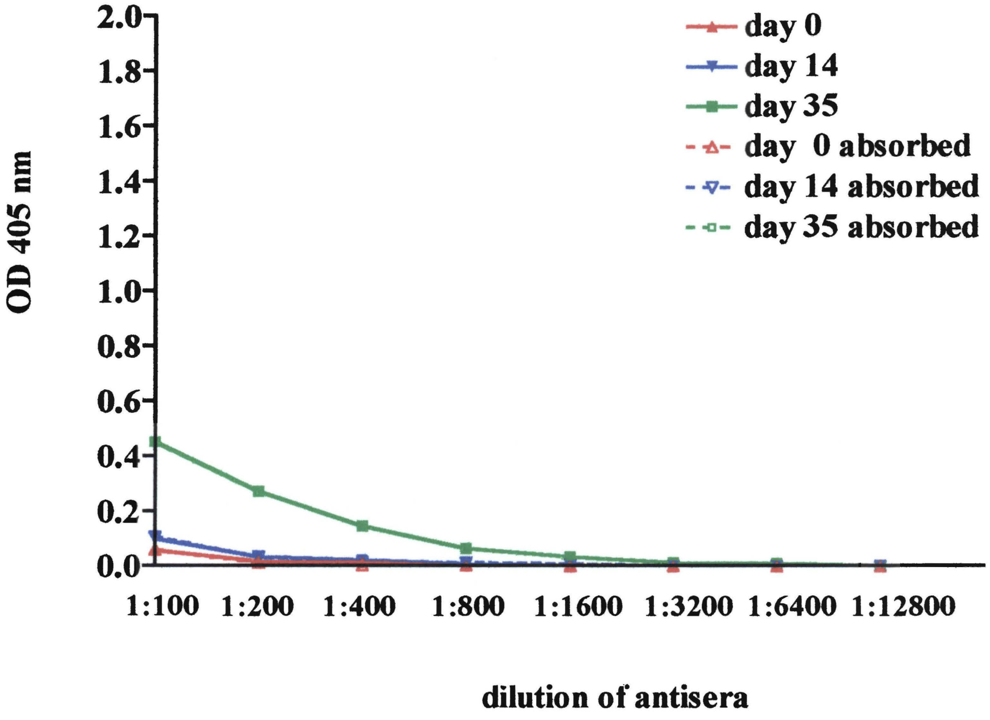

Figure 4.5: Binding response to HT-1 fusion protein - dog 29: example of a high response.

HT-1 fusion protein antigen (25 μg/mL) was immobilised to the wells of an EIA plate which were subsequently blocked with 3% BSA. Dog 29 antisera from days 0, 14 and 35, unabsorbed or absorbed with MBP and His proteins were added to the wells and serially diluted. The bound dog 29 antisera was detected with alkaline phosphatase conjugated anti-dog IgG and visualised by the addition of *p*-NPP. The absorbance was measured at 405 nm.

Table 4.5: Antibody titres in dogs

The end-point antibody titres for dogs 29, 35 and 36 were calculated based on ELISA readings (Figures 4.3, 4.4 and 4.5). A significant value was taken as being greater then OD of 0.1 at 405 nm. The antibody titres were recorded for antisera collected at days 0, 14 and 35. The titres were calculated for total immune sera values and immune sera where the binding to MBP and the histidine tag were absorbed.

Dog Number	*Immune Group*	***Antibody Titre***					
		Day 0 whole ab response	**Day 0** abs to HT-1 only	**Day 14** whole ab response	**Day 14** abs to HT-1 only	**Day 35** whole ab response	**Day 35** ab to HT-1 only
29	1 (10 µg/mL)	0	0	0	0	400	200
35	2 (100 µg/mL)	0	0	200	100	>3200	1600
36	3 (1000 µg/mL)	0	0	800	400	>12800	6400

which received the 1000 μg/mL dose had the highest antibody titres, while dog 29, receiving 10 μg/mL of antigen had the lowest titre.

4.5: DISCUSSION

The aim of producing a recombinant construct of the *I. holocyclus* HT-1 neurotoxin was to express it in a form where it would induce the production of protective antibodies. This in turn would provide the basic understanding required for the production of a recombinant vaccine which would gradually substitute the use of hyperimmunised dog sera for serotherapy, and to confer immunoprotection against tick toxin paralysis. This chapter presented results relating to the use of the recombinant HT-1 fusion protein as an antigen for immunisation in mouse and dog animal models with the aim of producing protective antisera against the native tick HT-1 toxin.

Mouse Assays: Toxicity

Injecting mice with varying concentrations of recombinant HT-1 fusion protein produced no symptoms of paralysis. Thus, the fusion protein was considered to be non-toxic. As toxin activity is most likely related to the native conformation, toxicity is one of the best tests for monitoring the correctly folded nature of HT-1. Although HT-1 appeared to be produced in a soluble form as part of a large fusion protein (Chapter 3) it is still unknown whether the formation of the specific four disulphide bonds were in the correct pattern as toxicity similar to that of the native toxin was not observed.

An alternative means of assessing the correctly folded nature of HT-1 would be to assess its binding to synaptosomes. Synaptosomes are an ideal *in vitro* model system for investigating pre-synaptic events. Such assays only require small amounts of material and provide a result quite rapidly (Miranda *et al.*, 1998). In previous studies (Thurn , 1992) partially purified *I. holocyclus* tick preparation was radiolabelled and incubated with synaptosomes isolated from rat brains. A polypeptide of approximately 5 kDa bound to the synaptosomes in a temperature dependent manner which correlated with the *in vivo* observations of Cooper and Spence, (1976).

There are a number of reasons why the HT-1 fusion protein may not have exhibited toxic activity. The most likely reason being that the MBP fusion partner constitutes a large proportion of the fusion and therefore could be hindering the exposure of HT-1 to its site of action on the hosts presynaptic membrane. Alternatively, the HT-1 portion of the fusion may not be folded correctly resulting in the toxic site being potentially buried within the core of the toxin structure. In addition, the amount of

HT-1 being injected into the mice as part of a fusion protein may not be sufficient to be toxic. This is because MBP is a bacterial protein which constitutes a large proportion of the fusion, and the total amount of the small eukaryotic sequence of HT-1 being expressed will potentially be much less.

Successful cleavage of the HT-1 toxin sequence from the remainder of the fusion may have made a difference in regard to its toxic activity. Successful expression of HT-1 in the absence of a fusion partner may have also produced as toxic protein as was attempted with the baculovirus expression system (Chapter 3). This toxicity has been demonstrated with other toxins and proteins expressed as part of a larger fusion. The gene encoding the neurotoxin charybdotoxin (ChTx) from the scorpion *Leiurus quinquestriatus* was expressed as a fusion protein to the gene-*9* product of phage T7 and was cleaved using factor Xa. The cleaved recombinant ChTx was found to be chemically and functionally similar to the native ChTx (Park *et al.*, 1991).

Similarly, in a study by Ducancel and colleagues (1989), the recombinant snake neurotoxin erabutoxin *a* (from *Laticauda semifasciata*) was expressed in *E. coli* fused to protein A. The recombinant fusion protein had lower functional affinity for its receptor and less toxicity than the native toxin. It was proposed that the non-native biological properties of the toxin were due to incorrect folding of the toxin and/or due to steric hindrance by the large protein A. Subsequently, the protein A moiety was removed and replaced with four methionine residues, N-terminal to the toxin, and cleaved using CNBr. This cleaved erabutoxin *a* had physiochemical and biological properties virtually identical to that of the natural toxin. It was therefore concluded that the low biological activity of the protein A construct was due to the presence of the bulky protein A and not due to incorrect folding of the snake neurotoxin (Boyot *et al.*, 1990). Thus the large MBP fragment of the HT-1 fusion protein may be having a similar effect as protein A on erabutoxin *a* whereby steric hindrance precludes the interaction of the toxic site in HT-1 with its receptor.

Many recombinant toxic proteins that retain the full biological activity of the native toxin/s require detoxification, usually chemically (formaldehyde, gluteraldehyde) or by site directed mutagenesis, prior to their use as immunogens (Henelne and Heneine, 1998). If recombinant HT-1 was produced as an active toxic protein it would be required in a non-toxic form for the purposes of vaccine development. Genetic alterations of specific residues would help to identify sites of the toxin responsible for receptor binding and activity and those responsible for antigenicity. These alterations could be based on studies performed for other arachnid neurotoxins which have led to the successful identification of residues important in the action of these neurotoxins.

In the case of sodium ion channel scorpion neurotoxins, the role of individual residues in toxicity and binding was tested by chemical modification of some residues and by mutational analysis, combined with data regarding the three dimensional structure of the toxin. For example, site directed alterations of LqhαIT from the scorpion *L. q. hebraeus* produced a selective decrease in its anti-mammalian toxicity thus improving its phylogenetic selectivity in favour of insects (Zilberberg *et al.*, 1996). Chemical modification of Lys 58 of AaH II from *A. australis* (Darbon *et al.*, 1983) resulted in only 1% of the original toxicity being retained. Similarly, active recombinant α-latrotoxin (α-LTX) from black widow spider venom was produced in a baculovirus expression system. The toxin was produced in an active soluble form and possessed all the properties of natural α-LTX. Mutagenesis studies are presently being carried out to study the functional domains of α-LTX (Volynski *et al.*, 1999).

The production of an active recombinant HT-1 toxin would not only prove useful for mutagenesis analysis and ensure that the toxin is correctly folded, but also for the determination of a correctly folded structure for the toxin by NMR or crystallographic analysis. HT-1 shares sequence similarity with arachnid neurotoxins in the number and arrangement of its cysteine residues, suggesting that it adopts a small, tightly folded structure. Analysis of the secondary and tertiary structures of HT-1 may extend the homology of this tick neurotoxin with other arachnid toxins whose tertiary structures have been elucidated.

Structural data in combination with site-directed mutagenesis alterations in the peptide sequence determined from an active toxin, can help to investigate the interaction of HT-1 with its receptor . Extensive electrophysiological studies have not been performed on HT-1 due to the lack of active material available. Therefore, although it is known that HT-1 acts in a presynaptic manner (Cooper and Spence, 1976), it is not yet known to what receptor it binds on the pre-synaptic membrane. In addition, once active recombinant toxin is produced, neuropharmacological studies can be performed so as to determine the activity of the toxin once it has bound to its receptor which results in the onset of the rapidly ascending flaccid paralysis in the host.

Mouse Assays: Immunogenicity and Protection

Although the HT-1 fusion protein was not toxic it was, however, immunogenic (Figures 4.1 and 4.2) as it was able to produce a high titre of specific antibodies. The presence of the large MBP fused to the toxin possibly enhanced its immunogenicity, as the antigen would have been more easily recognised as a large foreign molecule by immune cells (Harlow and Lane, 1988). This result is important in terms of vaccine production, as it is desirable to have a non-toxic but immunogenic antigen for immunisations in animals and humans (Henelne and Heneine, 1998).

The antisera against the HT-1 fusion protein was evaluated for its protective effect in mice when mixed with a paralysing dose of crude tick homogenate *in vitro*. Neonatal mice were chosen as the experimental model because they are sensitive to the effect of toxins and provide a convenient model to follow the development of a protective immune state against toxic antigens (Moreira-Ferreira *et al.,* 1998). The antisera against HT-1 fusion protein was shown to be partially protective against paralysis induced by crude tick homogenate (Table 4.3). The period of protection was between 8-18 hours at 1:2 to 1:50 dilutions of the antisera. Dilutions from 1:100 to 1:10 000, were protective for 4-8 hours. The control mice administered with a 1:2 dilution of normal mouse sera along with crude tick extract developed symptoms of paralysis almost immediately (between 0-4 hours). In contrast administration of the immune mouse sera delayed the onset of hind limb paralysis until 8-18 hours of incubation. The results presented indicate that a partial protective state of immunity was achieved due to the presence of antibodies that were able to neutralise toxic tick proteins *in vitro*. This neutralisation was effective up to a certain period, after which time the antibodies were no longer protective .

It is possible that the mouse immune sera had low neutralising antibodies due to the presence of low levels of tick neurotoxin/s in the recombinant HT-1 fusion protein immunogen. Two reasons to explain this are that, firstly, the MBP constitutes a large proportion of the fusion protein and therefore the expected dominating antibodies present will be to MBP. Secondly, the immune antisera was challenged with crude tick homogenate which potentially contains more than one tick neurotoxin (Thurn, 1994). Therefore, to confer complete protection it may be necessary to have circulating antisera that will neutralise all the toxins present.

It was previously established that toxic fractions of HT-1, HT-2 and HT-3 isolated from *I. holocyclus* share a similar amino acid profile and were each able to cross react with polyclonal dog antiserum, suggesting that they may share similar epitopes (Thurn, 1994). Therefore the development of a polyvalent vaccine based on conserved residues for an antigenic epitope of these toxins may be justified if they are proved to be present in the tick as distinctly expressed toxin genes.

The immune mouse sera was not as protective as the commercial AVSL hyperimmune dog-antisera, which was completely protective up to a 1:50 dilution for the entire 18 hour period, and partially protective from dilutions 1:100 to 1:1000 where hind limb paralysis developed between 8-18 hours. This commercial sera is used successfully without dilution in the treatment of animals presenting symptoms of tick paralysis. However it is still only effective in the early stages of paralysis (Concidine, 1985).

Dog Assays: Immunogenicity and Challenge Experiments

Clinical dog trials were performed using the HT-1 fusion protein as an immunogen. As these were experiments of commercial interest the 'all or nothing' principle was applied by the collaborator when designing the challenge experiments. Thus, dogs received only two immunisations of HT-1 fusion protein, and then were challenged with a maximum number of ticks.

ELISA results from dogs 29, 35 and 36 which represent each dog immunisation group were presented (Figures 4.3, 4.4 and 4.5). These ELISA results indicate that the antibody response increased with each immunisation. Specific antibodies to HT-1 were also detected when antibodies to MBP and His proteins were removed by absorption. It was established that dogs receiving the highest amount of fusion protein antigen during immunisation had the highest antibody titres (Table 4.4). It was not surprising, therefore, to see that from this group of dogs, 2 out of 8 did not develop paralysis symptoms (Table 4.3). The majority of dogs did develop paralysis symptoms (21/24) when they were challenged with 8 ticks. This result was expected as it is well known that one female *I. holocyclus* tick is sufficient to cause paralysis and death in a large dog (Ilkiw *et al.*, 1987). In addition, following primary immunisations the antibody levels are usually very low while after the second injection there is an increase in antigen specific antibodies. However, the response to the third and subsequent injections broadly mirror that of the second immunisation with the exception that they yield high affinity antibodies (Harlow and Lane, 1988). Therefore, one can speculate about what level of protection could have been obtained if the dogs had been immunised with a third dose of antigen and/or challenged with 1 or 2 ticks instead of 8. It must be reiterated that, as for the challenge experiments in mice with crude tick homogenate, the ticks may contain other toxins that cause paralysis. Therefore the antisera in the immune dogs to the fusion protein immunogen may not have a very strong neutralising capacity as it would not recognise all the native toxins and/or may not be at a sufficient level to neutralise the major toxin HT-1.

Comparison between the protective effects of the immune sera from mouse and dog assays are difficult as there are a number factors which need to be considered. These include the nature of the antigen, host factors such as age, weight and breed, route of injection and the use of adjuvant. The mice used (Balb/C) in this work were an inbred strain where the genetics of the immune response have been investigated and optimised for laboratory experimentation (Festing, 1979). The dogs used in this work were of mixed breed and weight. It is known that outbred animals such as these dogs have a wider range of immune response proteins than inbred dogs such as beagles, and therefore would expect to have varying immune responses to foreign antigen (Lewis *et al.*, 1995; Ohashi *et al.*, 1996).

The HT-1 fusion protein antigen used for immunisation of dogs and mice was identical. However it was difficult to estimate the level of antigen required to achieve the same antibody levels and protection results in dogs as seen in the mice assays. Therefore different amounts of fusion protein immunogen were administered to the dogs with the maximum level being determined by the amount of purified fusion protein that was available at the time. The recommended amount of soluble antigen required for immunisation of an adult rabbit is between 100-1000 µg per injection and for mice it is between 5-50 µg per injection (Harlow and Lane, 1988). The dogs in this study were injected with 10, 100 and 1000 µg of immunogen and the mice were injected with between 12.5 and 25 µg. Therefore if body weight was to be taken into account for antigen dose, the mice were immunised with a minimum of 12.5 µg/20g. Therefore to receive the same antigen dose a dog which weighs 20 kg would have required to be immunised with 12.5 mg of antigen. The immunisation of 24 dogs with this amount of antigen was not possible due limitations in availability of purified fusion protein antigen.

In summary, the results presented in this chapter have provided an understanding of the immunogenic and protective properties of the HT-1 fusion protein in mouse and dog animal models. To achieve a high level of protective antibodies to native *I. holocyclus* toxin a number of factors need to be considered and optimised. These include the possibility of producing a recombinant HT-1 toxin in the absence of a fusion partner, with the aim of obtaining a toxic protein. The production of an active toxic protein could lead to a number of further investigations such as characterisation of its toxic and functional sites and three-dimensional structure. Additionally, the question still remains of whether there are other tick neurotoxins present in *I. holocyclus* which are contributing to the poor neutralisation property of antisera raised against the HT-1 toxin, when challenged with native tick extracts or live ticks.

CHAPTER 5

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

The work presented in this thesis describes the determination of the complete gene sequence for the HT-1 neurotoxin of the Australian paralysis tick *Ixodes holocyclus*. In addition, it describes the successful expression and purification of the neurotoxin as a soluble fusion protein in an *E.coli* host system. This fusion protein was shown to be highly immunogenic, non-toxic and partially protective against the native toxin in mouse models. These results provide the basic information for the future development of a recombinant tick neurotoxin vaccine.

In the past, direct protein sequencing for a toxic purified fraction of HT-1 was not possible due to a blockage. Therefore, partial peptide sequence data was obtained following tryptic digest (Thurn, 1994). Due to the difficulties in obtaining sequence information for HT-1 using protein biochemistry techniques, an alternative strategy was employed to obtain sequence information whereby the existing partial amino acid sequence for HT-1 was used to design degenerate primers for PCR amplification.

PCR-amplification with degenerate primers yielded a product from which specific primers were designed and used in 5' and 3' RACE PCR reactions. A 3' sequence was deduced from this work (Masina, 1995). The confirmation of the 3' sequence formed part of the initial experiments presented in this thesis. The 5' sequence was subsequently derived. It was from the untranslated portion of this 5' sequence that a specific primer was designed and used to amplify a product in a 3' RACE reaction corresponding to that of the complete gene for the neurotoxin HT-1.

Several differences were observed when the translated amino acid sequence of HT-1 obtained by RACE-PCR was compared to the original partial peptide sequence obtained by Thurn (1994). The peptide sequence information obtained by tryptic digest was potentially inaccurate as the amount used for protein sequencing was below the level of detection of the instrumentation used at the time. Furthermore, the sequence information for the partial tryptic peptides was never confirmed. The sequence information obtained via RACE-PCR for HT-1, on the other hand, was repeated several times using different clones of PCR product. Each clone screened was identical, and the sequence information of complete HT-1 cDNA also correlated with that of the individual 3' and 5' RACE sequences.

Another factor which may contribute to the differences observed with the HT-1 peptide sequence is that the sequence obtained for HT-1 by RACE-PCR may represent the sequence for one of the other *I. holocyclus* toxic fractions, HT-2 or HT-3, identified by Thurn (1994). Peptide sequence information for these two fractions was never determined as they were purified in low quantities. However, it is possible that HT-2 and HT-3 may have unique transcripts in the tick RNA and therefore their DNA sequences may differ from that of HT-1. Minor differences in neurotoxin sequences arising from a single venom source are common among scorpion neurotoxins that have been characterised (Diniz, *et al.*, 1993; Vazquez *et al.*, 1995; Kalapothakis *et al.*, 1998; Xiong *et al.*, 1999). Regardless of this, the first complete neurotoxin sequence obtained for *I. holocyclus* was referred to as HT-1.

The HT-1 cDNA sequence contained all the classical features of fully functional, eukaryotic mRNA. These included transcription initiation sites, secretion signal sequence, start and stop codons, polyA tail and putative polyadenylation signal sequence (Perlman and Halvorsen, 1983; Kozac, 1984; von Heijne 1986). Additionally, the mature peptide sequence translated from the HT-1 DNA sequence has several characteristics indicating that it is an arachnid neurotoxin. These include the small size (5-6 kDa) of the mature toxin, the presence of 8 cysteine residues suggesting that it is potentially a tightly folded structure, and homologous motifs in the primary sequence with other arachnid neurotoxin sequences. For the purpose of structural homology analysis, HT-1 was compared to known neurotoxin sequences and structures of other arachnid neurotoxins.

Homology studies on scorpion neurotoxins have indicated that different species of scorpion toxins which have different modes of action share structural similarities (Gordon *et al.,* 1998). For example, the conserved motif consisting of an α-helix linked to a triple stranded β-sheet arrangement that forms a dense core supported by 3-4 well conserved disulphide bridges is found in all scorpion neurotoxins characterised to date (Bontems *et al.,* 1991). In addition, the conserved amino acid sequences of these scorpion neurotoxins, include two peptide stretches with the sequence C-X-X-X-C, located in an α-helix and C-X-C, contained in a β-sheet. The location of the cysteine residues of HT-1 were compared to a range of scorpion neurotoxins (Figure 2.8). HT-1 has 5 out of 8 cysteine residues in common with the scorpion sequences listed. In addition, homology is observed in the location of the C-X-X-X-C motif. The primary sequence homology observed between HT-1 and the scorpion neurotoxins listed may prove valuable for future disulphide bond assignment and tertiary structure prediction of HT-1.

The HT-1 amino acid sequence also conformed to the consensus sequence present in spider neurotoxins and other inhibitory polypeptides (Figure 1.7). All these toxins and inhibitory polypeptides have different modes of action on their targets, yet they share a consensus sequence $CX_{3-7}CX_{3-6}CX_{0-5}CX_{1-4}CX_{4-13}C$, determined by the gaps between the half cysteine residues. This consensus sequence is responsible for the formation known as the inhibitor cysteine knot, which forms the structural core of these proteins. The first six cysteine residues of HT-1 share this consensus and HT-1 may therefore also have tertiary structural similarity with the cysteine knot group.

It is well accepted that *I. holocyclus* contains the most potent tick neurotoxin (Ilkiw *et al.*, 1987). Knowledge of the sequence for the HT-1 neurotoxin will be useful for future homology studies with other yet unidentified paralysis tick neurotoxin sequences. This homology analysis would determine the structural features and specific residues of *I. holocyclus* which are responsible for its highly toxic activity when compared to the other ticks.

For this thesis, knowledge of the HT-1 sequence led to the development of a recombinant HT-1 fusion protein. This fusion protein was expressed in a soluble form in a bacterial host system. Milligram quantities of the expressed fusion protein were purified on a Ni-affinity column. Production of native recombinant HT-1 by cleavage of the purified fusion protein with factor Xa was not successful. Therefore the antigenicity, toxicity and protective properties of the HT-1 toxin were assessed using HT-1 in the form of a fusion protein.

Mice immunised with the HT-1 recombinant fusion protein produced antisera that were able to cross react with the recombinant fusion protein antigen as demonstrated by Western blot and ELISA. It is known that larger antigenic molecules produce a greater antibody response than small antigenic molecules as they are more easily recognised by cells of the immune system (Marston, 1986). Therefore it is probable that the majority of antibodies produced in the mouse antisera to the HT-1 fusion protein would be to the MBP portion of the fusion, with less antibodies being produced against the smaller HT-1 portion .

The protection experiments in mice indicated that the fusion protein antisera was able to produce antibodies to HT-1 which were partially protective. This suggests that as well as antibodies specific for MBP and other peptide epitopes in the fusion protein, antibodies were also produced against HT-1. It was encouraging to observe that these antibodies to HT-1 could confer some neutralising property when challenged with a toxic dose of native tick toxin.

When immunising animals, a number of factors need to be considered and optimised. One of the most important factors is the nature and dose of antigen that is being used as the immunogen (Harlow and Lane, 1988). The immunogen for the mouse and dog assays was the purified HT-1 fusion protein. The dose of this immunogen required for the mouse immunisations had been optimised with several separate antigenic and protection assays. These experiments were always performed with inbred strains of mice of similar weight and age. Therefore, the serum levels of antibody produced against the fusion protein immunogen and the level of protection observed with the antisera from these mice was consistent.

In contrast to the assays performed with the mice, immunisation and challenge experiments performed with the dog trial were not standardised due to the limited availability of purified fusion protein antigen and due to the expense of conducting clinical trials on a large number of dogs. The dogs that were used for this study were all of different breeds, sex and weight. These features correlate with those of a true population of dogs, but it is very difficult to control and assess the immune responses in such a mixed population of outbred animals (Festing, 1979). The antigen dose response had not been optimised for the dogs as it had been in the mice. Therefore the amount of immunogen used was estimated based on the availability of purified fusion protein at the time. The maximum amount of immunogen used was two 1 mL doses at 1 mg/mL. Within the group of dogs that received this dose, two did not develop paralysis symptoms upon challenge with live ticks. The 'immunised' dogs were challenged with 8 ticks, which is an extreme amount considering that one tick is sufficient to cause paralysis and death in a single dog (Ilkiw *et al.*, 1987). Because two dogs which received the highest dose of antigen were able to resist the challenge with 8 ticks, it is likely that a significant number of dogs would survive if they were challenged with only one or two ticks.

The combined results from the mouse and dog protection experiments suggest a number of ideas which can be considered for further investigation in the production of a recombinant tick vaccine. For example, it would interesting to know if there definitely are other tick neurotoxins present in the native material. If this was the case, these new toxins could potentially be purified, characterised and used as immunogens. This in turn could improve the protective properties of the antisera. In addition, successful expression without the presence of a large fusion partner, as was attempted with the expression of HT-1 in a baculovirus construct (Chapter 3) can be further investigated. The absence of a fusion partner may result in a decrease in immunogenicity as the foreign antigen will not be as large and easily recognised (Harlow and Lane, 1988). However, such a construct will have the ability to produce specific antibodies mostly to the toxin and, therefore, may be more protective.

The production of a correctly folded and toxic recombinant protein is another area of importance for future study. The toxin may prove to be toxic without the fusion protein, which would allow for mutagenesis studies to be performed so as to determine the toxic and antigenic sites of the protein. In addition determination of the three-dimensional structure may help to further establish the relationship between tick toxins and those of other arachnids based on conserved structural domains. Once an active recombinant protein is developed, analysis of the electrophysiology and receptor identification of toxin binding could also be investigated. In addition, neuropharmacological analysis on the mechanism of action of the toxin in the host once it has bound to its receptor will help to understand the onset and development of the ascending flaccid paralysis symptoms.

Paralysis by the Australian tick *I. holocyclus* is an important medical and veterinary disease. The work presented in this thesis has provided the groundwork necessary for future development of recombinant tick vaccines with the aim of having an effective preventative measure for the control of tick toxicoses.

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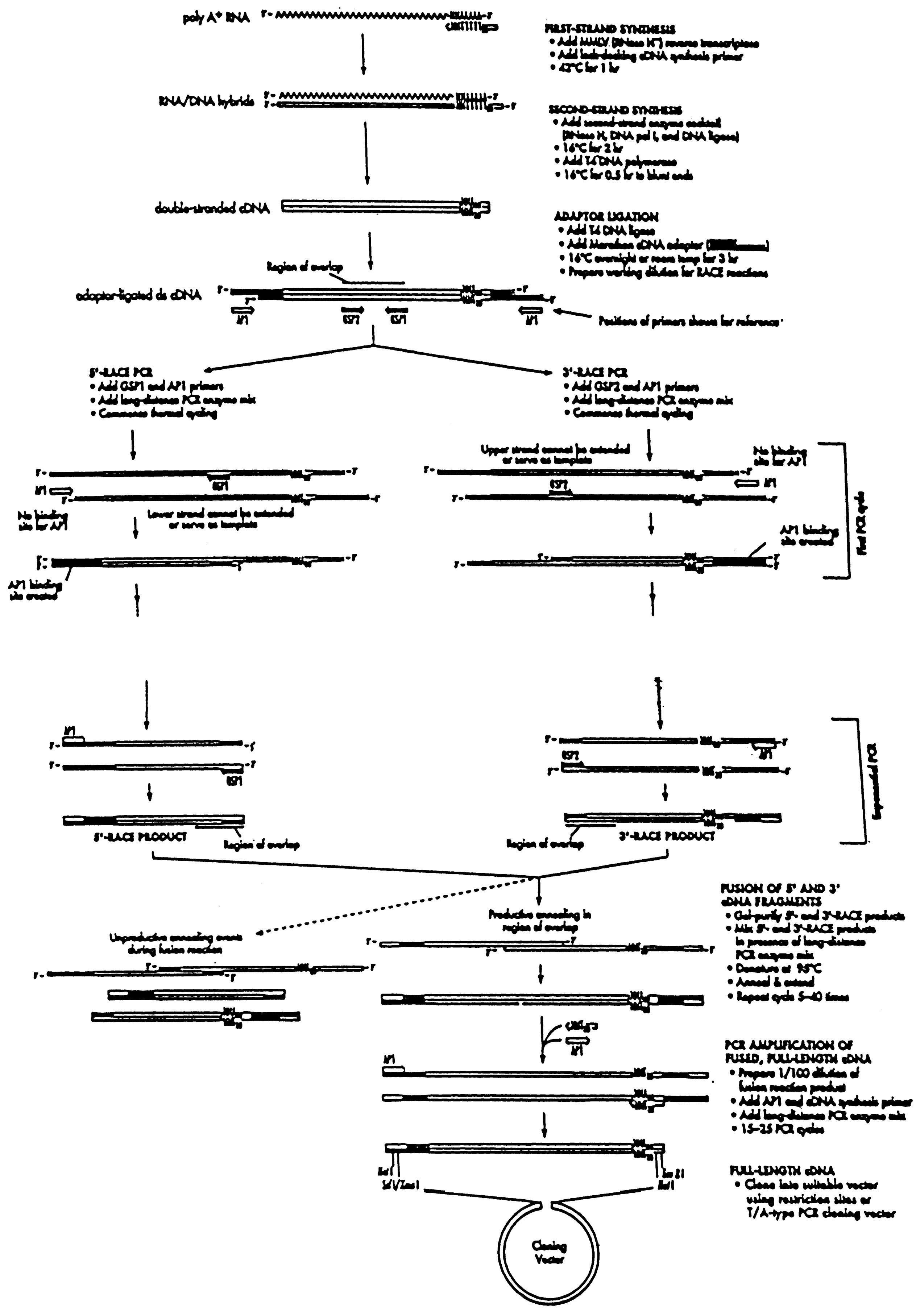
APPENDIX



Appendix 1: Overview of the Marathon cDNA amplification protocol