EXPRESSION OF A TICK TOXIN FOR THE DEVELOPMENT OF A CANINE VACCINE

BY

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September 2001
DECLARATION

CERTIFICATE OF AUTHORSHIP / ORIGINALITY

I certify that this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

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ABBREVIATIONS

(Abu)$_8$ AahII  *Androctonus australis hector* toxin analog
AaHIT  *Androctonus australis hector* insect toxin
AaIT  *Androctonus australis hector* insect selective toxin
AcNPV  *Autographa californica Nuclear Polyhedrosis Virus*
BCIP  5-bromo-4-chloro-3-indolyl phosphate
BM  *Boophilus microplus*
BmK  *Buthus martensill Karsch*
bp  base pair
BSA  bovine serum albumin
cDNA  complementary DNA
Da  Dalton
DMSO  dimethyl sulphoxide
DNA  deoxyribonucleic acid
dNTP  deoxynucleotide triphosphate
ED$_{50}$  paralytic unit
EDTA  ethylene diaminetetra-acetic acid
ELISA  enzyme linked immunoassay
Fab  fragment antigen binding
FBS  fetal bovine serum
Fc  fragment constant
GM  midgut membrane
GST  glutathione S-transferase
HAT  hypoxanthine, aminopterin, thymidine
HPLC  high pressure liquid chromatography
HPRT  hypoxanthine phosphoribosyl transferase enzyme
HT  holocyclus toxin
HWTX  Huwentoxin
Ig  immunoglobulin
IMAC  immobilized metal affinity column
IPTG  isopropylthio-β-D-galactopyranoside
kbp  kilobasepairs
kDa  kiloDalton
KTX2  kaliotoxin2
LB  Luria broth
LD₅₀  dose causing 50% death
LqhαIT  *Leiurus quinquestriatus hebraeus* alpha insect toxin
mA  milliamps
mAb  monoclonal antibody
MBP  maltose binding protein
mM  millimolar
MW  molecular weight
MWCO  molecular weight cut off
NaAz  sodium azide
NBT  nitro blue tetrazolium
Ni-NTA  nickel-nitrilotriacetic acid
OD  optical density
PCR  polymerase chain reaction
pmol  picomole
*p*-NPP  p-nitrophenyl phosphate
PVDF  polyvinylidenedifluoride
rpm  revolutions per minute
SDS-PAGE  sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSNAP  sparingly soluble non-antigenic protein
%T  total acrylamide concentration (w/v)
TBE  tris buffered ethylene diaminetetra-acetic acid
TEMED  N,N,N',N'-tetramethylenediamine
TFA  trifluoroacetic acid
TsNTxP  *Tityus serrulatus* nontoxic protein
U  unit
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<tr>
<td>UCH-L1</td>
<td>ubiquitin carboxyl terminal hydrolase-L1</td>
</tr>
<tr>
<td>USP</td>
<td>ubiquitin specific protease</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>V</td>
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ABSTRACT

Acute, ascending, flaccid motor paralysis of forelimbs and death due to respiratory failure is the dominant characteristic of tick toxicosis by the Australian paralysis tick, *Ixodes holocyclus*. A tick toxin vaccine has the potential to be an effective preventative measure against tick toxicosis that affects thousands of domestic and companion animals each year. In previous research for the development of an anti-tick vaccine, Masina (1999) used a maltose-binding protein (MBP) holocyclus toxin (HT-1) fusion protein as immunogen. It was partially protective against challenges with crude tick extract in neonatal mice but unable to protect dogs against paralysis caused by direct tick attachment.

Subsequently, four monoclonal antibodies generated in this study against the fusion protein were found to be incapable of binding to the native toxin in crude tick extract in Western blots. These results indicated that the HT-1 component of the MBP fusion protein was incorrectly folded compared to the native toxin.

An expression system using ubiquitin fusion protein was investigated. Ubiquitin is a 10 kDa carrier protein which is smaller than the 43 kDa MBP. A smaller fusion partner was considered as it is less likely to interfere with HT-1 folding. The ubiquitin-HT-1 fusion protein was expressed in both soluble and insoluble forms, corresponding to the cytoplasmic fraction and inclusion bodies. The soluble form could be purified under non-denaturing conditions utilising the incorporated His-6-tag and a Ni affinity column. The insoluble form, like the periplasmically expressed MBP-HT-1 fusion protein, could only be purified using denaturing conditions.

The purified soluble ubiquitin-HT-1 fusion protein appeared to have the correct conformational folding as it was recognized by commercial dog anti-tick serum in Western blots. The purified soluble ubiquitin-HT-1 fusion protein was used to immunise rabbits and mice for protection experiments, but was found to be unprotective. However, serum from immunized animals was able to detect a 5 kDa protein from crude tick extract in a Western blot. This 5 kDa protein was also recognized by the commercial dog anti-tick serum and
has the molecular weight corresponding to the HT-1 neurotoxin. Creation of monoclonal antibodies to this ubiquitin-HT-1 fusion protein may therefore aid future development towards a tick vaccine. These antibodies could in turn be used to isolate native HT-1 from the crude tick extract instead of a combination of conventional chromatography methods. This approach would allow isolation of HT-1 in more significant quantities than is currently possible to enable confirmation of the HT-1 sequence. Suspected problems associated with incorrect sequence or interference by the carrier protein to cause a non-protective response could also be resolved.
CHAPTER 1

INTRODUCTION
1.1 PREVIEW

Neurotoxins are molecules that are specifically targeted to neurons and interfere with electrical impulse transmission in the nervous system. Due to their high specificity, one neurotoxin type often disrupts only one function (i.e. ion channel) of the nervous system. Many animals, plants and fungi produce neurotoxins which primarily function as an offensive or defensive mechanism to immobilize and kill prey or prevent themselves from being eaten (Hucho, 1995). Humans, domestic animals and livestock are not normally perceived as prey or predators, however they are often the victims of envenomation (Hodgson, 1997).

Thirty-one species of seven *Ixodide* genera and six species of three *Argasid* genera can cause tick toxicosis. The Australian paralysis tick, *Ixodes holocyclus* is one of the most potent paralysis ticks in the world (Stone *et al.*, 1979). In Australia, tick paralysis is a significant veterinary problem where thousands of domestic and companion animals are affected or killed by the neurotoxin from the Australian paralysis tick (Stone and Aylward, 1987). A single female *I. holocyclus* has the ability to cause death of the host as a result of tick paralysis (Grattan-Smith *et al.*, 1997). Moreover, the cost of treatment is $220-300 for three days hospitalization (Coorey, 1998) and loss of livestock can be considerable. Incidence of tick paralysis in humans has also been reported, prior to the introduction of antivenom (Stone *et al.*, 1983b). Therefore, the development of effective antitoxins and vaccines would be useful to control tick paralysis.

This research is focused on the Australian paralysis tick. The first objective was to produce and characterize a panel of monoclonal antibodies against a toxin fusion protein. The fusion protein produced by Masina (1999), had been shown to be partially protective against tick paralysis in neonatal mice. Subsequent to the expression of this existing fusion protein, another recombinant toxin was expressed and evaluated for the ability to be protective against tick paralysis caused by the native toxin.
This introduction will provide a background on arachnid neurotoxins and different protein expression systems used for the production of recombinant scorpion, spider and tick toxins. Extensive research has focused on neurotoxin expression for vaccine development, and for characterizing a rising number of identified receptors and ion channels in the nervous system (Adams and Olivera, 1994). Parallel to the discussion of recombinant techniques, the application of monoclonal antibodies as an aid in the research of arachnid neurotoxins will be elucidated. The remainder of the review will focus on the biology of ticks, tick paralysis, information on current treatment protocols and current progress in the development of a vaccine for tick paralysis.

1.2 SCORPIONS

1.2.1 The structure of scorpion toxins

Prior to an extensive discussion of scorpion toxin expression, a basic understanding of scorpion toxin structure and function is necessary. It is known that scorpions express multiple neurotoxic polypeptides to immobilize their prey, with scorpion venom glands producing biologically-active complex mixtures consisting of nucleotides, lipids, peptides, enzymes, mucoproteins, biogenic amines and unknown substances. The most studied scorpion toxins are those polypeptides that are specific for receptors in excitable membranes and ion channels i.e. sodium, potassium, chloride and calcium channels. All of the known sodium-channel specific toxins are anti-mammalian toxins made up of 60-76 amino acid residues, whereas small peptides of less than 40 amino acid mainly act on potassium channels. Potassium channel specific toxins are able to bind to the extracellular region of the ion channel and prevent passage of ions (Possani et al., 1999). Chloride and calcium channel specific toxins are less abundant. It has been reported that chlorotoxin (chloride channel specific) has 33 amino acids with four disulfide bonds. Calcium channel specific toxins that modify the binding of ryanodine, which regulates calcium channel activation by binding to specific receptors, range in size from 27 to 104 amino acids (DeBin et al., 1993; Lippens et al., 1995; Possani et al., 1999).
As well as grouping scorpion toxins by their specificity against ion channels, they can also be divided by their biological specificity in vivo and by pharmacological characteristics. Mammalian toxins have been grouped into mammalian specific α neurotoxins and β neurotoxins that can also act on insects. Scorpion α neurotoxins which include insect and mammalian selective toxins are small (6-7 kDa) but extremely potent and act by slowing the inactivation of voltage dependent sodium channels, whereas β-neurotoxins modify sodium channel activation. Unlike α neurotoxins, β neurotoxins function in a voltage independent manner. Insect selective toxins are classified into excitatory and depressant neurotoxins (Herrmann et al., 1995). Excitatory toxins cause immediate, fast and prolonged spastic contractive paralysis with stimulation of repetitive channel action. In contrast, depressant toxins cause short transient phases of contraction which have a secondary effect of slow onset, progressive flaccid paralysis by depolarization of the axon and inhibition of excitability (Bouhaouala-Zahar et al., 1996). All scorpion toxins have a constant structural motif of a dense core formed by an α-helix, a triple stranded β-sheet and are stabilized by four disulfide bridges (Gordon et al., 1998).

### 1.2.2 Scorpion toxin expressions

Protein expression for further studies of protein structure/function relationships depend on the ability to efficiently express a cloned gene. Fusion proteins can be expressed by bacterial (prokaryotic), baculovirus (eukaryotic) or yeast (eukaryotic) expression systems. Scorpion neurotoxins are preferentially expressed in eukaryotic systems, mainly because prokaryotic systems are deficient in post-translational processing, unable to recognize regulatory sequences and, most importantly, the reducing environment of the bacteria cytosol can destroy disulfide bond formation (Higgins and Hames, 1999). However, the amount of active recombinant toxins expressed in eukaryotic system is often too low for further studies. Prokaryotic expression is more efficient in producing high levels of heterologous protein (Shao et al., 1999).

Successful scorpion toxin expression using a bacterial system has been demonstrated by Turkov et al. (1997). By using a strong promotor, large quantities of insoluble toxin were
expressed. Turkov and co-workers (1997) used the T7 promoter for massive expression of non-active depressant scorpion neurotoxin, LqhlIT. The LqhlIT was expressed without a fusion partner and contained no additional sequence to direct secretion. The expressed toxin (40 mg/L culture) accumulated in inclusion bodies in an insoluble, non-functional form. However, a functional recombinant toxin was reconstituted (500 µg HPLC purified toxin/L culture) by denaturing (solubilizing), oxidizing and refolding the insoluble recombinant protein with reductant. The paralytic unit (ED₅₀) for Sarcophaga falculata fly larvae was 70 ng for native toxin and 90 ng for the recombinant toxin. This functional, bacterially expressed, recombinant scorpion depressant neurotoxin will allow further study of structure-activity relationships and insect sodium channel gating (Turkov et al., 1997). Similar to the above study, a bacterial expression of scorpion toxin LqhlαIT produced recombinant toxin isolated from inclusion bodies that had chemical and biological properties equivalent to the native toxin after in vitro renaturation. A yield of 5 mg/L culture was achieved and 20 % was recovered as active recombinant toxin. Further studies of the toxin in relation to its selectivity in binding to sodium channels will be possible (Zilberberg et al., 1996).

Scorpion toxin expression is not restricted to use in structural studies. Use in production of antiserum against scorpion envenomation would also be useful for people in high risk areas. In Mexico, it is estimated that scorpion envenomation affects more than 200,000 people and is the cause of an average 700 deaths annually (Calderon-Aranda et al., 1995). Scorpion stings disturb nerve, cardiovascular and respiratory systems and cause dysfunction of the peripheral and central nervous system, which may lead to death. In the development of antiserum, a nontoxic protein (TsNTxP) from Tityus serrulatus venom has been isolated using gel filtration, ion exchange and reverse phase HPLC. T. serrulatus is responsible for most of the fatal cases of scorpion envenomation that occur in Brazil where approximately 10,000 incidences of scorpion stings have been recorded. The 63 amino acid residues of TsNTxP elicit antibodies against the protein in horses, mice and rabbits. Most importantly, these antibodies are capable of neutralizing the main toxic fraction (TstFG50) from T. serrulatus in vivo and in vitro. To express the non-toxic TsNTxP, the TsNTxP gene was cloned into c2pMal vector and expressed as a maltose
binding protein in *E. coli* BL21DE₃ cells. It was reported that the yield of the recombinant TsNTxP was 5 mg/L of bacterial culture. The recombinant fusion protein was purified by affinity chromatography (amylose column) and immunizations using the fusion protein produced antibodies that cross-react with TsNTxP and crude venom. It was found 20 LD₅₀ of *T. serrulatus* venom could be neutralized by 1ml of antiserum from mice immunized with the recombinant protein. Since the antiserum against the recombinant TsNTxP is protective, cleavage of the MBP was not required. This recombinant TsNTxP was seen as a potential candidate for the production of neutralizing polyclonal antibodies for treatment (Guatimosim *et al.*, 2000). Other practical expressions of scorpion toxins using MBP as a fusion partner have also been demonstrated such as the expression of charybdotoxin from *Leiurus quinquestriatus* (Park *et al.*, 1991) and kalotoxin 2 from *Androctonus australis* (Legros *et al.*, 1997).

In addition to toxin structure study or antiserum development, scorpion toxins have potential as insecticides. Unlike conventional insecticides, they would eliminate the problems of non-target specific toxicity, environmental pollution and development of insecticide resistant pests (Herrmann *et al.*, 1995). Scorpion toxin DNA coding for the insect toxin can be inserted into genome of insect pathogens, such as the baculovirus *Autographa californica* nuclear polyhedorsis Virus (AcNPV), to express the scorpion toxin as a biological insecticide and improve the time required for killing by the virus. Baculoviruses are non-infectious to vertebrates and plants, but are able to penetrate and infect lepidopterous pests making them the perfect vectors for mobilizing selective toxins into pests. Baculovirus are also capable of post-translational modifications to proteins, such as phosphorylation, glycosylation and signal peptide cleavage, and usually express protein in a soluble form, unlike prokaryotic expression systems (Dertzbaugh, 1998).

In a study of scorpion depressant and excitatory toxins, depressant toxin LqhIT₂ and excitatory toxin LqhIT₁ were expressed as recombinant proteins via AcNPV. LqhIT₁ expressing virus had effective paralysis time (ET₅₀) of 66 hours, and LqhIT₂ expressing virus had an ET₅₀ of 59 hours compared with a wild type virus ET₅₀ of 87 hours post infection against *Helicoverpa armigera* lepidopteran larvae. The pathogenicity of
AcNPV had improved by 30-40 % when anti-insect toxin was expressed within the recombinant virus. Similar in principle to the bacterially expressed protein, the expression of the scorpion toxin requires it to be regulated by a promoter such as p10 or polyhedrin (Gershburg et al., 1998). The choice of promoter can influence the level of expression, time and effective toxicity (McCutchen et al., 1991). From the above investigation, the AcNPV subgroup of baculovirus proved its usefulness as a biopesticide by restricting host range and being able to penetrate larva tissue and express the toxin with minimum environmental impact (Gershburg et al., 1998). Extensive research on the development of recombinant baculovirus expression of insect-selective neurotoxins have lead to several functional scorpion toxins being expressed including the expression of AaIT by McCutchen et al. (1991) and AaHIT by Stewart et al. (1991).

Another technique for protein expression was demonstrated by the production of the scorpion *Buthus martensill* Karsch (BmK) toxin BmK M1 in *Saccharomyces cerevisiae*. The gene sequence encoding BmK M1 was cloned into vector pVT 102 U/α prior to fusion with KEX2 linker which contains the yeast KEX2 protease site Lys-Arg. The vector consisted of an α-mating factor leader sequence to cleave the expressed protein and an alcohol dehydrogenase promoter. Secreted recombinant BmK M1 (rBmK M1) was purified by ion exchange (SP-Sepharose Fast Flow column) and gel filtration (HiTrap Desalting column) chromatography. The yeast expressed recombinant BmK M1 had similar biological activity to the native toxin, where the LD$_{50}$ of native BmK M1 is 0.75 mg/kg and 0.53 mg/kg for rBmK M1. In contrast to insect cells, the yeast system had higher yields of expressed protein than the insect cell system (5 mg/L for rBmK M1 expression). Compared to *E. coli* expression, the yeast system produced better protein folding, eliminating the need for refolding to correct the disulfide bonds, thus more functional protein is recovered ie 5 mg/L for rBmK M1 yeast expression and 500 ug HPLC purified TxNTp toxin/L bacteria culture. In addition, the growth medium used is relatively protein free and the purification of the expressed protein from culture media is much simpler than extracting protein from bacteria cells (Shao et al., 1999).
Chemical synthesis of proteins has also been used as an alternative to prokaryotic and eukaryotic expression systems. Previous studies have examined the feasibility of active protection against the potent and well characterized *Androctonus australis hector* toxin analog (Abu)$_8$ AahII. Since immunization of native AahII is detrimental to an animal and usage of native toxin as an immunogen is impractical, (Abu)$_8$ AahII was produced by chemical synthesis of peptides including replacement of eight half-cysteines by $\alpha$-aminobutyric acid to remove the disulfide bridges. Analogs were produced as monomers or polymers by glutaraldehyde. The analog is non-toxic and readily synthesized. When used to immunize mice or rabbits, it induces antibodies capable of binding to native AahII and preventing $[^{125}\text{I}]}$-AahII binding to rat brain synaptosomes. Mice immunized with polymerized analog six months prior to challenge survived an 8 LD$_{50}$ of native toxin challenge despite a decline in antibody titre (Zenouaki et al., 1997). This result demonstrated the capacity of chemically produced protein to act as an immunogen against the deadly *Androctonus australis hector* toxin.

### 1.2.3 Monoclonal antibodies against scorpion toxins

Parallel to scorpion toxin expression, monoclonal antibodies against scorpion toxins have also played an important role in toxin characterization, and their potential as an antiserum can not be overlooked. Scorpion venoms are composed of proteins with a common mode of action but diverse amino acid sequences, and antisera raised against one toxin often fails to neutralize other toxins from the same or a different scorpion venom. The potency of serotherapy can be retarded by the diverse toxins expressed and its effectiveness is restricted by the rapidity of the polyclonal antibodies capturing the diffused toxin (Devaux et al., 1996).

The current treatment for scorpion envenomation is administration of antiserum raised in horses. Replacements for the conventional treatment using mixtures of toxin-specific monoclonal antibodies can be valuable candidates for serotherapy (Zenouaki et al., 1997). One aim of immunotherapy is to administer high levels of specific, homologous antibodies allowing treatment to be more effective and tolerable (Chippaux, 1998). It has
been reported that 1 mg of mAb BCF2 neutralizes 28 LD\textsubscript{50} of \textit{Centruroides noxius} venom and 1 mg of the Fab fragment of BCF2 neutralizes 43 LD\textsubscript{50} of soluble \textit{C. noxius} venom. To obtain equivalent degree of neutralization by horse antiserum (polyclonal), ninefold more antiserum than Fab fragments would be required. Since BCF2 Fab fragments have high neutralization ability, the quantity of foreign protein needed to be injected into a patient would be reduced significantly. In addition, the adverse effect from the F\textsubscript{c} fraction of immunoglobin is eliminated (Licea \textit{et al.}, 1996).

Monoclonal antibodies are powerful tools for studying the structure-functional relationship of proteins and for the examination of the neutralization mechanism. For example, noxiustoxin produced by Mexican scorpion \textit{Centruroides noxius} acts on potassium channels in a voltage dependent manner. To examine the number of epitopes present within the noxiustoxin, a panel of six monoclonal antibodies has been raised against the 39 amino acid residue peptide. The mAbs were tested in competitive enzyme linked immunoassay (ELISA) and all found to bind to an overlapping epitope or in close proximity. In this study, the effect of the monoclonal antibodies on toxin-receptor interaction were also inspected by their ability to inhibit the binding of radiolabeled $[^{125}\text{I}]-\text{noxiustoxin}$ to rat membrane synaptomes. All the mAbs were able to inhibit the toxin binding to its receptors by inducing conformational changes in the toxin (Herion \textit{et al.}, 1995).

One of the most important functions of mAb in toxin research is the neutralizing mechanism, which is demonstrated in the examination of \textit{Androctonus australis hector} toxin. Low doses of AahII and non-toxic, synthetic (Abu)$_8$ AahIIls (64 residues) were used as immunogens for monoclonal antibody production. As the production of monoclonal antibodies using native toxin is difficult due to the limited quantity of native material and its toxicity, the use of synthetic preparations is more feasible. Monoclonal antibodies raised against (Abu)$_8$ AaII were able to recognize and neutralize the native toxin. In addition, peptides spanning the full AahII sequence were used to assess the binding sites of the mAbs. It was shown that the mAbs bound to several parts of the AahII sequence, which suggested (Abu)$_8$ AaII contained epitopes that mimic the native
Chapter 1: Introduction

AahII epitopes. Thus, this full-length analog could be used as a potential immunogen (Devaux et al., 1997). In another study, two monoclonal antibodies (3C5, 4C1) raised against low doses of Androctonus australis Hector (AahII) were shown to protect mice injected with AahII toxin. Mab 4C1 was used to investigate the mechanism of neutralization. The mAb was found to be able to dissociate labeled toxin from its receptor. The monoclonal antibodies had high affinity to the antigens, which prevented dissociation of the mAb/antigen complex thus protecting the animals from paralysis (Bahraoui et al., 1988; Yahi et al., 1992).

Combining the neutralization ability of mAb and recombinant technology, advances in antibody engineering have lead to the production of recombinant single chain antibodies (scFv). The cDNA encoding the variable domains of the mAb 4C1 (VH and VL) has been engineered as a single chain Fv, with the C termini of the VH joined to the N termini of the VL by a short, flexible peptide. ScFv possessed the parent antibody’s binding properties for neutralization in vitro and in vivo and had reduced adverse effects in allergic hypersensitivity due to reduced size. It also has the ability to penetrate tissues more readily than conventional antibodies due to its small size, and consequently it diffuses more quickly for detoxification. In vivo and in vitro assays demonstrated that one ml of scFv neutralizes 800 LD₅₀ of AahII. Furthermore, scFv can be removed from the patient via urine. The scFv has been successfully expressed as soluble and active recombinant protein in Escherichia coli (Mousli et al., 1999).

Expression of scFv by bacterial systems is not always without problems. Bacterial expression systems are favored for the mass production of recombinant proteins with low cost. Nevertheless bacteria are more likely to synthesize recombinant proteins as insoluble inclusion bodies. Hence solubilization and refolding are often necessary during which there is the possibility of a loss in protein activity. Moreover, proteins are susceptible to degradation by proteases released during protein extraction. In the case of scFv expression from mAb A58 raised against the hemocyanin of the scorpion A. australis, the scFv protein was found to be inactive and nonsoluble when expressed in the E. coli inclusion bodies. By incorporating a signal peptide sequence, the scFv was
redirected to accumulate in the periplasm in the native state. The periplasmic space is an oxidizing environment that is comparable to the lumen of the endoplasmic reticulum in eukaryotic cells and thus enhances disulfide bonding (Billiald *et al.*, 1995). Alternatively, expression of scFv can be achieved in a baculovirus expression system. This is exemplified in another 4C1 scFv expression using the baculovirus system for production of soluble scFv. ScFv secreted by insect cells would also avoid toxic contaminants (endotoxin) released from *E. coli*. The advent of scFv has opened new prospects in envenomation treatment with better features for immunotherapy (Lemeulle *et al.*, 1998).

### 1.3 SPIDERS

#### 1.3.1 Spider toxin structures

There are 40,000 types of spiders; 20,000 of these are venomous and 200 species affect humans. Nevertheless only a few spider toxins have been well characterized, mainly due to the difficulty in obtaining adequate amounts of toxin (Figueredo *et al.*, 1995; Grishin, 1999; Russell, 1991). Most studies have been confined to spiders with large venom glands such as those from the *Araneidae* family (Jackson and Parks, 1989). Broader research on spider toxins including species that are not medically significant are important because spider neurotoxins can be potential tools for neurochemical and electrophysiological studies and used as insecticides due to their highly specific action. For example, the discovery of *Argiope lobata* venoms that block neuromuscular transmission in frog and locust has lead to the isolation of glutamate receptors from crab muscle using active fractions of *Argiope lobata* to an coupled affinity column. (Jackson and Park, 1989)

Spider venoms are known to contain a mixture of components such as amino acids, nucleic acids, enzymes, inorganic salts, monoamines, polyamines and proteinaceous and nonproteinaceous toxins (Jackson and Parks, 1989). Complex mixtures of toxins are synthesised in order to kill various types of prey (Shu and Liang, 1999). The majority of spider neurotoxins have a molecular weight range from 4 to 10 kDa with intramolecular
disulfide-bridges formed by 6 to 14 cysteines. These low molecular weight toxins bind to excitable membrane cation channels such as sodium, calcium and potassium channels. The low molecular weight toxins affect voltage-gated ion channels and include sodium channel specific μ-agatoxins, calcium channel specific ω-agatoxins from the American funnel web spider (*Agelenopsis aperta*), and the less abundant potassium channel specific toxins, for instance, hanatoxin 1 and hanatoxin 2 from *Grammostola spatulata* and heteropodatoxins originating from *Heteropoda venatoria*. Finally, high molecular weight neurotoxins that interact with receptor sites of the presynaptic membrane and cause release of neurotransmitters are found in the most studied European black widow spider *Latrodectus tredecimguttatus* as the 130 kDa latrotoxins (Grishin, 1999).

### 1.3.2 Expression of spider fusion toxins

Huwentoxin-I (HWTX-I) is a neurotoxin from the venom of the Chinese bird spider *Selenocosmia huwena*. It consists of 33 amino acids with six cysteines that form three disulfide bridges. The structural motif of HWTX-I is a triple stranded antiparallel β-sheet and a cystine knot. The HWTX-I sequence is similar to the four-disulfide bonded μ-agatoxin from the funnel web spider, *Agelenopsis aperta*. However, the two possess different biological activities. To investigate the effect of the disulfide bonds on the structure-function relationship, the production of active HWTX-I was required (Zhang and Liang, 1993). Functional expression of HWTX-I was achieved by ligating a synthetic gene encoding HWTX-I to the C-terminal end of the glutathione S-transferase (GST) gene in the fusion vector pGEX-KT followed by expression of the protein in *E coli* DH5α cells. The fusion protein was partially purified via GSH-Sepharose before being cleaved by thrombin (sequence specific protease) and the recombinant HWTX-I purified by HPLC. Bacterial synthesized HWTX-I had incorrect disulfide bridge formation that caused low activity. However with reduction and renaturation treatment, full biological activity was produced and the protein was found to block neuromuscular transmission in mice, resulting in paralysis and respiratory failure. This demonstrated that successful expression can be accomplished in a bacterial system and that production of active
recombinant neurotoxin can further assist in the characterization of ion channels and receptors.

As well as the bacterial expression demonstrated with HWTX-I toxin, the baculovirus expression system has also provided efficient synthesis of protein from genes of spider toxins. The use of baculovirus insect cell expression was successfully demonstrated in the expression of α-latrotoxin. Black widow spider venom contains various neurotoxins including two high molecular weight proteins and an eight-kDa protein. The two identified high molecular weight (130 kDa) neurotoxins are the vertebrate specific α-latrotoxin and invertebrate specific α-latroinsectotoxin. It is known that these neurotoxins enhance neurotransmitter release from synaptic vesicles at nerve endings and in endocrine cells (Kiyatkin et al., 1995). To further study the mechanism of α-latrotoxin action, it was essential to express the protein as a recombinant toxin that mimics native α-latrotoxin. The α-latrotoxin gene was cloned into baculovirus and the recombinant protein synthesized in insect cells. However, injection of Trichoplusia ni larvae with recombinant α-neurotoxins from the insect cells had no toxic effect (Kiyatkin et al., 1995). Volynski and coworkers (1999) later discovered that a functional and soluble recombinant α-latrotoxin can be generated by adding a strong signal peptide from melittin to the N-terminus of mature α-latrotoxin. The successful production of recombinant α-latrotoxin via the baculovirus system has produced a protein that is indistinguishable from the native toxin enabling functional research to occur by mutational analysis of the toxin. The recombinant α-latrotoxin has also assisted in the discovery of presynaptic toxin receptors (latrophilin and neurexin Ia).

1.3.3 Monoclonal antibodies to spider toxins

The first report employing monoclonal antibodies for quantifying arachnid venom is in a study of venom dose and prey size. Monoclonal antibodies raised against the venom of the wandering spider Cupiennius salei were able to quantify venom volumes as little as 0.02 μl by ELISA. The study of spider venom using mAb 9H3 demonstrated that the
quantity of the venom injected by the spider corresponds to prey size, supporting the
theory that spiders lower the cost of energy for venom production by injecting smaller
quantities of venom when immobilizing smaller prey (Malli et al., 1998).

Based on the antibody-antigen binding mechanism, the use of antibodies has been
explored in immunoassays for the development of diagnostic tests. These immunoassays
would allow the identification and quantification of circulating venom. This is important
due to an increasing incidence of spider bites and the variety of choice for treatment. For
instant, *Loxosceles* spider bites can cause a wide spectrum of effects from mild
dermonecrotic lesions (loxoscelism) to serious systemic reactions and death in severe
cases (Chavez-Olortegui et al., 1998; Chippaux and Goyffon, 1998; Walter et al., 1999).
Various treatments are available including administration of antibiotics, corticosteroids,
antiserum, surgical excision of the envenomation site or blood transfusion. Thus,
determining the level of antigen can be used to correlate clinical symptoms and evaluate
the effectiveness of antivenom treatment. In a study of *Loxosceles intermedia* spider
venom detection in experimental mice, it was shown that ELISA had the capacity to
differentiate circulating antigens of *L.intermedia* venom from the mice inoculated with
*L.intermedia*, *L.gaucho*, *L.laeta*, *Phoneutria nigriventer* spider venoms, *Tityus serrulatus*
scorpion venom and *Bothrops jararaca, Crotalus durissus terrificus, Lachesis muta muta*, and *Micrurus frontalis* snake venoms. The assay system was able to measure as
low as 0.8 ng of venom/assay and identify antigens from the sera of a *L. intermedia*
envenomed patient (Chavez-Olortegui et al., 1998; Chippaux and Goyffon, 1998).

To further improve ELISA systems as diagnostic tests of envenomation, better
preparation of specific antibodies is required. In the *L.intermedia* study, a homogenous
population of IgG F(ab')² was obtaining by purifying IgG from the sera of *L.intermedia*
hyperimmunized horses using an immunoaffinity (CNBr-Sepharose) column. IgG was
then subjected to pepsin digestion and the digested IgG F(ab')² purified by protein
A-Sepharose chromatography and then further purified using a *L.intermedia*-Sepharose
affinity column (Chippaux and Goyffon, 1998; Chavez-Olortegui et al., 1998).
Alternatively, with the advance of diagnostic tests, panels of monoclonal antibodies can
be generated as diagnostic tools for detection of the toxin. This can eliminate the procedures for immunopurification of IgG populations and enhance the quality of antibodies. Hauer and Clough (1999) and Noah et al. (1995) suggested that application of a collection of monoclonal antibodies for quality control of biological vaccine production would reduce the number of animals required for measuring the protective antigen using an *in vivo* assay. In addition, *in vitro* assays reduce the biological variability of *in vivo* assays, the cost of labor and time for the test. Similarly, the movement toward *in vitro* tests for spider envenomation may be advantageous since they can be used as an aid to assess the type and level of venom in the patient and as proof of envenomation in young children whose clinical symptoms may be misdiagnosed.

1.4 TICKS

1.4.1 Cattle tick *Boophilus microplus*

Ticks are obligate ectoparasites of mammals and with the major families being *Ixodidae* (hard ticks) and *Argasidae* (soft ticks). Members of the *Ixodidae* family contributed 80% of species of economic or medical significance (Jongeljan and Uilenberg, 1994). *Boophilus microplus* also known as the cattle tick, is responsible for the majority of veterinary parasite infestations in the cattle industry in tropical and subtropical areas ie Australia or Cuba (Fuente et al., 1998). *B. microplus* is a one-host tick (Lee and Opdebeeck, 1999) and it is known that cattle can attain partial immunity against the tick species through extensive natural exposure. However, this tick often causes a hypersensitivity reaction in cattle (Rodriguez et al., 1994), with clinical symptoms of weakness, blood loss and reduction in milk production. The ticks also act as vectors for the transmission of babesiosis or anaplasmosis. Traditionally tick infestations have been controlled by chemical acaricides and as a result chemical residues can be introduced into the milk or meat. The development of chemical resistant tick strains has also resulted from prolonged chemical exposure. Thus, the development of a vaccine was proposed as a cost-effective approach for treating the problem (de la Fuente et al., 1998). Vaccination against *B. microplus* was the first and most successful study on vaccine-induced
immunity in a host using a single antigen. Therefore work on B. microplus will provide the basis for the further development of tick vaccines against B. microplus and illustrate the potential of vaccines against other tick species (Willadsen, 1990).

1.4.2 Fusion protein expression for vaccine studies

Willadsen (1989) was the first to demonstrate that 90% of ticks failed to survive when attached to cattle immunized with three doses of 2 μg of a membrane-bound glycoprotein (Bm 86) purified from cattle ticks. Rabbit antisera against native Bm86 indicated that the targeted antigen was located in the midgut epithelium of the cattle tick. Despite the potential of the native immunogen, only 20-100 μg of Bm86 can be purified from 40,000-60,000 ticks. To produce a vaccine in commercial quantities, many more ticks would be required. To conquer this problem, the Bm86 gene has been cloned and expressed as a recombinant protein in bacteria, baculovirus and yeast expression systems.

The gene sequence encoding Bm86 was first discovered by Rand et al. in 1989. By ligating the PCR product coding for Bm86 into pBTA224 vector containing the β-galactosidase coding region function as fusion partner, the vector pBTA708 was formed. The vector was then transformed into E. coli to produce recombinant Bm86 as inclusion bodies. The 143 kDa fusion protein possesses the 651 amino acid of β-galactosidase, 599 residues of Bm86 and 19 amino acid residues encoded by the vector. The inclusion bodies can be recognizing by antiserum from native Bm86 vaccinated cattle. Cattle immunized with a crude preparation of inclusion bodies had a 24% reduction in the number of ticks attached and 77% of the surviving ticks had reduced weights with damaged egg-laying capability (Rand et al., 1989).

To overcome incorrect protein folding in the bacterial expression system, baculovirus expression system was investigated. The cDNA sequence identified by Rand et al. (1989) was shown to contain a high proportion of cysteines. To improve the production of correctly folded protein, the cDNA sequence for truncated Bm86 (without the last 30 amino acids of Bm86 which code for a putative GPI anchor recognition sequence) was
cloned into a baculovirus vector and expressed in virus infected insect cells (*Spodoptera frugiperda*, Sf9). The truncated form of Bm86 (Bm86trun) was expressed by the cell and secreted into the culture medium. Bm86trun was then purified from the medium using DEAE-Sepharose ion exchange chromatography. A vaccination trial showed that Bm86trun induced a 77 % reduction in egg production and a 45 % decrease in the number of ticks feeding on vaccinated animals (Richardson *et al.*, 1993).

Recently, more efficient expression has been reported by a South American group (Rodriguez *et al.*, 1994) using the methylotrophic yeast *Pichia pastoris*. Yeast are attractive species for use as an expression system because of their non-pathogenic properties and the unicellular eukaryotes can grow rapidly in simple defined medium (Higgins and Hames, 1999). The expression vector pPBm consists of 608 residues encoding the Bm86 gene, excluding 23 amino acids coding for the carboxyterminal transmembrane region and 19 amino acid residues for the signal peptide from the native Bm86 glycoprotein. The Bm86 gene isolated from the mRNA of South American cattle ticks were found to have three nucleotide differences to the DNA sequence published from Australian cattle ticks (Rand *et al.*, 1989; Rodriguez *et al.*, 1994). The effect of the variation in the DNA was investigated using monoclonal antibodies (further discussed in section 1.4.3). Despite some nucleotide variations, the rBm 86 from yeast expression still caused a 31 % reduction in cattle ticks feeding on vaccinated animals and a 70 % decrease in their reproductive ability. These results are essentially the same as with *E. coli* expressed rBm86. However, the level of expression in yeast was much higher (1.5 g/L) than any other expression system reported so far (Rodriguez *et al.*, 1994). The *P. pastoris* expressed rBm86 is marketed as recombinant vaccine Gavac™ and used against the cattle tick in Cuba and Latin America (Garcia-Garcia *et al.*, 1998)

Overall, vaccination by recombinant Bm 86 has been shown to effectively control tick infestations by increasing the production of IgG in the host to destroy cells in the gut of cattle ticks. (Fuente *et al.*, 1998; Brossard, 1998). Leukocytes from the hosts can also attack the reproductive glands of male ticks and consequently the population of ticks is
reduced. More importantly, acaricide resistant ticks are sensitive to the antibodies generated in the cattle immunized with recombinant Bm 86 (Angus, 1996).

1.4.3 Characterisation of antigens from *Boophilus microplus*

The success of the research into the Bm 86 vaccine has lead to the study of the diversity of protective antigens in the field population of cattle ticks. It is known that antigenic variance occurs within protozoan parasites that effects the efficacy of vaccines, but this has never been examined for arthropod parasites. In the investigation, three murine monoclonal antibodies, and antiserum from vaccinated cattle and naturally immune cattle were used to probe gut antigens from *B. microplus*. The Western blot data suggested that the gut antigens from the ten field isolates of *B. microplus* have identical reactivity with all mAbs and antiserums. One of the mAbs (QU13) raised against a native midgut extract of *B.microplus* was able to protect cattle from tick infestation. QU13 was also shown to recognize a carbohydrate epitope on all ten isolates of *B. microplus* adult and larval membrane gut extracts (Lee and Opdebeeck, 1991). In addition to the vaccination experiments, four mAbs raised against embryo (designated BrBm 1, 3, 4) and gut tissue (designated BrBm 2) have contributed to the knowledge about the damaging effects of anti-tick antibodies to *B. microplus*. Inoculation of the mAb into the ventral surface of fully engorged female ticks with a microsyringe showed that BrBm1 and BrBm2 reduce reproduction efficiency by 50% and 70% while BrBm3 and BrBm4 have no affect on oviposition (Toro-Ortiz et al., 1997). These results indicate that the vaccine could be effective against *B.microplus* in the field at different life cycle stages. The application of monoclonal antibodies from this research demonstrates the diverse role mAb can play in research.
1.5 THE AUSTRALIAN PARALYSIS TICK, \textit{IXODES HOLOCYCLUS}

The native environment for \textit{Ixodes holocyclus} is the eastern seaboard of Australia, extending from North Queensland to Lakes Entrance in Eastern Victoria (Figure 1.1) (Roberts, 1970). In southern regions the prevalence of the tick is restricted to where conditions are warm and humid (Ross, 1935) or by the existence of native hosts including kangaroos, koalas, possums, the long nosed or the giant brindled bandicoot (Albiston, 1968), and the principle accidental hosts are domestic or companion animals including cattle, horses, dogs and cats. Concurrent with abundance, paralysis occurs more frequently in spring and summer (Grattan-Smith \textit{et al}., 1997). In their native environment, bloodmeals are mainly obtained from native hosts, which acquire immunity against heavy infestions. The ability to cause paralysis does not pose any survival issues for the ectoparasite. Stone \textit{et al}. (1989) have suggested that paralysis may be a coincidence of nature, which overlaps with primary functions such as anticoagulation or local anesthesia. Alternatively, at one point all tick species may have been predators that paralyze their prey and through evolution most ticks have lost their ability to paralyze, adapting to a parasitic life style (Thurn, 1994; Grattan-Smith \textit{et al}., 1997).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1_1.png}
\caption{\textit{Ixodes holocyclus} distribution.}
\end{figure}
1.5.1 Parasitic life cycle

The life cycle of *I. holocyclus* consists of three stages, larvae, nymph and adult, each of which engorge on separate hosts. Unlike other arachnids, *Ixodes* ticks have adapted to long term survival by their ability to maintain water balance by uptake of moisture from the atmosphere. Their life span is measured by years, where temperature or humidity variation can often delay development at any stages of the life cycle (Grattan-Smith *et al.*, 1997).

The tick life cycle (Figure 1.2) initiates with eggs laid by the female tick hatching after 40-60 days to develop into six legged larvae on moist leaf litter. The larvae attach to their first host for a bloodmeal and this engorgement stage lasts for 5-6 days. The immature larvae abandon the host after completion of feeding by dropping on to moist vegetation. The engorged larvae moult, developing into eight-legged nymphs, which climb up vegetation and seek a second host. The nymph feeds for 5-6 days and then falls to the ground to moult to become an eight-legged adult tick. The unfed female tick again crawls up vegetation attaches to a third host and engorges on this host for 7-12 days (Grattan-Smith *et al.*, 1997). Male ticks have small mouth parts that make it incapable of feeding on the host. They are found wandering on the host, seeking and feeding from female ticks (Cooper *et al.*, 1976). Fully engorged female ticks, when fertilized by the male tick, lay 2000 to 3000 eggs on the ground as the final stage of the life cycle (Cooper *et al.*, 1976; Grattan-Smith *et al.*, 1997).

**Figure 1.2 I. holocyclus life cycle.**
(Taken from New South Wales Agriculture web site)
1.5.2 Tick attachment and feeding

In the parasitic life cycle, tick attachment commences with the tick grasping the host with its legs and raising its body to a position that allows the chelicerae to penetrate the skin with a horizontal cutting action. The chelicerae are rigid cutting digits, which function as a penetration device on the hypostome (Figure 1.3). The long hypostome of *I. holocyclus* can perforate the skin to a depth of 980 microns and function as substitute for internal or external cement protein for securing its attachment (Stone, 1990). The host's tissues and fluid are then sucked up by the pharyngeal pump, through the hypostome and stored in multiple sac-like diverticulae in the midgut (Kemp *et al*., 1983). Excess fluid from the bloodmeal and the neurotoxins are returned to the host via the salivary gland.

Figure 1.3 Electron micrograph of the mouthparts of *Ixodes holocyclus*. The teeth of the hypostome (H) secure attachment of the tick upon insertion. The pedipalps (b) protect the hypostome from damage prior to attachment (Kemp *et al*., 1983)
The salivary gland plays a crucial role in completion of prolonged feeding. To achieve the required extended feeding time, the salivary gland produces antiplatelet aggregatory, anti-vasoconstrictory and anticoagulatory factors to suppress the host’s immune, inflammatory and haemostatic responses (Bowman et al., 1997). In contrast to other blood feeding ectoparasites, *Ixodidae* have relatively long feeding period of 7-12 days when compared to mosquitoes (<3 min), Kissing bugs (<20 min), argasid ticks (5-25 min) and leeches (2 h) (Bowman et al., 1996; Grattan-Smith et al., 1997). Corresponding to the feeding stages, unfed ticks have a lower level of toxicity than half fed ticks in which the peak amount of toxin injection occurs (day 5-6) (Stone et al., 1986) (Figure 1.4). During the initial feeding phase (day 1-4), digestion of the host tissues allows for the development of reproductive tissues, salivary glands, organs and growth of the cuticle for body expansion. At the final stage of engorgement, female *Ixodid* ticks can increase by 100-200 times their body weight during feeding (Kemp et al., 1983; Sonenshine, 1991; Wang et al., 1999). The rate of feeding increases in the later phase (Kemp et al., 1983).

![Tick activity scale](Taken from New South Wales Agriculture web site)

**Figure 1.4 Level of toxicity (Tick activity scale) at each stage of life cycle.**

(Taken from New South Wales Agriculture web site)
1.5.3 Features of tick paralysis vs current treatments of paralysis

The dominant characteristic of tick toxicoses is hindquarter wobbling followed by acute, ascending, flaccid motor paralysis involving the forelimbs (Stone et al., 1989). Consequently the animal becomes uncoordinated and lies in lateral recumbence. Other symptoms observed in dogs during tick intoxication include vomiting, regurgitation, loss of voice and appetite, ocular irritation, and terminates in death due to respiratory failure (Stone et al., 1989; Malik and Farrow, 1991; Goddard, 1998).

The first step in the treatment of tick toxicosis is usually the elimination of the tick. For instance, removal of North American ticks *D. andersoni* and *D. variabilis* will allow the patients to recover within one day (Malik and Farrow, 1991; Grattan-Smith et al., 1997). However in the case of *I. holocyclus* tick paralysis, the condition of the affected animal may worsen if the tick is detached physically. This is due to salivary proteins including the paralyzing toxin being released from the attachment site into circulation when ticks are physically removed (Stone, 1990). To prevent patients deteriorating, tickicidal chemicals suitable for animal or human use can be applied to kill the ticks. Tickicidal chemicals that are currently available include Pyrethrin/pyrethroids, which disrupt nerve transmission in ticks to stop salivation, acaricides for tick detachment, surgical ether, chloroform, kerosene, tickicidal repellent (Stone, 1990) or fipronil for adulticide effect (Searle et al., 1995).

Following tick removal, the most common treatment for tick paralysis in animals is the injection of commercial polyclonal antiserums and veterinary support, ie monitor fluid balance, oxygenation/ventilation, core temperature, and blood pressure. With advanced intoxication, animals require administration of the α-adrenoreceptor antagonist phenoxybenzamine and the phenothiazine tranquilizer acepromazine as vasodilators to decrease peripheral vascular resistance, thus easing respiratory distress (Malik and Farrow, 1991; Fitzgerald, 1998).
A hyperimmune serum obtained by feeding large numbers of ticks on dogs is used to treat tick toxicosis. In human cases, antiserum treatment is only suitable for severe paralysis due to the risk of acute reactions and serum sickness (Grattan-Smith et al., 1997). The use of antiserum is also restricted in several ways. Firstly antiserum is expensive, and the cost of treatment can be greater than the value of livestock (Stone et al., 1983b). Secondly, the antiserum is relatively heterogeneous (Boulain et al., 1982), and foreign serum proteins in the antiserum can cause side effects, particularly anaphylactic reactions (Stone and Aylward, 1987). Antiserum is also only effective in the early stages of paralysis (Stone and Wright, 1981). The neurotoxins secreted by the tick can diffuse throughout the whole body and therefore the effectiveness of antiserum treatment depends on the rapidity with which the antiserum is administered to bind to the neurotoxins (Zenouaki et al., 1997). It is conceivable that the toxin can become internalized in cells, preventing neutralization by the antiserum (Grattan-Smith et al., 1997). Moreover, variation between individual hyperimmunized animals and the methods of obtaining antiserum have led to difficulties in the standardization of each antiserum sample (Stone et al., 1982). Finally, a constant supply of ticks is necessary to hyperimmunise dogs and this is not feasible for large scale antiserum production. Hence, the development of an effective antitoxin and vaccine is important for the treatment of tick paralysis.

1.5.4 Isolation and purification of paralysis toxin

To develop a vaccine, antigen or antigens that are the cause of disease must be identified (Willadsen, 1990). Since 1960, efforts have been made by a number of research groups to purify the protective antigen from the complex mixture of proteins produced in the tick salivary gland. In the past, this has been difficult due to lack of technology for isolating the very small amounts of toxin present in a limited supply of engorged ticks. In addition, conditions used to purify the paralysis toxin in the past were believed to be too harsh and the biological assays were ineffective with small quantities of toxin (Stone et al., 1979; Thurn, 1994).
Kaire (1966) was the first to partially purify the paralysis toxin from the salivary gland of *I. holocyclus* using DEAE cellulose chromatography. This toxic fraction was able to produce paralysis in dogs that was indistinguishable from native paralysis. Dogs also acquired protective immunity when immunized with the toxic fraction. In addition, anti-tick serum from dogs immunized with this fraction protected mice against lethal doses of the toxin. The toxic fraction was found to be resistant to heat up to 75 °C, pH changes (3-9) and enzyme digestion by pepsin, trypsin and papain. However, the short supply of ticks prevented the development of a vaccine or the isolation of pure toxin from this fraction.

Since the discovery of the toxic fraction, numerous studies have concentrated on isolation of the tick toxin. Stone *et al.* (1979) separated a number of toxic fractions (referred to as SX-vv, -I, -II, -III) by gel filtration and affinity chromatography. These toxic fractions contained proteins of molecular weights ranging from 60-80 kDa. Biological assays indicated that while SX vv, I and II all produced paralysis, SX II was the main paralyzing component. SX III had a molecular weight less than 20 kDa and was associated with other symptoms such as cardiovascular failure. Its presence was stated to be lethal but non-paralyzing. Other biologically active fractions identified by this group (Stone, 1979; Stone *et al.*, 1983a) include a lower MW (40-60 kDa) toxic fraction purified from a Sephacryl 200S column. The major electrophoretic band had a MW of 50 kDa when the fraction was digested by pronase. The paralyzing fraction also had an isoelectric point around the range of pH 4.5-5.0.

Parallel to the attempted isolation of paralysis toxins, the immunity to tick paralysis was investigated. Dogs were found to develop protective immunity artificially. This was achieved by feeding a single female tick on an unexposed dog for 14 weeks (Figure 1.5) until the dogs were able to withstand a full tick feeding cycle. This was followed by an increase in the number of ticks feeding on the dog over a period of time to a maximum of 32 ticks per dog. The maximum antiserum potency corresponds to an increase in the number of ticks. Despite the success in inducing protection, immunity declines when the dog is kept tick free, as demonstrated by a decline of 1/3 of the maximum antitoxin serum...
within 10 weeks (Figure 1.5). Similarly, native hosts such as bandicoots can lose their resistance to tick paralysis and be killed by one tick when kept free from ticks for a period of time (Ross, 1926; Grattan-Smith et al., 1997). Thus, immunity against tick paralysis is considered to be short-lived (Stone and Wright, 1981; Stone et al., 1983b). This illustrates the necessity of multiple immunizations to restimulate immune response by ticks with the labor cost for animal care being significant when using ticks as a vaccine. However, the study indicated the potential of developing immunity against tick paralysis.

Figure 1.5 Dog antibody responses against ticks by feeding ticks on dogs. Numbers on the curve represent the number of ticks on the dog. (Taken from Stone and Wright, 1979; Stone and Wright, 1981)

In a continuation of immunisation studies, Stone and Neish (1984) attempted to immunize animals with partially purified toxin instead of live ticks. As this fraction could be toxic to some animals, the antigen was detoxified by treatment with glutaraldehyde to produce the vaccine (Stone et al., 1983b). This toxoid was found be immunogenic and more potent in provoking an immune response when compared to immunization by the
native fraction. This is possibly due to an increase in the molecular weight of the antigen or decreases in the immunosuppressive effects of paralyzing toxin (Stone et al., 1986). Therefore, fewer injections of the detoxified, but potent paralyzing fraction are required for the development of protective immunity in rabbits and dogs (Stone et al., 1986). Despite some success in these studies, it is not feasible to use the native toxin as a commercial vaccine because of the difficulties in isolation of the toxin, short supply of ticks, cost and quality control for a modified biological vaccine is remarkably difficult (Licea et al., 1996).

Before the application of recombinant technology, Stone and coworkers (1982) undertook one of the most innovative ways for toxin isolation by recovering the soluble toxin from tick feeding medium. The ticks were artificially fed on tissue culture medium (TC199) through a thin membrane. Proteins of a MW between 60-80 kDa from the medium were purified on Ultrogel AcA44 gel filtration column and shown to be toxic. The soluble toxic component isolated by this method has been shown to contain similar toxinological characteristics to toxin extracts from tick salivary gland. However, the soluble toxin yield per tick feeding on artificial media is less than for ticks feeding on rats then extracting from the salivary glands. In addition, the survival rates for ticks' feeding on artificial medium is lower than ticks feeding on rats (Stone et al., 1982; Stone and Binnington, 1986).

Efforts to isolate paralysis toxin using gel filtration by Davey and coworkers (1989), produced one toxic fraction with a molecular weight of 40-60 kDa. This finding was similar to Stone (1979). Later, Thurn (1994) performed an experiment using a semi-purified toxic fraction of tick extract in which the protein had been radio-labelled with $^{125}$I to identify a protein band binding to rat brain synaptosomes. This band was shown to be composed of 3 low molecular weight neurotoxins. In SDS-PAGE using Tricine gels the neurotoxin appeared as three bands (8, 9 and 11 kDa) under non-reducing conditions and as a single 5 kDa band under reducing conditions. The three homogeneous Holocyclus neurotoxins designated HT-1, HT-2, HT-3 were also purified by Thurn (1994), using hydrophobic interaction chromatography and reverse phase HPLC,
showing apparent molecular weight of 5 kDa. Unlike the toxins previously isolated, these neurotoxins were quite small. These neurotoxins were also found to contain a common epitope as indicated by the ability of mAb 96B 1G6 (a mAb against native toxin) to bind to the three neurotoxins. Unfortunately, this non neutralizing antibody is no longer available for study. Furthermore, *I holocyclus* neurotoxins were able to bind to synaptosomes in a temperature dependent manner. This observation supported the electrophysiological studies by Cooper and Spence (1976) that showed animals were paralyzed more pronouncedly at elevated temperature.

Using Edman degradation of tryptic digests, partial peptide sequences were obtained by Thurn (1994). Tryptic digestion was used since blockage at the N-terminus prevented the direct sequencing of the HT-1 gene. Peptide sequences of HT-2 and 3 were never generated because only low quantities of purified neurotoxins, insufficient to perform N-terminal sequencing, could be isolated. Using molecular techniques Masina (1999) designed primers based on the partial sequence from Thurn (1994) to isolate the complete HT-1 cDNA sequence from the tick mRNA (see Appendix 1). The strong homology between this tick toxin sequence and that of other arachnid toxin from scorpion and spiders described in detail by Masina (1999). The HT-1 gene was expressed as a recombinant fusion protein (Maltose binding protein- *Holocyclus Toxin* I fusion protein or MBP-HT-1-fusion protein). The advantages of expression as a fusion protein were the ease of purification by incorporation of a His tag and stabilisation of HT-1 by MBP. Two factor Xa cleavage sites were also incorporated at both ends of HT-1 for the release of free HT-1. A disadvantage of the fusion system was that the carrier protein, in this case MBP, may impede the subsequent characterisation of the protein and cause incomplete cleavage (Baker, 1996). Production of cleaved HT-1 was unsuccessful due to incomplete cleavage by factor Xa. However, high levels of the expressed recombinant protein (10 mg/L) permitted further research on the immunogenicity, protective ability of the recombinant MBP- HT-1 fusion protein and its feasibility as a vaccine (Masina, 1999).

In the recent study by Masina (1999), recombinant MBP-HT-1 fusion protein was examined in protection experiments using immunised mice challenged with crude tick
extract and immunized dogs challenged by eight engorged ticks. *In vivo* experimentation showed that antiserum against the fusion protein from mice was able to partially protect neonatal mice from tick paralysis. While control mice developed tick paralysis within 0-4 hours after injection of a mixture of a 1:2 dilution of normal mouse serum and crude tick extract, 1:2 to 1:50 dilutions of serum from recombinant toxin vaccinated mice delayed paralysis in neonatal mice for 8-18 hours. In the dog trial, dogs were challenged with eight ticks after two immunizations of 1ml doses of 10, 100, or 1000 µg/ml of the HT-1 fusion protein. However, the recombinant protein failed to protect the dogs against tick paralysis. Thus, it is necessary to explore other methods of toxin production and further investigate whether the absence of a fusion partner would also allow production of more specific antibodies against the neurotoxin and, more importantly, explore different ways of producing active recombinant protein or pure HT-1 (Masina, 1999).

### 1.6 AIMS OF THIS PROJECT

The specific objectives for this research are to generate and characterize monoclonal antibodies against the recombinant HT-1 protein produced by Masina (1999). Secondly, HT-1 sequence data will be used to clone and produce a ubiquitin-HT-1 fusion protein in bacteria. Following the expression of recombinant protein, cleavage of HT-1 by deubiquitin enzyme will be examined. Finally, immunisation with ubiquitin HT-1 will be used to investigate whether protective antibodies against native toxin can be elicited by the fusion protein.
CHAPTER 2

PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES AGAINST MALTOSE BINDING PROTEIN-HT-1 FUSION PROTEIN
2.1 INTRODUCTION

Previously, three neurotoxins from the Australian paralysis tick *Ixodes holocyclus* were isolated and designated as HT-1, HT-2 and HT-3. Partial amino acid sequence of the HT-1 neurotoxin was obtained but due to only minute quantities of the neurotoxins HT-2 and HT-3 being available, the amino acid sequence of these neurotoxins could not be identified (Thurn, 1994). Degenerate primers based on the partial peptide sequence were designed to isolate the complete HT-1 sequence using the RACE-PCR technique (Masina, 1995). The expression of the HT-1 neurotoxin as a MBP-HT-1 fusion protein in a bacterial system was established for the further investigation of the fusion protein as an anti-tick vaccine (Masina, 1995).

Parallel to the expression of maltose binding HT-1 fusion protein carried out by Masina 1999, monoclonal antibodies against the fusion protein were raised for the potential application in immunopurification of the expressed maltose binding HT-1. Also, in this chapter, the methodology involved in the production of monoclonal antibodies and their characterization specifically the determination of the region within the fusion protein to which the antibodies bound will be discussed.
2.2 MATERIALS AND METHODS

2.2.1 Production of monoclonal antibodies

2.2.1.1 Mouse immunisation protocol

Six week old, female Balb/C mice (−20 g) were injected intraperitoneally with 25 μg of maltose binding HT-1 fusion protein in complete Freund’s adjuvant (Sigma, St Louis, MO, USA). Booster injections of 12.5 μg fusion protein in Incomplete Freund’s adjuvant were given at 2, 4 and 6 weeks. Mice were euthanased by cervical dislocation and the spleens were collected for fusion 3 days after the final boost (Harlow and Lane, 1988).

2.2.1.2 Preparation of myeloma cells

Myeloma cell line Sp 2/0 (obtained from Dr S. Mahler, Department of Biotechnology, University of New South Wales) were grown to log phase at 37 °C in complete media [RPMI 1640 (Sigma, St Louis, MO, USA) plus 10 % fetal bovine serum (FBS) (Trace, NSW, Australia) and 1 % L-glutamine (200 mM)].

2.2.1.3 Fusion

Fusion technique was followed as described in Harlow and Lane (1988) with some modification. An immunized mouse was anaesthetized with 1 part O₂, 2% halothane, 2 parts N₂O then a terminal bled via cardiac puncture was performed for serum collection. Blood was allowed to clot for 30 minutes at 37 °C and at 4 °C overnight for the clot to contract. The clot was removed by centrifugation at 10,000 g for 10 minutes at 4 °C then the serum collected using a Pasteur pipette and stored at −20 °C.

After bleeding, the spleen was removed under sterile conditions and a single cell suspension in RPMI-1640 medium was made by teasing the spleen apart to release the cells. Trypan blue cell counts were performed. Spleen cells were mixed with myeloma [Sp
2/0] cells in 4:1 ratio and pelleted by centrifuge at 1000 rpm for 5 minutes. The pellet was washed three times with RPMI 1640 by centrifugation as before. One ml of 67% polyethylene glycol (PEG) (AJAX, NSW, Australia) was added to the pellet slowly over 45 seconds and further incubated for 45 seconds. PEG was diluted by slowly adding 10 ml of RPMI 1640 over 3 minutes, then a further 20 ml of RPMI 1640, followed by incubation at 37°C for 5 minutes.

After the incubation, the cell suspension was centrifuged for 10 minutes at 1000 rpm. The pellet was washed with the addition of RPMI 1640 (50 ml) and inverted twice without disrupting the pellet to remove any remaining PEG present in the pellet. This was then followed by centrifugation for 5 minutes as before and the supernatant then discarded. The cell pellet was resuspended in 50 ml complete medium and distributed into 24 well plates at 1ml per well. The plates were incubated overnight at 37°C in 5% CO₂ environment.

2.2.1.4 HAT selection

HAT medium which is used to select for hybridomas, consists of 100 ml RPMI 1640 supplemented with 27.22 mg hypoxanthine, 7.6 mg thymidine and 0.0352 mg aminopterin (Sigma, St Louis, MO, USA). This medium (1 ml) was added to each well and the cells were further fed at day 7. Wells containing confluent growth were tested for specific antibody production using an enzyme linked immunoassay (ELISA).

2.2.1.5 Enzyme linked immunoassay

Each antigen preparation (50 μl; 25 μg/ml) in carbonate buffer (15 mM Na₂CO₃, 35 mM NaHCO₃ and 0.02 % NaAz) was aliquoted into 96 well EIA microtitre plates and incubated at 37°C for one hour. The wells were washed 3x with PBS/Az containing 0.05 % Tween-20 (200 μl/well) and blocked with 3 % BSA/PBS-Az (200 μl/well) for 1 hour. The plates were washed and culture supernatants (50 μl) were added and further incubated for 1 hour then washed again. Antibodies from immunized mouse serum or culture supernatant specific for the fusion proteins were detected with biotinylated rabbit anti-mouse immunoglobulin
(Dakopatts, Denmark) at 1:2000 dilution and 50 µl/well. Extravidin alkaline phosphatase conjugate at 1:5000 dilution was added at 50 µl/well and incubated for 1 hour. After the incubation, the wells were washed three times with PBS/Az, once with carbonate buffer and once with glycine buffer (0.1 M glycine, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4). The substrate p-nitrophenyl phosphate (1 mg/ml) (Sigma, St Louis, MO, USA) in glycine buffer was added and the plates left to develop for 10-30 minutes at 37 °C. The reaction was stopped with 3 M NaOH and the absorbance at 405 nm measured in a microelisa plate reader (Organon Teknika).

2.2.1.6 Cloning

The growth from wells testing positive in the ELISA assay were cloned using the limiting dilution procedure (Zola, 1987) in 96 well plates. Wells were routinely checked by microscopy for clonal growth and supernatants tested in the ELISA assay. Clones expressing high levels of specific antibody were recloned 2-3 times. At each stage of the cloning procedure cells were collected for cryopreservation.

2.2.1.7 Cryopreservation

Cells were grown to log phase (1-2 x 10⁵ cells/ml), followed by removal of culture supernatant by centrifugation for 5 minutes at 1000 rpm. The pellet was resuspended in 5 ml sterile-filtered freezing medium which consisted of 50 % RPMI 1640, 10 % dimethyl sulfoxide (DMSO) (Research Organics Inc, Ohio, USA) and 40 % FBS. Cells (1 ml) were aliquoted into cryovial tubes and frozen overnight at −80 °C then moved to liquid nitrogen.

2.2.1.8 Thawing cells

Cryovials were removed from liquid nitrogen and placed in a 37 °C water bath to thaw. The vials were gently flicked to resuspend the cells and the mixture transferred to 5 ml of undiluted FBS, then centrifuged at 1000 rpm for 5 minutes. The supernatant was removed.
and the cells resuspended in 3 ml of complete media. Cells were plated at 1.5, 1.0, 0.5, 0.1 ml per well in a 24 well plate and incubated at 37\(^\circ\)C in 5 \% CO\(_2\).

### 2.2.2 Characterisation of monoclonal antibodies

#### 2.2.2.1 SDS gel electrophoresis

Protein samples were electrophoresed in 10 \% or 12 \% SDS-PAGE gels as described by Laemmli (1970). Prior to loading, non reduced protein samples were boiled for 5 minutes with 2x SDS-PAGE sample buffer, which contained 2 \%, SDS, 10 \% glycerol, 0.01 \% bromophenol blue, 250 mM Tris-HCl pH 6.8. For reducing conditions, 5 \% 2-mercaptoethanol (Sigma, St Louis, MO, USA) was added. A mini-Protean II (BioRad) apparatus was used for electrophoresis at 180-200 V in a 24 mM Tris, 200 nM glycine, 0.1 \% SDS, pH 8.3 running buffer.

#### 2.2.2.2 Western blots

SDS-PAGE gels containing electrophoresed proteins were soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20 \% methanol, pH 8.3) for 5 minutes. The gels were then assembled in a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad) and electroblotted onto PVDF membranes in transfer buffer for 1 hour at 250 mA. Thereafter, the membrane was stained with 0.5 \% Ponceau S in 1 \% acetic acid for 5 minutes to confirm the quality of protein transferred, then cut into strips. The Ponceau S stain was removed by washing with Milli Q water (Towbin et al., 1979).

#### 2.2.2.3 Immunodetection

The PVDF membrane strips were blocked with 3 \% BSA and 0.05 \% Tween-20 in TBS-Az (150 mM NaCl, 27 mM KCl, 50 mM Tris, pH 7.2 and 0.02 \% NaN\(_3\)) for 1 hour at room temperature or overnight at 4 \(^\circ\)C and then washed three times with 0.05 \% Tween-20 in TBS for 15 minutes. These washes were also carried out at each step prior to the addition of
antibodies or substrate. Each strip was incubated with a different hybridoma culture supernatant for 1 hour at room temperature. Secondary antibody (anti-mouse polyvalent IgG or anti-mouse IgM conjugated with alkaline phosphatase) (Sigma, St Louis, MO, USA) was diluted in TBS-Az Tween (1:5000 dilution) and incubated with strips for 1 hour at room temperature. The bound antibodies were detected by incubation in 0.17 mg/ml of bromochloroindolyphosphate (BCIP) and 0.33 mg/ml of nitroblue tetrazolium (NBT) (Sigma, St Louis, MO, USA) in AP buffer (5 mM MgCl₂, 100 mM NaCl, 100 mM Tris-HCl, pH 9.5). The colour development was stopped by washing the strips in Milli Q water before air drying.

2.2.2.4 Isotyping

Monoclonal antibody isotyping was carried out by an ELISA technique where the hybridoma supernatants were used to coat the plate, followed by the addition of goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA isotyping kit (Sigma, St Louis, MO, USA) and anti-goat IgG alkaline phosphatase conjugate (Sigma, St Louis, MO, USA) were used to detect the antibody reactions.

2.2.2.5 Inhibition ELISA

Monoclonal antibody in the culture supernatants from each clone was serially diluted. Diluted monoclonal antibodies were then pre-incubated with the MBP or an irrelevant recombinant histidine-tagged protein separately at 37 °C for 1 hour. To the wells coated with the fusion protein (25 µg/ml), the absorbed culture supernatants as primary antibody were added to the wells and reactions detected as described in section 2.2.1.5.
2.3 RESULT

2.3.1 Production of monoclonal antibodies

The protocol for the production of monoclonal antibodies was followed as in Figure 2.1. Serum collected from the immunised mouse was tested for the presence of polyclonal antibodies specific for the immunizing antigen (Figure 2.2). It showed high levels of specific antibody when compared to the unimmunized control and significant reactivity was still seen at a serum dilution of 1/64,000. Since the immune mouse was producing specific antibodies against the fusion protein, the spleen was isolated from the mouse to fuse with cultured myeloma (Sp2/0) cells. To select the fused cells, wells containing the fusion of spleen cells and Sp2/0 cells were grown in HAT medium where only fused cells survive. Viable cells (fused cell) were examined by microscopy. It was found that 55% of the wells contained fused cells following HAT selection. A resulting fusion colony is illustrated in Figure 2.3 where the fusion cells are transparent and rounded. Surrounding dead cells are the unfused myeloma cells killed during HAT selection. This represents the majority of the myeloma cells used in the hybridization.

As many of the hybrids may secrete antibodies against antigens that are not of interest, all the culture supernatants from the wells containing fused cell were ELISA screened for specific antibodies against the fusion protein prior to cloning. This ensures that only wells containing antibody-secreting hybrids were further investigated. Supernatants from these wells were screened for specific antibody production using the ELISA. As Figure 2.4 indicates, only three hybridomas were found to secret significant levels of antibodies against the fusion protein with absorbance approximately at 0.5 to 0.9. These readings suggest considerable amount of antibodies were secreted when compare to the -ve control (non immune mouse serum) which had zero absorbance, also any readings above 0.1 was consider positive for antibody production in the primary screen.

These hybridoma cells were then cloned by limiting dilution to generate a population of identical cells producing antibodies that are monoclonal. During this initial cloning, hybrid
Figure 2.1 Schematic diagram for the production of monoclonal antibodies.

(Taken from Goding, 1986)
Figure 2.2 Polyclonal antibodies binding response to HT-1 fusion protein.

HT-1 fusion protein (25 μg/ml) was immobilized in the assay plate. Dilutions of immunized and non-immunized mouse sera were added to fusion protein and subsequently detected with biotinylated rabbit anti-mouse immunoglobulin and visualized by colour development with p-NPP substrate using Extravidin-alkaline phosphatase.
Figure 2.3 Photomicrograph of a large fusion cell colony growing in HAT media at seven day post fusion.
HT-1 fusion protein (25 μg/ml) was immobilized in the assay plate. Dilutions of positive immunized mouse sera (+ve control) and undiluted non-immunized mouse sera (-ve control) were used as controls. 106 supernatants from wells containing hybridomas were added to the fusion protein coated plate and developed with biotinylated rabbit anti-mouse immunoglobulin and visualized by colour development with p-NPP substrate using Extravidin-alkaline phosphatase. Only the results for the three wells with absorbances above 0.1 are shown. Absorbance readings above 0.1 are considered as positive result.
16 was subcloned into clones 16A9 and 16F11. To ensure the clones are stable and monoclonal, three to four cloning procedures were carried out. Figure 2.5 shows the antibody production in the primary and final screening for the four clones. Each clone was found to increase its antibody production two-fold in comparison to the +ve control (immune mouse serum), this is possibly due to a higher proportion of cells in each well producing antibody. From the graph, clone 15, 16A9 and 16F11 produced much higher level of antibodies (OD at 2.2-2.8) than clone 1 (OD at 0.5). This suggests clone 1 could produce a different antibody class or bind to a different epitope than the other clones. This dissimilarity between the clones was revealed in the characterization of monoclonal antibodies.

2.3.2 Characterisation of the monoclonal antibodies

In addition to the ELISA results which showed the binding of the monoclonal antibodies to the fusion protein (Figure 2.5), Western blot analysis (Figure 2.6) was carried out. The monoclonal antibodies recognized the 50 kDa fusion protein under both reducing and non-reducing conditions. High molecular weight bands were also detected by the mAbs and probably represent aggregates of the fusion protein. The mAb also bound to some lower molecular weight protein corresponding to (43 kDa) MBP.

The Western blot (Figure 2.6) shows the binding to the whole fusion protein. To further identify which part of the fusion protein that the mAbs bind to, these monoclonal antibodies were tested for their ability to bind to maltose binding protein alone, and an irrelevant His-6-tagged protein, since the fusion protein is composed of the maltose binding protein (43 kDa) plus the HT-1 toxin (5 kDa) and 6 histidine residues at C-terminal (Figure 2.7). Figure 2.8 shows the binding responses of the mAbs to MBP and an irrelevant His-6-tagged protein. In graph A, monoclonal antibody 1 was found to bind to both MBP and the irrelevant His-6-tagged protein coated plates when compared to the –ve control (non immune serum). Since mAb 1 reacted against both the His-6-tag and maltose binding protein, it suggests that mAb 1 lacks any specificity for the fusion protein, even though the interaction occurred in a concentration dependent manner. The other mAbs, mAb 15, 16A
Figure 2.5 Absorbance readings for the monoclonal antibody secreting clones.

HT-1 fusion protein (25 μg/ml) was immobilized in the assay plate. Immunized and non-immunized mice sera were used as controls and supernatants from the desired antibody secreting hybridomas were added to the fusion protein. The bound antibodies were detected with biotinylated rabbit anti-mouse immunoglobulin and visualized by colour development with p-NPP substrate using Extravidin-alkaline phosphatase. Positive hybrids from Figure 2.4 were cloned four times to verify single cell grown into a clone. Clone 16 was subcloned into 16 A9 and 16 F11 after the first cloning.
Figure 2.6 Western blot of mAb 1, mAb 15, mAb 16A9 and mAb 16F11 binding to HT-1 fusion protein under reducing and non-reducing conditions.

The HT-1 fusion protein was electrophoresed in a 12 % SDS PAGE and blotted onto PVDF membrane. The membrane strips were probed with culture supernatants followed by anti-mouse polyvalent IgG conjugated with alkaline phosphatase. The bound secondary antibodies developed color changes with incubation of BCIP and NBT in AP buffer. The fusion protein is the 50 kDa band and aggregates of the fusion protein with 100 kDa molecular weight.

Lane 1, 2: mAb 1  
Lane 3, 4: mAb 15  
Lane 5, 6: mAb 16A9  
Lane 7, 8: mAb 16F11  
Lane 1, 3, 5, 7: Reduced fusion protein  
Lane 2, 4, 6, 8: Non reduced fusion protein  
Lane 9: Marker 10
Recombinant toxin (unscaled)

Maltose binding protein 43 kDa

Xa factor recognition Site: Ile-Glu-Gly-Arg

Figure 2.7 Schematic diagram of recombinant toxin.
Figure 2.8 Binding responses of mAbs to maltose binding protein and irrelevant His-6-tagged protein.

Maltose binding protein (76 µg/ml) and irrelevant His-6-tagged protein (18 µg/ml) were immobilized in the assay plates separately. Culture supernatants and non immune mouse serum as -ve control were serial diluted than added to the MBP and His-6-tag protein coated plates. Plates were developed with secondary antibodies (biotinylated rabbit immunoglobulin to mouse immunoglobulin), enzyme (extravidin) and visualized by p-NPP substrate.
and 16F11 reacted very weakly if at all, to MBP or the irrelevant His-6-tagged protein coated plates (Figure 2.8), indicating that these 3 monoclonal antibodies were not directed against MBP or His-6-tagged moiety of the fusion protein, they were reacting to the HT-1.

These observations were also confirmed in inhibition ELISA assays (Figure 2.9) where the monoclonal antibody culture supernatants were absorbed with maltose binding protein or the irrelevant His-6-tagged protein. Absorption with either antigen was able to absorb the monoclonal antibody 1 completely as OD readings were zero through out all the antibody dilutions (Graph A). In contrast, the unabsorbed antibody was able to bind to the fusion protein as a +ve control (Graph A). For the other mAbs (Graph B, C, D), the absorption with either antigen had no discernible affect on the other three monoclonal antibodies. This is shown by the same pattern of the curves for the bindings to the fusion protein with unabsorbed antibodies, antibodies absorbed with MBP or His-6-tagged protein. These results support the contention that mAb 15, mAb 16A9 and mAb 16 F11 react with the HT-1 portion of the fusion protein.

When the four monoclonal antibodies were isotyped (Table 2.1). Those three antibodies that reacted with HT-1 were all IgG1 isotypes while mAb 1 which reacted with all three antigens tested was found to be an IgM. It is not uncommon for IgM antibodies to show polyreactivity (Rousset and Wolff, 1980). This antibody isotype result is reflected in the level of binding to the fusion protein described earlier, where mAb 1 consistently had lower levels of reactivity (OD 0.5) to the fusion protein than the other three mAb (OD 2.2-2.8). IgM antibody often has lower affinity to antigen than IgG1 (Goding, 1986).

The four monoclonal antibodies (of which three could react with the HT-1 moiety of the MBP-HT-1 fusion protein) were further tested to determine whether they could detect the native toxin in crude tick extract. Crude tick extract was separated by SDS-PAGE and electroblotted onto nitrocellulose membrane then incubated with the four monoclonal antibodies and the commercial dog anti-tick serum (as positive control) (Figure 2.10). The positive control using commercial dog anti-tick serum clearly shows the binding to a 5 kDa protein band and a smear of low molecular weight proteins. As has been shown previously
(Thurn, 1994) the dog antiserum detects a large number of components in the crude tick extract including a band with a molecular weight of 5 kDa which was demonstrated to contain the paralysing toxins HT-1, HT-2 and HT-3. The four monoclonal antibodies produced in this study did not detect this 5 kDa band rather a series of bands which corresponded to the approximate size of myoglobin (17 kDa) and myoglobin aggregates (34 kDa and larger). These bands are believed to be myoglobin present in the crude extract of blood engorged tick as the serum proteins were visible as brown coloured bands in the gel prior to the immunostaining process.
Figure 2.9 Inhibition ELISA for the binding responses of mAbs to maltose binding protein, irrelevant His-6-tagged protein and fusion protein.

Monoclonal antibody in the culture supernatants were serial diluted and absorbed separately with maltose binding protein and irrelevant His-6-tagged protein by pre-incubation for 1 hour. The pre-incubated mixtures were added to fusion protein coated plate. The secondary antibodies (biotinylated rabbit immunoglobulin to mouse immunoglobulin) followed enzyme (extravidin) were added and visualized by p-NPP substrate. For +ve control, monoclonal antibodies in the unabsorbed culture supernatant were tested against the fusion protein coated plate.
Table 2.1 Immunoglobulin class determination.

The antibody subclasses for the monoclonal antibodies were determined by the ELISA assay. Hybridoma supernatants were used to coat the plate, followed addition of anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. The bound antibodies from isotyping kit were detected with anti-goat IgG alkaline phosphatase conjugate and visualised when \( p \)-nitrophenol phosphate substrate added.
Crude tick extract (0.1 µl/well) was separated on a 10 % Tricine SDS-PAGE and then blotted to nitrocellulose membrane. The membrane trips were incubated with monoclonal antibodies and commercial anti-tick dog serum separately. The blot was visualized by p-NPP substrate after addition of relevant secondary antibodies conjugated with alkaline phosphatase.

Lane 1: marker 12
Lane 2: mAb 1 detected with anti-mouse IgM
Lane 3: mAb 15 detected with anti-mouse polyvalent IgG
Lane 4: mAb 16A9 detected with anti-mouse polyvalent IgG
Lane 5: mAb 16F11 detected with anti-mouse polyvalent IgG
Lane 6: Commercial dog anti-tick serum detected with anti-dog polyvalent IgG
Lane 7: No 1° antibodies control
Lane 8: No 2° antibodies control

Figure 2.10 Monoclonal antibodies' reactivities to native toxin.
2.4 DISCUSSION

In 1975 Köhler and Milstein discovered the hybridoma technique to produce cell lines that secrete endless quantities of monoclonal antibody. This technology has revolutionized antibody applications in areas such as immunopurification, immunotherapy and clinical diagnosis. But most importantly, monoclonal antibodies are a powerful tool for the investigation of the structure-activity relationship of proteins and their neutralization mechanisms by epitope mapping (Bahraoui et al., 1988; Devaux et al., 1997; Herion et al., 1995; Stiles et al., 1994). The approach using monoclonal antibodies to study the structure of protein is illustrated in the production of monoclonal antibodies against scorpion toxins 2 and 3 from *Centruroides noxius*. The monoclonal antibodies lead to the identification of four neutralising epitopes which protected mice from intoxication and were used to study cross reactivity with several other scorpion toxins (Zamudio et al., 1992).

One aim of this project was to generate monoclonal antibodies against HT-1 in order to study the structure-activity relationship and to facilitate immunopurification of HT-1. In the production of monoclonal antibodies, the form of the antigen used to generate the monoclonal antibodies can influence the downstream application of the monoclonal antibodies. In the above mentioned study of *Centruroides noxius* toxins, the monoclonal antibodies were generated using native toxins. This was not practical in the current study due to the limited availability of native toxin for downstream monoclonal antibody screening and also due to its toxicity. Devaux et al. (1997) pointed out, using native toxins for the production of monoclonal antibodies can often create problems by killing the immunized animals and low and non-lethal doses of toxin may not be sufficient to induce antibody production. Clot-Faybesse et al. (1999) immunised mice with Aahl toxin and found that it was insufficient to induce an antibody response.

It has been reported that tick bites have been used to inoculate Borrelia into mice for monoclonal antibody production against proteins of *Borrelia burgdorferi*. This strategy allows presentation of Borrelia antigens to the mice through a more natural route of immunization (Gilmore and Mbow, 1998). The technique was not feasible in the current
study, because of the toxicity of *Ixodes* tick toxin and the intensive labour required in monitoring and caring for the animals. The procedure is essentially that used in the production of commercial dog anti-tick serum (polyclonal antibodies against tick paralysis), where ticks are engorged on dogs under strict conditions, with blood samples tested for immunity and number of ticks, engorging time regulated (Stone and Wright, 1979; Stone et al., 1983b).

Additional problems involved in using native protein mixes for monoclonal antibody production have been demonstrated in the study by Wozniak *et al.* (1996), where all the monoclonal antibodies produced were specific to concentrated high molecular weight proteins. Thus, the use of crude tick extract to generate monoclonal antibodies directly to HT-1 could be extremely difficult, as HT-1 is only present in relatively minute amounts compared to other high molecular weight tick and serum proteins. Moreover, it would be difficult to select clones producing the desired monoclonal antibodies due to the limited quantities and impure state of HT-1 available for monoclonal antibody screening.

Recombinant HT-1, developed by Masina (1999) as a candidate tick toxin vaccine was chosen for the production of monoclonal antibodies. If the dog trial by Masina (1999) proved to be successful with the MBP-HT-1 fusion protein, large-scale production of this protein would be necessary, and an alternative purification method for recombinant toxin would be critical, since the established purification required the use of expensive Ni-NTA agarose. Successful application of monoclonal antibodies in immunopurification has been demonstrated previously by Lee and Opdebeeck, (1991) where the monoclonal antibody (QU13) against midgut membrane (GM) antigens of *Boophilus microplus* is able to precipitate GM antigens as a further purification step, with the precipitate pure enough to be used as a vaccine.

If monoclonal antibodies against the MBP-HT-1 fusion protein could be generated, it is possible to use the monoclonal antibodies to isolate the native HT-1 from the crude tick extract in a larger quantity for further study of the structure and activity of native HT-1. Using monoclonal antibodies to detect and isolate proteins is often a more efficient
approach than combinations of conventional chromatography procedures. This is shown in the use of a monoclonal antibody (4B12) to the paralysis toxin of *Rhipicephalus evertsi evertsi*. It was found 4B12 was able to cross react with crude tick extract of *Argas walkerae*. This monoclonal antibody was used to immunoprecipitate the larval extracts of *A. walkerae* then further purify a 11 kDa paralysis toxin via chromatography (Maritz et al., 2000). This low molecular weight *A. walkerae* paralysis toxin is similar to the HT-1 in that both associate with large proteins. Thus, using monoclonal antibodies to isolate such proteins have been proved to be a more sensitive procedure in detecting minute quantities of toxins and could also be useful in isolating native HT-1 from other large molecular weight proteins in crude tick extract.

In this study, three hybridomas (three wells) were found to secrete antibody reactive with MBP-HT-1 fusion protein. This number of positive hybrids seems low given that 106 wells contained fused cells. According to the literature, only a small number of hybrids produce the preferred antibodies (Goding, 1986 and Zola, 1987) as only 1 % of splenocytes actually secrete antibodies and during the fusion process, only 1 % of starting cells are fused and only 1 in $10^5$ form viable hybrids. This has proved to be a difficulty in making monoclonal antibodies with only a small number of hybrids secreting the desired antibodies.

Western blotting was used to detect mAb binding to the fusion protein. It revealed that all the monoclonal antibodies were binding to the 50 kDa fusion protein under reduced and non-reduced conditions (Figure 2.6) which indicates that the monoclonal antibodies bind linear epitopes. This result was unwelcome, since Thurn, (1994) suggested antibodies that could neutralize the HT-1 toxin should be to a conformational epitope, as the neurotoxic fraction prepared by DEAE Affi-Gel Blue chromatography can only be detected by commercial polyclonal anti-tick sera under non-reducing conditions on Western blots. The Western blot analysis only showed the binding of the monoclonal antibodies to the whole fusion protein. The fusion protein used in this monoclonal antibody production (Figure 2.7) is composed of the maltose binding protein, HT-1 and a His-6-tag. As part of the characterization of the monoclonal antibodies, each monoclonal antibody was tested in inhibition ELISA to determine which part of the fusion protein they bound to.
MAb 1 was found to have non specific reactivity and bind to both the His-6-tag and MBP portion of the fusion protein. It was not surprising for the monoclonal antibody to bind to the 43kDa MBP as Knuth et al. (2000) suggested that when the carrier protein is larger than the protein of interest, it is possible to generate the majority of the immune response to the carrier protein and thus have the monoclonal antibodies bind to MBP. However, it is unexpected for a monoclonal antibody to bind to two separate epitopes (Figure 2.8). One possible explanation for this result is that the MBP and the His-6-tag folded to form a conformational epitope. However, this is unlikely to occur because mAb 1 was found to bind to a linear epitope under denaturing conditions as mentioned.

Alternatively, the IgM subclass mAb 1 (Table 2.1) can weakly bind to maltose binding and the His-6-tag in a less specific manner but compensates for this low affinity binding by multivalent binding sites that confer high avidity. Rousert and Wolff (1980) have reviewed the issue of multiple specificity of monoclonal antibodies to unrelated antigens. They suggest that with the enormous number of biological macromolecules, it is to be expected that antigens-antibody complexes can be formed between functionally unrelated molecules when an adequate number and strength of short range-interactions occur.

In contrast to mAb 1, all of the other monoclonal antibodies (mAbs 15, 16A9, 16F11) bind to the HT-1 epitope (Figure 2.8 and Figure 2.9). The HT-1 protein elicits antibody responses to produce IgG1 subclass antibodies (Table 2.1), which is a frequent hybridoma product (Zola, 1987).

As these antibodies bind to HT-1, they could potentially neutralize its toxic effects. However, when binding to the native toxin in crude tick extract was examined by Western blot, these monoclonal antibodies could not detect the 5 kDa HT-1 (Figure 2.10) and therefore it is suspected that these mAbs cannot neutralize the native HT-1. This outcome is supported by Masina (1999) who hypothesizes that the MBP-HT-1 fusion protein may be folded incorrectly since the recombinant protein was found to be unable to protect immunized dogs from tick paralysis. If the HT-1 portion of the recombinant fusion protein were not in the correct conformation, then the neutralizing epitope would not be present to
allow the production of monoclonal antibodies capable of preventing its toxic action. A similar problem was encountered by Azorsa et al. (1999), where 800 hybridomas were raised against tetraspanin (EC2 region) GST fusion protein expressed in *E. coli* but none of the monoclonal antibodies recognized the native tetraspanin epitopes. As the binding of the monoclonal antibodies to the native toxin is dependent on the recombinant antigen mimicking the native conformation (Clot-Faybesse, 1999), it was important to assure that the recombinant antigen used did indeed have the correct conformation. As a result, a new effort was directed toward the construction of a different HT-1 recombinant toxin.
Chapter 3

Ubiquitin fusion protein expression and toxicity assays
3.1 INTRODUCTION

Previous investigations into the development of an anti-tick vaccine (Masina, 1999) used a maltose binding protein (MBP) *holocyclus* toxin (HT-1) fusion protein as an immunogen. It was found to be non-protective against tick paralysis in dogs and partially protective against challenges with crude tick extract in neonatal mice.

Subsequently, a ubiquitin fusion protein expression system was examined. Ubiquitin is a smaller (10 kDa) carrier protein than MBP (43 kDa) and thus less likely to interfere with HT-1 folding. The system also has a specific cleavage site, which may be used to generate free HT-1 toxin.

The bacterial expression system produces the ubiquitin fusion protein expressed in both a soluble (cytoplasmic) and insoluble (inclusion body) form. Purification of the soluble form under non-denaturing conditions utilizing the incorporated His-6-tag via Ni-NTA agarose affinity chromatography is illustrated, as well as the purification of fusion protein from the insoluble form.

Two strategies for examining the possible neutralizing ability of antibodies produced to the HT-1 fusion protein in immunized animals are then investigated. Firstly, immunizing rabbits to obtain antibodies in quantities equal to the commercial dog anti-tick serum (positive control) for protection assays, and secondly, immunizing mice without the use of adjuvant in order to induce antibodies to the soluble fusion protein whilst avoiding possible denaturation of the antigen.
3.2 MATERIALS AND METHODS

3.2.1 Ubiquitin HT-1 fusion protein expression

3.2.1.1 Primer design for pMal-p2 vector

HT-1 genes in TBL cells were obtained from Dr. Slavica Masina, Immunobiology Unit, University of Technology, Sydney. The oligonucleotides used in PCR amplification of HT-1 gene were produced by Gibco BRL, Australia at 50 nmol for scale of synthesis. (CGC GGT GGA) from below are incorporated Arg-Gly-Gly ubiquitin cleavage site.

Forward primer

\[ \text{GTC C(CG CGG TGG A)TG TAC CA} \text{A CCC TGG CCG CGG} = \text{Sac II restriction site} \]

Reverse primer

\[ \text{GTC CTG CAG TT} \text{A TCA TTG TTT ACA G CTG CAG} = \text{Pst I restriction site} \]

3.2.1.2 PCR amplification

For a 50 µl PCR reaction mix, 1X Taq polymerase (Perkin Elmer Corporation, CT, USA), 10 ng of template, 0.2 mM dNTP (Invitrogen, NSW, Australia), 1X Taq buffer, 15 pmol of forward and reverse primer were used. The first PCR cycle contained a 95 °C denaturation step for 2.5 minutes, 1 minute DNA extension step at 55 °C and an annealing step for 1 minute at 72 °C. The remaining PCR proceeded for 30 cycles of 95 °C for 30 seconds, 55 °C for 1 minute and 72 °C for 1 minute in a Hybaid Omnigene thermal reactor (Ashford, UK).

3.2.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using a Mini Sub DNA cell horizontal electrophoresis system (BioRad), in which a 1 % agarose gel was used in TAE buffer (6 µl
of 10 mg/ml of ethidium bromide, 0.04 M Tris, 0.02 M glacial acetic acid and 1 mM EDTA pH 8.0). DNA samples were diluted with 6 X DNA sample buffer which contained 0.25 % bromophenol blue and 40 % w/v sucrose and electrophoresed at 100 V for 30 minutes.

3.2.1.4  **Ligating HT-1 gene into pGEM-T vector**

The PCR product was gel extracted according to the manufacture’s protocol (Qiagen, Germany) and then ligated into pGEM-T vector (Promega, Madison, WI, USA). The standard ligation reaction contained 1x rapid ligation buffer, 50 ng pGEM-T vector, 150 ng of insert and 3 Weiss units of DNA ligase in a 10 µl reaction volume.

3.2.1.5  **Electroporation**

Competent *Escherichia coli* cells (DH5α) were prepared by the Ausubel (1987) technique. The Gene Pulser® apparatus (Bio-Rad) was used to transform 4.5 µl of ligation mix into 50 µl of competent cells in a cold electroporation cuvette (Bio-Rad). The electroporation was set at 200 Ohms, 25 µF and 1.8 kV. After the pulse, cells were resuspended in 0.5 ml SOC® medium and incubated at 37 °C for 1 hour with agitation by shaking at 200 rpm. Each agar plate of Luria broth (LB) containing ampicillin (50 µg/ml) was coated with 100 µl of IPTG and 50 µl of X-gal then plated with 50 µl of the transformed cells.

3.2.1.6  **Plasmid restriction**

White colonies were randomly picked and grown into 5 ml cultures and the plasmids from the bacterial cells were extracted using QIAprep spin minprep kit (Qiagene, Germany). The plasmids (10 µg) containing the HT-1 gene were restricted by incubating overnight at 37 °C with 40 U of Sac II, Pst I and 1X NEB buffer (New England BioLabs, Beverly, MA, USA) in a 50 µl mix to check the incorporated restriction sites and the insert size in a 1 % agarose gel.
3.2.1.7 **Sequencing**

DNA sequencing reaction mix was prepared as per the protocols from Sequitherm EXCEL II Long-Read DNA sequencing (Epicentre Technologies, Madison, WI, USA) and the electrophoresis was performed using the Li-Cor 4000L protocol. The SequiTherm EXCEL II kit was used to make forward and reverse M13 primer master mix, each containing 7.2 µl of sequencing buffer, 2 pmoles of the IRD41 labeled M13 primer, 700 ng of plasmid, 5 U of DNA polymerase and sterile water for a final volume of 17 µl. The mix (4 µl) was added to each of four tubes containing 2 µl of one of the termination mixes (G, A, T, C) on ice. The PCR reactions were then started with a 95 °C DNA denaturation for 5 minutes, 30 cycles of 95 °C for 30 second, 60 °C for 30 second and 70 °C for one minute in a Hybaid Omegene thermal reactor. Loading buffer (3 µl) was added to each reaction tube and stored on ice.

A 4 % polyacrylamide gel was made up with 31.5 g of urea, 6ml of Long Ranger™ 50 % gel concentrate solution, 9 ml of long run 10 X TBE buffer (1.34 M Tris, 45 mM boric acid, and 25 mM EDTA) and MilliQ water for a volume of 75 ml. The gel solution was filtered, then 350 µl of 10 % (w/v) APS and 40 µl of TEMED were added and the gel poured between glass plates to set. The gel was heated to 40 °C and pre-run at 2000 V, 25 mA, 45 °C in 1 X Long Run TBE buffer then the electrophoresis was carried out overnight with samples denatured at 95 °C for 3 minutes prior to loading. The forward and reverse DNA sequences were analysed using LiCor’s Image Analysis software (Base ImagR v4.0) and compared to the HT-1 DNA sequence determined by Masina (1999).

3.2.1.8 **Insertion of HT-1 gene into ubiquitin vector**

Gel extraction was carried out as described in Qiaquick gel extraction kit (Qiagen, Germany) to isolate the HT-1 gene from an agarose gel (section 3.3.1.5). The ubiquitin vector pRB 493 was obtained from Dr. R. Baker, (Australia National University) and consisted of a tac promoter, His-6-tag and ubiquitin sequence. Ubiquitin vector (50 ng) was ligated with insert (120 ng) using 1X ligation buffer and 3 Weiss units of DNA ligase.
3.2.1.9 **Transformation**

The ligation mix was used to transform the ubiquitin HT-1 vector into (TG1) bacterial cells for expression. Transformation was carried out as described in the electroporation section (3.3.1.4). Plasmids from the transformed TG1 were re-isolated and restricted to confirm the transformation and size of the HT-1 insert.

3.2.1.10 **Expression protocol**

The expression protocol was similar to the expression of MBP / HT-1 fusion protein described by Masina, (1999) with some modifications. A 5 ml volume of ubiquitin HT-1 expressing bacterial culture was grown overnight and inoculated into 500 ml of superbroth [5 g yeast extract, 10 g tryptone, 2.5 g NaCl, 1.25 g K2HPO4, 1 g MgSO4, 0.1 μg/ml biotin, 0.4 % glucose and 1.5 ml of trace elements (1.6 g FeCl2, 0.2 g ZnCl2, 0.2 g CoCl2, 0.2 g Na2MoO4, 0.1 g CaCl2, 0.1 g CuCl2, 0.05 g H3BO4, 10 ml 8 M HCl for 100 ml stock solution)]. The bacterial culture was grown in a shaking incubator (Gallenkamp, UK) at 37 °C and 200 rpm for approximately 3 hours to reach an OD600 of 0.8-1.2. After the initial growth, the culture was placed on ice for 15 minutes to stop bacterial growth then centrifuged at 15,000 g for 15 minutes at 4 °C. The pellet was resuspended in 1 L superbroth without glucose (20 ml) and grown under the same condition as before for 30 minutes (recovery period) then induced by the addition of IPTG to a concentration of 0.3 mM for 3 hours. The final step was to pellet the bacterial cells at 15,000 g for 15 minutes and store the pellet at -80 °C.

3.2.1.11 **Affinity purification**

The procedures for protein purification followed the instruction from The QIAexpressionist™ from Qiagen, (Vic, Australia) using Ni-NTA IMAC agarose. The fusion protein could be purified under native or denaturing conditions.
For denaturing conditions, the bacterial pellet was resuspended in 60 ml of lysis buffer (8 M urea, 0.1 M NaH₂PO₄, and 0.01 M Tris-Cl at pH 8.0) then sonicated on ice for six 1-minute bursts at 200-300 W by an Ultrasonic homogenizer (Cole Parmer Instrument, Chicago, USA) for each 30 ml of sample with a 1 minute cooling period between each burst. The lysate was centrifuged at 15,000 g for 15 minutes to remove cell debris. Total lysate was added to 7 ml of the Ni-NTA slurry and mixed on a rotary mixer for 60 minutes at 4 °C. The lysate-resin mixture was then loaded into a glass column and the unbound material collected. The column was washed with 2 x 60 ml of wash buffer (8 M urea, 0.1 M NaH₂PO₄, and 0.01 M Tris-Cl at pH 6.3) and the bound protein eluted with 60 ml of elution buffer (8 M urea, 0.1 M NaH₂PO₄, and 0.01 M Tris-Cl at pH 4.5). Eluted protein (10 ml) was dialysed against PBS/Az pH 7.2 (1 L) overnight (120 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 10 mM KH₂PO₄ and 3.1 mM NaN₃).

The purification procedure for native conditions was the same as the denaturing conditions except that the lysis buffer consisted of 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl and 10 mM imidazole and the wash buffer and elution buffers each contained 50 mM NaH₂PO₄ pH 8.0, 300 mM NaCl with additional imidazole at 20 mM and 250 mM respectively. No dialysis to remove urea was required for the native purification.

**3.2.1.12 Enzyme expression and Ni-NTA purification**

Deubiquitin enzyme YUH1 (vector pRB 492) was obtained from Dr. R. Baker, (Australian National University). The expression and purification protocols were the same as the expression and purification protocol for the fusion protein under native condition except 2 mM 2-mercaptoethanol (Sigma, St Louis, MO, USA) was added to lysis, wash and elution buffer.

**3.2.1.13 Concentration of fusion protein**

The eluate from the Ni-NTA agarose was further purified by ultrafiltration in an Amicon stirred cell system (NA, USA) using YM 30 (30 000 MWCO) cellulose triacetate
membrane (Sartorius, Gottingen, Germany) to remove protein with molecular weight greater than 30 kDa. The flow through was concentrated with a YM 10 membrane (10 000 MWCO) to a 10 ml volume.

3.2.1.14 Protein estimation

Protein concentrations were determined using the Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, Illinois, USA) following the manufacturers' instructions. Bovine serum albumin was employed as standards.

3.2.1.15 Deubiquitin enzyme cleavage

Cleavage of ubiquitin fusion protein by UCH-L3 deubiquitin enzyme was carried out essentially as described by Pilon, et al. (1997). Similarly, the digestion using YUH1 deubiquitin enzyme was carried out at a concentration ratio of 1:3 enzyme:fusion protein in PBS at room temperature for two hours.

3.2.1.16 Reverse Phase HPLC

The fusion protein / enzyme digest (500 μl) was separated by a Sephasil C18 (1.0 mm x 100 mm) reverse phase column (Applied Biosystems) by Matthew Padula. The column was equilibrated with 0.1 % Trifluoroacetic acid (TFA) at a flow rate 100 μL/min. Each fraction was eluted with a gradient to 80 % acetonitrile containing 0.08 % TFA, 0-60% over 90 minutes and 60-100% over 5 minutes. Eluted fractions were lyophilized and each fraction resuspended in 50 μl of Milli Q water for SDS-PAGE analysis, liquid chromatography mass spectrometry (LC-MS) and MALDI-MS.

3.2.1.17 Tricine SDS-PAGE

Samples were separated in a 10% Tricine SDS PAGE (Schagger and Von Jagger, 1987) containing 30 % T (T for the total monomer concentration) and 3 % C (C for the weight %
of crosslinker) for both stacking and separating gel with cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1 % SDS, pH 8.25) and anode buffer (0.2 M Tris-HCl, pH 8.9). Non-reduced samples were boiled for 5 minutes with 2x SDS-PAGE sample buffer prior to electrophoresis. Samples were run at 90 V until the dye reached the interphase of the two gels when voltage was then increased to 150 V.

3.2.1.18 Silver staining

Proteins in the gel were fixed in 50 % ethanol and 8 % acetic acid for 30 minutes followed by 5 % ethanol and 1 % acetic acid for 15 minutes. The gel was rinsed with MilliQ water for 3 x 20 seconds, pretreated with Na₂S₂O₃ (0.02 g/100 ml) for one minute then washed as before. The gel was impregnated with AgNO₃ (0.2 g/100 ml) and 150 µl of 40 % w/w formaldehyde for 20 minutes and rinsed with MilliQ water for 2 x 20 seconds. Finally, the gel was developed with Na₂CO₃ (6 g/100 ml), and 50 µl of formaldehyde then the reaction stopped with 30 % methanol and 8 % acetic acid (Blum et al., 1987).

3.2.1.19 Western blotting

Western blotting technique was carried out as previously described in chapter section 2.2.2.2 and PVDF membrane blotted proteins were detected with the antibodies specified in section 3.3.

3.2.1.20 Protein A column purification

IgG was purified from sera by Matthew Padula using a POROS Protein A affinity column connected to a Biocad SPRINT preparative high performance liquid chromatography (HPLC) system (Perseptive Biosystems, USA). Briefly, the column was equilibrated with PBS at a flow rate of 5 ml/min. Sera was injected and the unbound proteins collected by an automated fraction collector. After washing with PBS, the bound IgG was eluted from the Protein A by 0.1 % HCl/0.15 M NaCl, collected and the acid neutralized by addition of 3-4
drops of 1 M Tris-HCl pH 8.0. The purified serum was then further concentrated to 70 mg/ml by removing the liquid by rotary evaporation.

3.2.2 Protection assay

3.2.2.1 Crude tick homogenate preparation

Engorged *Ixodes holocyclus* were homogenized with cold MilliQ water (100 µl/tick) in a manual glass homogeniser. The homogenate was centrifuged at 13 000 g for 20 minutes at 4 °C. The pellet was then resuspended into 2 X volume of cold MilliQ water and further homogenized and pelleted as before. Supernatants from both homogenization processes were combined and stored at –20 °C.

3.2.2.2 Toxicity assay

The crude tick homogenate was serially diluted from 1:2 to 1:32 in order to evaluate the toxicity of the native material. Each dilution was injected sub-cutaneously into 7 day old Balb/C neonatal mice. The mice were observed over an 18 hour period for the characteristic symptoms of paralysis.

3.2.2.3 Rabbit immunization and protection assay

New Zealand white rabbits were injected intramuscularly with 62 µg/500 µl of ubiquitin HT-1 fusion protein in complete Freund’s adjuvant. Secondary and tertiary boosters of fusion protein were administered 2 week apart in incomplete Freund’s adjuvant. Rabbits were then ear bled one week after the immunization was completed to examine the antibodies raised against the fusion protein using Western blotting.

Rabbit serum Ig was purified on a protein A column to eliminate unwanted serum proteins. Purified antibodies were tested against the fusion protein in a Western blot to ensure purified antibodies were anti-ubiquitin HT-1 antibodies.
Purified Ig was then tested for neutralization activity against native toxin. The purified Ig (70 mg/ml) was pre-incubated for one hour with equal volume of a 1:2 dilution of crude tick homogenate, to make a final concentration of crude tick homogenate of 1:4 dilution. The mixture was injected into a neonatal mouse (100 μl/mouse) sub-cutaneously using a Hamilton syringe. Commercial dog anti-tick serum (North Coast Serum Products, NSW, Australia) was used as a positive control while non-immune mouse sera was used as a negative control. The mice were observed for 18 hours to assess the degree of protection by the immune serum. The experiment was also repeated with a crude tick homogenate at a final concentration of 1:8 and a higher concentration of purified rabbit Ig (70 mg/ml).

### 3.2.2.4 Mouse immunization and protection assay

Six week old, female Balb/C mice were injected intraperitoneally and sub-cutaneously with the soluble ubiquitin HT-1 fusion protein (25 μg/mouse in 200 μl) weekly. After ten days the third immunization, mice were tail bled and the immune serum tested against ubiquitin HT-1 fusion protein in a Western blot to determine whether the mice have developed anti-ubiquitin HT-1 antibodies without using adjuvant. A final boost of immunization was given prior to terminally bleeding the mice by cardiac puncture. The immune serum was used for the mouse protection assay, using the same procedure described for the rabbit protection assay (section 3.2.2.3).
3.3 RESULTS

3.3.1 Ubiquitin fusion protein expression / purification

Forward and reverse primers containing Pst I and Sac II cleavage site sequences (section 3.2.1.1) were used to replicate the HT-1 sequence from the pMAL-p2 expression vector created and investigated by Masina (1999) (see Appendix 1). The incorporated Pst I and Sac II sites allowed the HT-1 gene to be subsequently restricted and inserted into the ubiquitin vector. To confirm the incorporation of the restriction sites and the correct HT-1 sequence, the PCR products were ligated into the pGEM-T vector, then transformed into DH5α E. coli cells for sequencing. Both forward and reverse sequences contained the sequences for the Pst I and Sac II sites as well as the HT-1 sequence (data not shown). The HT-1 insert was restricted from the pGEM-T vector and ligated into the ubiquitin vector then transformed into TG1 bacterial cells. Bacterial cells containing successfully transformed plasmids with the HT-1 insert were verified by isolating the plasmids for restriction analysis. Figure 3.1 shows that four randomly selected clones all contained digested plasmids with the correct sized (160 bp) HT-1 insert. The ubiquitin vector is seen as a very bright band at 3.8 kbp and supercoiled ubiquitin vector, having a more compact structure (2kpb) is also present.

After a clone containing the desired plasmid was isolated, protein expression was studied. Previously, the MBP-HT-1 fusion protein was found to express maximally following induction with 0.3 mM IPTG for 4 hours at 37 °C (Masina, 1999). However, due to the differences in the vector and bacterial cell type used, the expression conditions were reviewed for the optimum protein expression. Expression conditions were varied for IPTG concentration, induction period and temperature. It was found that the 15 kDa fusion protein was expressed in the cytoplasm as a soluble protein (Figure 3.2) and in inclusion bodies as an insoluble protein (Figure 3.3) with various concentrations of IPTG (blot A), induction times and temperatures (blot B). Figure 3.2 shows that ubiquitin fusion proteins were successfully expressed in the soluble fraction, in comparison to the pre-induced
Figure 3.1 Confirmation of HT-1 insert that was cloned into the ubiquitin vector in TG1 bacterial cells.

Plasmids from TG1 bacterial cells were restricted with Pst I / Sac II then electrophoresed on a 1 % agarose gel and visualized by Ethidium Bromide staining under UV illumination.

Lane 1: 1 Kbp marker
Lane 2 - 5: Colonies 1 - 4 containing 3.8 Kbp ubiquitin vectors and 200 bp HT-1 inserts.
Lane 6: 100 bp marker
Figure 3.2 Western blot of the ubiquitin fusion protein expressed (soluble fraction) under various IPTG concentrations and induction periods.

The bacterial expression was optimized by inducing cultures for 4 hours at various IPTG concentrations (blot A) inducing for various time periods and applying a heat shock upon induction of 0.5 mM IPTG for 4 hours (blot B). Fusion protein from the soluble fraction was electrophoresed under reducing conditions in 12 % SDS PAGE then blotted onto PVDF membrane. The fusion protein on the membrane was detected by 1/1000 dilution of anti-His antibody followed by 1/3000 dilution of alkaline phosphatase conjugated anti-mouse IgG.
Figure 3.3 Western blot of the ubiquitin fusion protein expressed (insoluble fraction) under various IPTG concentrations and induction periods.

The optimal expression was determined by growing cultures at different IPTG concentrations for 4 hours (blot A), induced for various time periods and heat shock upon induction of 0.5 mM IPTG for 4 hours (blot B). Fusion protein from the insoluble fraction was electrophoresed under reduced condition in 12% SDS PAGE then blotted onto PVDF membrane. The fusion protein on the membrane was detected by 1/1000 dilution of anti-His antibody followed by 1/3000 dilution of alkaline phosphatase conjugated anti-mouse IgG.
samples (Lane 2 blot A and B), where the 15 kDa band was not observed. The ubiquitin fusion protein from the soluble fraction appeared to be a doublet under non-reducing conditions, which could indicate the fusion protein is folded in two different ways, or different processing of the expressed protein has occurred. From Figure 3.2 blot A, 0.3-0.5 mM IPTG inductions have the most intense fusion protein bands, while Figure 3.2 blot B indicates the optimal induction time was 1 hour. The additional immunoreactive bands seen at approximately 10 kDa in the Western blots (Figure 3.2 blot A and B) must be expressed as result of the inducing gene construct as they were not present in the pre-induced sample. This combined with the bands reacting with the anti-His $^2$ antibodies indicates these bands represent either a breakdown product of the full protein or incomplete translation of the gene product. As for the fusion proteins expressed in the insoluble fraction, (Figure 3.3) the pre-induced samples (Lane 2 blot A and B) did not express any fusion protein. From Figure 3.3 (blot A), the optimum induction condition was 0.1-0.3 mM IPTG with 3 hours induction time (blot B) as indicated by the intensity of the bands. From the Western blots (Figure 3.2 and 3.3), the most favorable condition for soluble and insoluble protein expression was to induce the culture for 3 hours with 0.3 mM IPTG. The previous study on the ubiquitin system for peptide expression by Pilon et al. (1996) showed that heat shock treatment could maximize protein yield. Thus, heat shock treatment was carried out at 42°C upon induction for 4 hours in this experiment. However this treatment did not enhance protein expression in either soluble (Figure 3.2, blot B, Lane 7) or insoluble fractions (Figure 3.3, blot B, Lane 7), in fact the amount of fusion protein in the soluble fraction was significantly reduced.

The His-6-tagged fusion proteins were purified using Ni-NTA agarose affinity chromatography. This purification was also used previously for the purification of the His-6-tagged MBP-HT-1 fusion protein (Masina, 1999). Protein expressed in the soluble fraction could be purified under native (non-denaturing) conditions using NaH$_2$PO$_4$ buffer, whereas the insoluble fraction required denaturing conditions to solubilize the protein for purification. Figure 3.4 (blots A & B) shows SDS-PAGE of the purification process under denaturing and native conditions respectively. Lane 2 and 3 show the complex mixture of proteins in the crude cell lysate from pre-induced and induced bacterial cultures prior to
puration. The His-6-tagged fusion protein in the crude cell lysate (Lane 3) was purified using Ni-NTA column chromatography. The majority of proteins present in the cell lysate were collected in the flow-through (Lane 4) as unbound material. The column was washed to remove any impurities remaining in the column as shown in Lane 5. The 15 kDa ubiquitin fusion protein product (Lane 6) was obtained in the elution. The 15 kDa band (Lane 6) was also present in the flow-through indicating the affinity column had been saturated in this experiment. This material was retained for further purification of the fusion protein.

The Ni-NTA affinity purified material when concentrated by Amicon ultrafiltration and examined by SDS-PAGE revealed bands additionally to the 15 kDa fusion protein (blot A and B, Lane 7). Specifically, a small amount of a high MW band at approximately 30 kDa was seen in the soluble fraction. These were thought to be fusion protein aggregate. The aggregation was most likely caused by high protein concentration present in the concentrated elution of the fusion protein (Figure 3.4, blot B, Lane 7) as this aggregation was not observed in the unconcentrated elution fraction (Lane 6). The two bands around 50-60 kDa in lane 6 are regularly observed artifacts of DTT reduction. The fusion protein from insoluble fraction was not aggregated (blot A), as this fusion protein, purified under denaturing conditions, was stored in elution buffer containing 8M urea.

The low molecular weight bands (~10 kDa) however are still present in proteins purified under both native (blot B) and denaturing conditions (blot A), suggesting that these His-6 containing proteins are the result of the expression process rather than a breakdown of fusion protein during purification and storage. These low molecular weight bands present in both blot A and B, Lane 7 are most likely some form of truncated His-6-tagged ubiquitin protein. The bands have an apparent molecular weight of 10 kDa on the gel while the theoretical mass of His-6-tagged ubiquitin is 9.6 kDa and Western blot of this protein was also detected by anti-His monoclonal antibody (data not shown). This protein proved to be difficult to remove during purification as the truncated fusion protein still contains a His-6-tag and thus could not be removed by affinity chromatography. Amicon™ ultrafiltration also did not remove the protein because the 9.6 kDa protein had a molecular weight close to
Figure 3.4 Expression and purification of ubiquitin fusion protein from soluble and insoluble fractions using native and denaturing conditions.

Samples of expressed proteins and purified fusion proteins, purified under denaturing (gel A) and native (gel B) conditions. Electrophoresis was carried out on a 12 % SDS-polyacrylamide gel under non-reducing conditions. The reduced fusion protein is visible by silver staining at 15 kDa. Band of interest
the 10 kDa cut off for the Amicon membrane. Purified ubiquitin fusion protein without the ~10 kDa protein could be achieved by HPLC purification, however HPLC purification is not feasible on a large scale, and also produced denatured protein. Both the soluble and insoluble fractions of the fusion protein were optimally expressed at a concentration of 2-5 mg/L culture as measured by micro-BCA assay (data not shown). After the optimization for both expression and purification, subsequent experiments on the fusion protein were carried out using the soluble fraction only, because refolding was not required.

### 3.3.2 Cleavage

It was desirable for the bacterially expressed protein to mimic the native form of the HT-1. The first approach was to cleave off the carrier protein, in order to assess the bacterial expression protein for its toxicity and protectiveness. The unique feature of the ubiquitin expression system is that specific cleavage of the ubiquitin fusion partner from the desired protein can be achieved using ubiquitin specific proteases (USPs) or deubiquitin enzymes (Baker, 1994). The yeast deubiquitin enzyme has been cloned and is available for this procedure. Yeast deubiquitin enzyme (YUH1) was expressed in TG1 bacteria (E coli) transformed with the expression vector pRB492 kindly donated by Dr. Baker from Australian National University. Figure 3.5 shows a silver stained gel containing pre-induced (Lane 2) and IPTG induced crude cell lysate samples (Lane 3) in which the deubiquitin enzyme cannot be distinguish from other cell proteins. Purification of the His-6-tagged recombinant enzyme using Ni-NTA agarose column was carried out. Figure 3.5 shows the affinity column elute (Lane 4) containing a protein band at ~30 kDa which was the expected size of the His-6-tagged enzyme (calculated mass from protein sequence 27.85 kDa).

The purified enzyme was used to cleave the ubiquitin-HT-1 fusion protein. The digestion was carried out by incubating the fusion protein and deubiquitin enzyme in PBS (3:1 ratio in concentration) for 2 hours at room temperature. This deubiquitin enzyme cleaves the peptide bond following the amino acid sequence of Arg-Gly-Gly. This Arg-Gly-Gly
Figure 3.5 Deubiquitin enzyme expression and purification.

Deubiquitin enzyme (28 kDa) was expressed and purified under native conditions using Ni-NTA agarose. The purified protein was visualized by silver staining of a 12 % polyacrylamide gel under reducing condition using glycine buffer.

Lane 1: Marker 10
Lane 2: Pre-induced sample
Lane 3: Induced sample
Lane 4: Elution
The sequence was integrated into the fusion protein when creating the gene construct by the forward primer upstream of HT-1 and downstream of ubiquitin (section 3.2.1.1).

The digestion mixture was separated and analysed by C\textsubscript{18} reverse phase HPLC and electrospray mass spectrometry. Fractions representing each protein peak were also collected and analysed by Tricine SDS-PAGE (Figure 3.6).

The protein peak eluting at 61 mins (collected as fraction 30) revealed a mass of 14,965 Da that corresponds closely to that of the ubiquitin-HT-1 fusion protein (calculated theoretical mass of 14,980 Da). Similarly the protein peak eluting at 85 mins (collected as fraction 39) revealed a mass of 27,950 Da that corresponds closely to that of the deubiquitin enzyme, YUH1 (calculated theoretical mass of 27,850 Da). All the other peaks observed in the HPLC run did not produce a signal by electrospray mass spectrometry.

When the fractions collected from the HPLC separation were analysed by Tricine SDS-PAGE (Figure 3.6 insert), fraction 30 contained bands consistent with the presence of the ubiquitin-HT-1 fusion protein, confirming the MS data. It is interesting to note that the low molecular weight bands (~10 kDa) are still observed although they were not detected by MS. Fraction 39 contained a protein of approximately 30 kDa, consistent with the presence of the deubiquitin enzyme; again confirming the MS data. However SDS-PAGE indicated the presence of other proteins not detected by ESI-MS in this instance. Fraction 31 (Figure 3.6 insert, Lane 3) contained a prominent protein of approximately 6 kDa, possibly the cleaved HT-1, while fraction 37 (Lane 4) contained a prominent band at 10 kDa, possibly the cleaved ubiquitin. Although these proteins can be detected easily in silver stained gels they were not detected by ESI-MS suggesting that these proteins were not being ionised by the electrospray procedure. Thus it was decided to analyse these fractions by MALDI-TOF MS at the Australian Proteome Analysis Facility.

MALDI-TOF MS analysis of fraction 37 revealed a mass of 9,611 Da, consistent with the presence of His-6-tagged ubiquitin (calculated theoretical mass of 9,608 Da). This result indicates that the deubiquitin enzyme had successfully cleaved the fusion protein and at the
Figure 3.6 Silver stain of HPLC purified fusion protein, enzyme and cleavage product.

The fusion protein was digested by the deubiquitin enzyme and the cleavage reaction was then analysed by reverse phase HPLC. Fractions collected from the HPLC were run in a 10% reducing Tricine gel and visualized by silver staining.

**Insert**

Lane 1: Marker 12
Lane 2: Fraction 30 ubiquitin HT-1 fusion protein (A)
Lane 3: Fraction 31 suspected HT-1 (B)
Lane 4: Fraction 37 ubiquitin (C)
Lane 5: Fraction 39 deubiquitin enzyme (D)
specific cleavage site. Analysis of fraction 31 revealed a mass of 4,816 Da which did not match the expected mass of the cleaved HT-1 (calculated theoretical mass of 5,382 Da). Several possibilities could account for this discrepancy. The low molecular weight protein may result from non-specific cleavage of the recombinant toxin by the USP enzyme or be impurities or breakdown protein fractions from the semi-purified recombinant USP enzyme preparation. To confirm whether the 4816 Da was the result of cleaved protein, sequencing using the digestion mix was applied. The sequence for both ubiquitin and USP were detected, however no sequence corresponding to HT-1 was seen (Matthew Padula, personal communication).

3.3.3 Immunogenicity

As purified cleaved HT-1 could not be obtained, a Western blot was performed to determine whether commercial dog anti-tick serum could detect the HT-1 ubiquitin fusion protein. Figure 3.7 shows the reaction of the commercial dog anti-tick serum to the ubiquitin fusion protein, MBP-HT-1 fusion protein and deubiquitin enzyme. The commercial antiserum was found to bind to the fusion protein, indicated by the presence of a dark band at approximately 15 kDa in Lane 2 and Lane 4. Also in Lane 4, a band at ~5 kDa was not detected, indicating that either the deubiquitin enzyme was not cleaving at all or it was unable to produce sufficient amount of HT-1 for the detection by the commercial antiserum. In contrast to the binding to the ubiquitin fusion protein, the commercial dog anti-tick serum did not bind to MBP-HT-1 fusion protein (50 kDa, Lane 5) or deubiquitin enzyme (28 kDa, Lane 3). The faint bands in these lanes were due to non-specific binding by the secondary antibody caused by protein overloading as shown by the non immune mouse sera control (Lane 7). An anti-His monoclonal antibody was used to detect the mixture of fusion protein and deubiquitin enzyme as a +ve control (Lane 6). His-6-tagged ubiquitin (9.6 kDa), ubiquitin enzyme (30 kDa) and fusion protein doublet (15 kDa) were all detected. As mentioned earlier, the fusion protein from soluble fraction often appeared to be doublet in the non-reducing gel. Also, a protein band at 50 kDa was shown to bind non-specifically to the anti-His monoclonal antibodies in this +ve control. It was probably the impurity from the enzyme expression as the band was not detected by the commercial
Figure 3.7 Western blot of commercial dog anti-tick serum binding to ubiquitin fusion protein.

Ubiquitin fusion protein, deubiquitin enzyme and MBP-HT-1 fusion protein were electrophoresed under non-reducing conditions in a 10% Tricine gel and blotted onto PVDF membrane. Blotted proteins were probed with the commercial dog anti-tick serum at 1:1000 dilution except for the -ve control membrane strips. 1:5000 dilution of secondary antibody (alkaline phosphatase conjugated anti-dog IgG) was used to detect the bound commercial dog anti-tick serum.

Lane 1: Marker 12
Lane 2: Fusion protein detected by commercial dog anti-tick serum
Lane 3: Deubiquitin enzyme detected by commercial dog anti-tick serum
Lane 4: Protein/ enzyme mix detected by commercial dog anti-tick serum
Lane 5: MBP-HT-1 fusion protein detected by commercial dog anti-tick serum
Lane 6: Protein/ enzyme mix detected by anti-His antibodies (+ve control)
Lane 7: Protein/ enzyme mix detected by non immune mouse sera (-ve control)
Lane 8: Protein/ enzyme mix with no 1° antibodies (-ve control)
Lane 9: Protein/ enzyme mix with no 2° antibodies (-ve control)
dog anti-tick serum (Lane 4). As the commercial dog anti-tick serum did not detect the His-6-tagged ubiquitin band at 9.6 kDa observed with the anti-His antibody it can be inferred that the dog antibodies are reacting specifically with the HT-1 portion of the fusion protein. Overall, this result indicated that the commercial dog anti-tick serum was able to recognize the recombinant protein.

To further study this result, rabbits were vaccinated using ubiquitin fusion protein / adjuvant and mice were vaccinated using ubiquitin fusion protein alone. The antibodies produced in the immunized animals against the fusion protein were confirmed in the Western blots. As Figure 3.8 shows, the immune rabbit serum bound to the 15 kDa fusion protein, 30 kDa aggregates and some low molecular weight impurities (10 kDa). While non immune sera (Lane 5) weakly bound to the fusion protein by non-specific binding. Purified immune rabbit IgG (Lane 3) bound to the fusion protein more significantly than the unpurified immune rabbit serum (Lane 2) or the positive control anti-His antibodies. Western blots using immune mouse gave a similar pattern of binding to the proteins in the fusion protein preparation (Figure 3.9). However it was interesting to note that, immunization without adjuvant was still able to induce antibodies against the fusion protein (appeared as doublet under non-reducing condition), fusion protein aggregate and some low molecular weight impurities. These two polyclonal antisera are likely to contain antibodies reacting to all regions of the fusion protein including the HT-1 toxin, thus it was thought important to investigate whether these sera would react with the native toxin present in crude tick extract.

Crude tick extract was run on 10 % Tricine gel and detected by the serum from purified immune rabbit IgG and immune mouse serum (Figure 3.10). These sera appeared to recognize the 5.4 kDa native HT-1 with apparent molecular weight of 6 kDa in the Western blot. The same band is observed with the commercial dog anti-tick serum (Lane 5). It was very interesting to note that protein A purified dog IgG did not recognize the suspected HT-1 band. This suggests that the antibody detecting the band in Lane 5 is not IgG since protein A purification should retain all the IgG. This observation requires further investigation. It should be noted that the dog antibodies were detected with a commercial
Figure 3.8 Western blot of protein A column purified immune rabbit serum binding to HT-1 fusion protein.

Serum from rabbits immunized with fusion protein / adjuvant emulsion were purified via protein A column. The fusion proteins were detected by the purified rabbit serum at 1:2000 dilution followed by probing with 1:5000 dilution of alkaline phosphatase conjugated anti-rabbit antibodies. The fusion protein is visible as a 15 kDa protein, 30 kDa aggregates and some of the breakdown product (> 10 kDa) that were unable to be eliminated by ultrafiltration. Band of interest ➔

Lane 1: Marker 10
Lane 2: Rabbit sera
Lane 3: Protein A column purified rabbit sera
Lane 4: Anti-His antibodies
Lane 5: Non immune rabbit sera
Lane 6: No 1° antibodies control
Lane 7: No 2° antibodies control
Figure 3.9 Western blot of immune mouse sera binding to HT-1 fusion protein.

The fusion proteins were detected by the serum from mice immunized without adjuvant at 1:2000 dilution. The blot was further probed with 1:5000 dilution of alkaline phosphatase conjugated anti-mouse polyvalent antibodies. The 15 kDa fusion protein is visible on the blot as double bands in the non-reduced condition, as well as a 30 kDa fusion protein aggregate and some low molecular impurities that were unable to be removed by ultrafiltration.

Lane 1: Marker 12
Lane 2: Fusion protein 4 µg
Lane 3: Fusion protein 8 µg
Lane 4: Fusion protein 12 µg
Lane 5: Fusion protein 16 µg
Lane 6: Fusion protein 20 µg
Lane 7: No 1° antibodies control
Lane 8: No 2° antibodies control
Figure 3.10 Western blot of commercial dog anti-tick serum, immune rabbit and mouse serum binding to crude tick homogenate.

Samples of the native toxin (0.1 μl/well) were run in a 10 % Tricine gel then blotted onto PVDF membrane. The fusion protein immunized rabbit and mouse serum were used at 1:1000 dilution to detect the native toxin under non-reducing conditions. Moreover, the unpurified and purified commercial dog anti-tick sera were used as controls. The 1° antibodies were further detected with relevant anti-mouse, anti-rabbit and anti-dog antibodies conjugated with alkaline phosphatase at 1:5000 dilution.

Lane 1: Marker 12
Lane 2: Cytochrome C
Lane 3: Protein A column purified immune rabbit sera (immunized with adjuvant)
Lane 4: Immune mouse sera (immunized without adjuvant)
Lane 5: Commercial dog anti-tick sera
Lane 6: Elution from protein A column purified commercial dog anti-tick sera
a: No 1° antibodies control
b: No 2° antibodies control
anti-dog IgG reagent which may still detect dog IgM due to cross reactivity between the light chains of each immunoglobulin class. Each antiserum also detected a number of high molecular weight bands in the crude tick extract. The commercial dog anti-tick serum recognized six high molecular weight bands and a smear of unknown low molecular weight bands (Lane 5 and Lane 6) while the immune sera recognized four high molecular weight bands (Lane 3 and Lane 4) in the Western blot. This is expected as the dog serum recognizes more high molecular weight proteins than immune serum produced against the purified fusion protein, as it is a polyclonal antiserum produced by engorging ticks on dogs and antibodies will be produced against many salivary proteins. In addition, the high molecular weight bands detected by the immune sera were also detected by the primary antibody control (Lane 3a, 4a, 5a and 6a) suggesting that several tick proteins are reacting non-specifically with the secondary antibody or the substrate.

### 3.3.4 Protection assays

In addition to the Western blot results from above, the immune sera were investigated for their protection against tick paralysis using an *in vivo* protection assay. In order to determine the amount of crude tick extract to use in the *in vivo* assay, crude tick extract was serially diluted and injected into neonatal mice. As shown in Table 3.1, 1:4 and 1:8 dilutions proved to cause characteristic paralysis symptoms with minimal amounts of crude tick extract used. Therefore these amounts were chosen for the paralysis dose in the protection assay. Purified serum from the rabbits was concentrated to 70 mg protein/ml, similar to that of the commercial dog anti-tick serum for the protection assay to prevent a possible non-protective result due to insufficient specific antibodies available. The final dilutions of 1:4 and 1:8 crude tick homogenate were pre-incubated with rabbit serum and commercial dog anti-tick serum separately then injected into neonatal mice for the assay. The rabbit serum against the fusion protein did not protect neonatal mice from paralysis (Table 3.2). A delay of paralysis from 6th hour to 10th hour was observed when compared to crude tick extract alone but the paralysis symptoms and the time to paralysis onset were similar to that with serum from non immune rabbits for both 1:4 and 1:8 of crude tick extract injected (Table 3.2).
It is possible that the fusion proteins used to immunize rabbit were denatured by the adjuvant so antibodies raised would not recognize the conformational epitope from the toxin and thus not be protective. To assess this possibility, serum from mice immunized without adjuvant (Figure 3.9) was also examined for its protectivity against paralysis in neonatal mice. However, serum from these mice was also found to be non-protective against the paralysis in the protection assay (Table 3.3). In this experiment, the neonatal mice responded to the native toxin in the same pattern as the protection assay using rabbit serum. A delay of paralysis from 6\textsuperscript{th} hour to 10\textsuperscript{th} hour was observed but immune mouse serum did not significantly affect the symptoms in comparison to the non-immune mouse serum.

While the immune rabbit and mouse sera were unable to protect neonatal mice from paralysis, the positive control using commercial dog anti-tick serum was able to provide full protection as shown in Table 3.2 and 3.3. Interestingly, when the purified IgG from the commercial dog anti-tick serum was pre-incubated with crude tick extract, it was found to be unable to protect mice from paralysis (Table 3.4). This corresponded to, the purified IgG dog serum not binding to the $\sim$5 kDa HT-1 in the Western blot (Figure 3.10).
### Table 3.1 Toxicity assay.

One week old neonatal mice were sub-cutaneously injected with 100 μl of crude tick homogenate in serial dilutions. Each dilution was carried out in duplicate. Mice were monitored for the progress of tick paralysis for 18 hours.

<table>
<thead>
<tr>
<th>Dilution of crude tick extract</th>
<th>Symptoms</th>
<th>Paralysis initiate time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:2</td>
<td>complete body paralysis, breathing difficulties</td>
<td>6&lt;sup&gt;th&lt;/sup&gt; hour hind leg paralysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8&lt;sup&gt;th&lt;/sup&gt; hour front leg paralysis</td>
</tr>
<tr>
<td>1:4</td>
<td>front &amp; hind leg paralysis, breathing difficulties</td>
<td>6&lt;sup&gt;th&lt;/sup&gt; hour hind leg paralysis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8&lt;sup&gt;th&lt;/sup&gt; hour front leg paralysis</td>
</tr>
<tr>
<td>1:8</td>
<td>hind leg paralysis</td>
<td>8&lt;sup&gt;th&lt;/sup&gt; hour hind leg paralysis</td>
</tr>
<tr>
<td>1:16</td>
<td>normal</td>
<td></td>
</tr>
<tr>
<td>1:32</td>
<td>normal</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2 Protection assays using purified immune rabbits serum.

One week old neonatal mice were sub-cutaneously injected with 100 μl mix of crude tick homogenate pre-incubated with immune serum. Crude tick homogenates were diluted by the serum to final dilution of 1/4 and 1/8 in triplicate. Mice were monitored for the progress of tick paralysis for 18 hours.

<table>
<thead>
<tr>
<th>Time</th>
<th>1/4 dilution of crude tick extract</th>
<th>1/8 dilution of crude tick extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS</td>
<td>NRS</td>
<td>IRS</td>
</tr>
<tr>
<td>6th hour</td>
<td>H</td>
<td>N</td>
</tr>
<tr>
<td>8th hour</td>
<td>F, H, B</td>
<td>N</td>
</tr>
<tr>
<td>10th hour</td>
<td>F, H, B</td>
<td>H</td>
</tr>
<tr>
<td>12th hour</td>
<td>F, H, B</td>
<td>H</td>
</tr>
<tr>
<td>15th hour</td>
<td>F, H, B</td>
<td>F, H</td>
</tr>
<tr>
<td>18th hour</td>
<td>F, H, B</td>
<td>F, H</td>
</tr>
</tbody>
</table>

NS: No serum control
NRS: Non immune rabbit serum
IRS: Immune rabbit serum
CDS: Commercial anti-tick dog serum
N: Normal
H: Hind leg paralysis
F: Front leg paralysis
B: Breathing difficulties
### Paralysis symptoms

<table>
<thead>
<tr>
<th>Time</th>
<th>1/4 dilution of crude tick extract</th>
<th>1/8 dilution of crude tick extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS</td>
<td>NMS</td>
</tr>
<tr>
<td>6th hour</td>
<td>H</td>
<td>N</td>
</tr>
<tr>
<td>8th hour</td>
<td>F, H, B</td>
<td>N</td>
</tr>
<tr>
<td>10th hour</td>
<td>F, H, B</td>
<td>H</td>
</tr>
<tr>
<td>12th hour</td>
<td>F, H, B</td>
<td>H</td>
</tr>
<tr>
<td>18th hour</td>
<td>F, H, B</td>
<td>F, H, B</td>
</tr>
</tbody>
</table>

**Table 3.3 Protection assays using immune mouse serum.**

One week old neonatal mice were sub-cutaneously injected with 100 μl mix of crude tick homogenate pre-incubated with immune mouse serum. Crude tick homogenates were diluted by the serum to final dilution of 1/4 and 1/8 in triplicate. Mice were monitored for the progress of tick paralysis for 18 hours.

- **NS:** No serum control
- **NMS:** Non immune mouse serum
- **IMS:** Immune mouse serum
- **CDS:** Commercial anti-tick dog serum
- **N:** Normal
- **H:** Hind leg paralysis
- **F:** Front leg paralysis
- **B:** Breathing difficulties
3.4 DISCUSSION

3.4.1 Fusion protein expression

Gene fusion technology has advanced the possibility of developing vaccines by efficiently expressing proteins for a constant supply of antigen. Often the antigen is expressed as a carrier protein fused to the protein of interest, which stabilizes the expressed protein, retarding proteolytic degradation of the fusion protein. The carrier protein may also assist in protein purification (Nair et al., 1994; Rian et al., 1993). Expression of functional recombinant neurotoxins as fusion proteins has been demonstrated to be possible in several studies. Charybdotoxin and kaliotoxin 2 from scorpion venom were expressed in Escherichia coli with a cleavable factor Xa cleavage site so that the active toxins could be released from the fusion carrier gene 9 protein of T7 bacteriophage. This method overcame the problem of costly purification and the small yield of toxin from the native sources (Park et al., 1991). The Clostridium botulinum neurotoxin F has also been produced as a fusion protein, with maltose binding protein as a carrier protein and found to be protective against toxicosis upon challenge (Holley et al., 2001). This is also the case for the expression of recombinant alpha scorpion neurotoxin LqhαIT. This recombinant toxin exhibited biochemical properties such as HPLC elution profile, synaptosome binding, induction of paralysis in blowfly larvae and modification of the sodium currents of cockroach axons that was indistinguishable from the native toxin (from Leiurus quinquestriatus hebraeus) (Zilberberg et al., 1996). These expressions illustrate the potential of recombinant technology in expressing recombinant toxin as opposed to obtaining native toxin (often from a limited source) for downstream studies.

The first research employing the recombinant technology to develop a recombinant vaccine against Ixodes holocyclus tick paralysis used a bacterial expression system to generate a maltose binding HT-1 fusion protein based on the tick neurotoxin peptide sequence (Masina, 1999). This MBP-HT-1 fusion protein encountered the same problem as a recombinant scorpion toxin fusion protein, MBP-kaliotoxin 2 (MBP-KTX 2) produced by Legros et al. (1997). Factor Xa was unable to cleave MBP-HT-1 and MBP-KTX 2 under a
variety of conditions. However, after refolding, MBP-KTX 2 was indistinguishable from
the native KTX 2 with respect to binding to rat synaptosomes. In contrast, MBP-HT-1 did
not resemble the native toxin, in that the commercial anti-tick dog serum which neutralizes
the native toxin did not recognize the MBP-HT-1 fusion protein in the Western blots.
Furthermore, the fusion protein did not induce protective antibodies against paralysis. This
was shown in experiments where serum from mice immunized with the MBP-HT-1 fusion
protein was mixed with crude tick extract and injected into neonatal mice. The commercial
dog anti-tick serum under similar conditions was protective (Masina, 1999).

Expression systems which use maltose binding protein, β-galactosidase or glutathione
S-transferase as a fusion partner often have problems related to the large fusion partner.
These fusion partners may interfere with the protein folding by weakly interacting with the
nascent unfolded protein (Varshavsky, 2000). This can prevent protease recognition for
subsequent release of the protein resulting in the loss of cleaved product (Baker et al.,
1994). Li and Singh (1999) reported a yield of only 0.5 mg/L of cleaved toxin from 10
mg/L of MBP fused Botulinum neurotoxin light chain. Large carrier proteins can also
compete with the protein of interest in the development of an immune response such that
the majority of antibodies are generated to the carrier protein (Knuth et al., 2000). These
problems were suspected to be associated with the 43 kDa MBP for the inability of
MBP-HT-1 to be cleaved by factor Xa and to not induce a protective immune response.

The ubiquitin fusion protein system was investigated as a means of improving the
recombinant protein folding and cleavage. Ubiquitin as a fusion partner is believed to have
the function of stabilizing the peptide chain when its released from the ribosome during
translation and works as a chaperon in folding the fusion protein (Butt et al., 1989).
Ubiquitin itself is also highly stable to high temperature and proteases making it an ideal
fusion partner (Pilon et al., 1996). It has been reported that the ubiquitin system is efficient
in producing small protein or peptides (Baker et al., 1994, Pilon et al., 1996; Pilon et al.,
1997) and that the other amino acids downstream to ubiquitin are insensitive to the
deubiquitin enzymes thus the cleavage should be specific (Baker, 1996). Also, in these
experiments the His-6-tag required for purification was positioned at the N-terminus of
ubiquitin rather than having a His-6-tag incorporated at the tail end of HT-1 which would require a second cleavage site to produce the intact toxin (Figure 2.7). With the MBP-HT-1 fusion protein, the enzyme needed to cleave twice to remove MBP and the His-6-tag. Incorporation of only one cleavage site allows a simpler cleavage procedure.

### 3.4.2 Protein expression and purification

To optimize the expression of the His-ubiquitin-HT-1 fusion protein, the expression conditions were varied with regard to IPTG concentration, induction period and temperature. Among the numerous studies reported to obtain high yields of ubiquitin fusion protein expression, the use of heat shock conditions was particularly interesting. Pilon *et al.* (1996) suggest that heat shock at 42 °C for induction gave a maximal yield of 90% total soluble protein content per cell (709 mg/L). However, this phenomenon was not observed for the expression of ubiquitin HT-1 fusion protein when the amount of protein expressed was compared by the intensity of protein bands on SDS-PAGE and Western blot. As Figure 3.2 shows, fusion protein expression in the soluble fraction decreased with heat shock treatment when compared to 4 hours induction without heat shock, while the amount of fusion protein in the insoluble fraction remained the same (Figure 3.3). In contrast to the heat shock treatment, Power *et al.* (1990) recommended induction at low growth temperatures (≤ 30 °C) for the production of truncated chicken progesterone receptor because of the loss of hormone binding activity with heat shock expression. This was suspected to be associated with the release of *E. coli* proteases at high temperature. Another reason for growing cells without heat shock was that high temperature can promote the formation of insoluble protein (Koken *et al.*, 1993) and has also been associated with protein degradation (Baillie *et al.*, 1998).

The HT-1 fusion protein was successfully expressed in soluble and insoluble forms using the ubiquitin system (Figure 3.2 and Figure 3.3), but most importantly, the soluble form of the fusion protein was able to be purified under non-denaturing conditions. A purified fusion protein yield of 2-5 mg/L from the soluble fraction was obtained. Kohno and co-workers (1998) reported a decahistidine-tagged ubiquitin fusion protein was expressed
in both soluble and insoluble forms but the insoluble fusion protein required refolding for subsequent use. However, in this study only the soluble fusion protein was used to avoid any possibility of incorrect folding, since the main problem suspected to be associated with MBP-HT-1 fusion protein was incorrect folding that caused non-protective response in the protection assay.

### 3.4.3 Ubiquitin cleavage

Previously, ubiquitin cleavage has been shown to be a very efficient process. Pilon et al. (1997) reported the production of 1.08 g of cleaved peptides from 3 L of bacterial culture, using the ubiquitin expression system. Deubiquitin enzyme was expressed (28 kDa YUH1) to cleave the fusion protein. Expression of the deubiquitin enzyme offered the advantage for reducing the cost in comparison to the expensive commercial proteases (Kohno et al., 1998). However, in these experiments efficient cleavage was unable to be achieved for the ubiquitin HT-1 fusion protein with YUH1. The cleavage that did occur also appeared to be inaccurate with the production of N-terminally truncated toxin. Following USP cleavage of the fusion protein, the toxin molecular weight was expected to be 5382 Da. However, the protein detected has a mass of 4.8 kDa. If the USP was cleaving the fusion protein between residues G-K (5 residues downstream to R-G-G) rather than cleaving after R-G-G which is the reported specific cleavage site of this enzyme, the calculated mass would be 4.785 kDa which corresponds closely to that observed by mass spectrometry.

To prove that mis-cleavage had occurred, the N-terminal sequence of the product needed to be sequenced. However, difficulties were experienced in obtaining enough material of the 4.8 kDa protein for sequencing. In an attempt to overcome these difficulties, the cleavage reaction mixture was protein sequenced instead of the purified 4.8 kDa protein. Since ubiquitin and the deubiquitin enzyme were both tagged with His-6, their sequences are identical for the first 11 amino acids. Therefore, protein sequences would be the identical for the first 11 cycles (1 amino acid per cycle) and would leave HT-1 with a different, and detectable, sequence. However, only the ubiquitin sequence was detected (Matthew Padula, personal communication). This result was probably due to high concentration of uncleaved
fusion protein relative to the cleaved HT-1. The inefficient cleavage of HT-1 prevented further experimentation with purified recombinant toxin. An alternate strategy using whole ubiquitin-HT-1 fusion protein was applied.

### 3.4.4 Immunological characterization

The expressed fusion protein was further evaluated with the commercial dog anti-tick serum for the possibility of using the whole recombinant protein as a protective immunogen. Several studies have demonstrated that expression of fusion proteins that are protective against toxins is possible, and cleavage of the fusion proteins may become unnecessary. Serum (1ml) from animals immunized with whole recombinant *Tityus serrulatus* nontoxic fusion protein, without any cleavage to release the toxin, was enough to induce protective antibodies to neutralize 20 LD$_{50}$ of toxin (Guatimosim *et al.*, 2000). Also, LaPenotiere and colleagues (1995) proposed the production of the nontoxic fragment of botulinum neurotoxin serotype A fused to maltose binding protein as a potential replacement for the current vaccine toxoid. A non-toxic fusion protein would also be a good immunogen by avoiding the need for inactivation of the toxin and reduce the hazards of handling a potentially active toxin.

The evaluation with the commercial dog anti-tick serum in a Western blot (Figure 3.7) showed the first evidence of the fusion protein’s antigenicity. Antibodies in the dog serum were able to recognize the fusion protein. Purified ubiquitin HT-1 fusion protein was then used to vaccinate rabbits for the production of anti-ubiquitin HT-1 serum in a large volume. Rabbit serum was purified via protein A column and concentrated to a protein concentration similar to that of the dog serum. This rabbit serum purification was carried out because the dog serum appears to have been fractionated, in that when analysed by polyacrylamide gel electrophoresis the serum mainly contained bands with molecular weight corresponding to antibodies (data not shown). The purified serum from the immunized rabbits contained antibodies developed against the fusion protein, fusion protein aggregates and low molecular weight impurities such as ubiquitin (Figure 3.8). These antibodies were IgG since they were purified by protein A column. Despite detecting the
fusion protein on Western blots, the purified rabbit serum did not provide full protection against tick paralysis in the neonatal mice (Table 3.2).

In summary, antibodies in the protective dog anti-serum could detect the ubiquitin-HT-1 fusion protein on Western blots. However, when using this ubiquitin-HT-1 fusion protein to immunize rabbits, the rabbit antibodies were not able to provide any significant protection against the native toxin in crude tick extract, even when the antibodies were purified and concentrated.

At this point, the possibility that adjuvant was affecting the immunogenicity of the fusion protein was investigated. Although it is well known that adjuvants enhance the immune response to antigens (Dertzbaugh, 1998) and induce higher and longer lasting responses (Ferro and Stimoson, 1996), Todryk et al. (1998) has pointed out that adding adjuvant to the antigen may modify antigen processing and presentation to T cells. This in turn can affect T and B cell epitope responses. Additionally, it was revealed previously that specific antibody response of immunized mice could be generated with the 10 kDa sparingly soluble non-antigenic protein (SSNAP) fusion protein alone to a level comparable to immunogen immunized with adjuvant (Knuth et al., 2000). Not using adjuvant also prevents potential side effects such as non-permanent tissue damage or granulomatous lesions (Ferro and Stimoson, 1996). Thus, animals were immunized without adjuvant.

Mice were chosen for the immunization without adjuvant because less antigen is required for immunization when compared to larger animals such as rabbits. It was observed that mice developed antibodies against the fusion protein without the adjuvant (Figure 3.9). Due to the limited serum volume available from mice, mouse serum was not further purified or concentrated for the protection assay, as had been the rabbit serum in the previous assay. The mouse antiserum also proved to be ineffective in the protection assay (Table 3.3).

Although both the rabbit and mouse antisera raised against ubiquitin-HT-1 were unable to neutralize the tick toxin present in crude engorged tick extract in a manner analogous to the commercial dog anti-tick serum, these antisera reacted strongly to a 5 kDa protein from the
crude tick extract in Western blots which was also detected by the dog anti-tick serum (Figure 3.10). This contrasts with the observations of Masina (1999) where the dog anti-tick serum did not react strongly with the MBP-HT-1 fusion protein and antisera raised against the MBP-HT-1 fusion protein could not detect any component of crude tick extract in the size range of the toxin. These results suggest that the anti-ubiquitin-HT-1 antibodies were able to react with the native toxin in Western blots, but cannot neutralize the native toxin.

Several explanations may be advanced for these results. Firstly, a much higher level of antibody may be required for neutralization, even though concentrating rabbit antiserum did not improve protection. Secondly, the structure of the immunizing antigen may be altered by the addition of adjuvant. It should be remembered that the dog anti-tick serum is produced by engorging ticks on the dog and the toxin would be in its native state. This possibility was investigated by immunizing mice with ubiquitin-HT-1 fusion protein without adjuvant. While this process induced significant levels of specific antibody, again no protection against the native toxin was afforded by these antibodies. The reaction of dog anti-tick serum with fusion protein on Western blots and the reaction of rabbit and mouse anti-fusion protein with a presumptive native toxin band in crude tick extract (Figure 3.10) suggested that protein folding is not a likely problem.

Another important phenomenon observed in these studies (Figure 3.10) was that protein A column purified commercial dog anti-tick serum, consisting mainly of IgG class antibodies, did not detect the 5 kDa band suspected to be HT-1. This purified dog IgG also failed to protect neonatal mice from paralysis (Table 3.4). It would be expected that binding to the 5 kDa protein was crucial for the neutralization of the protein but, as mentioned, the rabbit or mouse serum that could recognize the bands were unable to neutralize toxin. This leads to the possibility that other classes of antibodies such as IgM or substances present in the commercial dog anti-tick serum other than IgG may play an important role in neutralizing the toxin.
The lack of protection provided by ubiquitin-HT-1 immunized serum may also be due to the presence of other toxins in the crude tick extract. Thurn (1994) found three toxic proteins in crude tick extract, HT-1, HT-2 and HT-3. These proteins all possessed similar molecular weights, caused the same physiological response in neonatal mice, and were all detected by a monoclonal antibody (96B 1G6, from Commonwealth Serum Laboratories, no longer available) raised to crude tick extract. However, whether they are isoforms, vary through their post-translational modifications or if they are three distinct proteins is yet to be determined.

3.4.5 Future directions

From the present research, it is not known if the non-protective immunity with the fusion protein immunization is due to the presence of other paralysis toxins, error in the sequence of HT-1 or if the fusion protein was simply unable to induce the required antibody isotype. It is important to investigate all the potential causes by analysing the native materials for other toxins i.e. HT-2 or HT-3. Despite the similarity of the HT-1, -2, and -3 in molecular weight, toxicity and structure observed by Thurn (1994), the possibility that HT-2 and 3 are immunologically distinct should not be excluded.

HT-1 cleaved from the carrier protein needs further investigation with regards to its toxicity and structure. The cleavage process with YUH1 needs to be optimized or other deubiquitin enzymes could be employed. Koken et al. (1993) showed that ubiquitin specific protease expressed by UBP1 was able to cleave human ERCC1 (a DNA excision-repair protein) while ubiquitin carboxyl terminal hydrolase 9UCH-L1 or the hydrolase expressed by YUH1 were unable to cleave the fusion protein. Toxicity assays with HT-1 alone is the best method currently available to confirm that HT-1 is folded correctly, or if the non-protective response is due to fusion carrier interferences or an incorrect sequence. For instance, in the expression of Clostridium perfringens epsilon-toxin for vaccine development against enterotoxemic diseases (Goswami et al., 1996), the addition of a His-6-tag to act as fusion carrier at the N-terminus and to assist in purification was sufficient to convert the toxin into an inactive form, however the antigenicity was retained. This indicated that a slight
modification of the toxin sequence could disrupt the function of native toxins, presumably by incorrect polypeptide chain folding (Kohno et al., 1998).

Finally, other means of purifying tick toxins need to be investigated. Although the commercial dog anti-tick antiserum reacts with the toxins, it also reacts with all the other tick salivary proteins (Thurn, 1994) and would be of little use in immunoaffinity procedures. It should be possible to develop monoclonal antibodies against ubiquitin-HT-1 using methods similar to those used to produce monoclonal antibodies against MBP-HT-1 fusion protein described in this thesis. These antibodies may not be protective but as the mouse serum against the ubiquitin HT-1 fusion protein was able to detect the 5 kDa native toxin, they could be used in immunoaffinity purification. Alternatively, a monoclonal antibody (4B12) against Rhipicephalus evertsi evertsi toxin has been used to isolate a 11 kDa tick paralysis toxin from Argas walkerae (Maritz et al., 2000). This cross-reacting antibody may react with the I. holocyclus toxin and could be used for purification purposes.

Once the toxins have been identified, it would be useful to establish an expression system to reproduce active toxins, as a constant supply of toxin is a prerequisite for further studies. A sufficient source of active toxin would allow resolution of the three-dimensional structure for designing a non-toxic, but protective, vaccine. This could be achieved by site-directed mutagenesis and chemical modification, which would also provide insights into the structurally and functionally important residues. Concurrent with toxin expression, development of monoclonal antibodies would facilitate investigation into the structure-function relationship of the toxins. Monoclonal antibodies could also lead to a better understanding of neutralization mechanisms and the toxin’s physiological effects, by investigation of the interaction of the antibody with the toxin, and the effect of monoclonal antibodies on toxicity and on inhibition of rat synaptosome binding. Furthermore, these studies could aid in the elucidation of the structure and function of many other arachnid toxins through homology studies and comparison of their differences.


Appendix 1: Sequence of HT-1 cDNA and translated protein.