

Proteomic analysis of the humoral antifungal immune response of the soft tick, *Ornithodoros savignyi* Audouin (1827)

by

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List of abbreviations

AmpB	Amphotericin B
AMPs	Antimicrobial peptides
ATCC	American type culture collection
B	Broth (blank)
B2	β -1,3-glucan induced, 2 hours challenged
β GRP	β -1,3-glucan recognition protein
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
BSM	Bovine submaxillary mucine
B+Y	Broth with yeast
B-Y	Broth without yeast
C	Control
cDNA	Complementary DNA
CFU	Colony forming units
cys	Cystein
1DE	One dimensional gel electrophoresis
2DE	Two dimensional gel electrophoresis
DIF	Dorsal-related immunity-factor
DIGE	Difference gel electrophoresis
DMF/HCl	Dimethylfluoride/ Hydrochloric acid
DNA	Deoxyribonucleic acid
DTT	1,4-dithiothreitol



E	Eluted proteins
ESTs	Expressed sequence tags
FAB	Fast atom bombardment
g	Gram
GNBP	Gram-negative binding protein
HA	Hemagglutination
HeLP	Hemelipoprotein
HL	Hemolymph
HL+HC	Hemolymph with hemocytes
his	Histidine
HIV/AIDS	Human immunodeficiency virus/ Acquired immunodeficiency disease
IAA	Iodoacetamide
IEF	Isoelectric focusing
Imd	Immunodeficiency
INT	Para-iodonitrotetrazolium violet
IPG	Immobilized pH gradient
kDA	Kilo-Dalton
l	Litre
LMM	Low molecular mass marker
LGBP	Lipopolysaccharide and β -1,3-glucan binding protein



LGRP	Lipopolysaccharide and β -1,3-glucan recognition protein
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
MALDI-TOF-MS	Matrix assisted laser desorption time of flight MS
Min	Minutes
mM	Micromolar
ml	Milliliter
mRNA	Messenger ribosomal nucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MG	Midgut
MGE	Midgut extract
MIC	Minimum inhibitory concentration
MW	Molecular weight
MyD88	Myeloid-differentiation factor 88
NanoLC-Q-TOF MS/MS	Nanoscale liquid chromatography quadrupole time of flight tandem mass spectrometry
ng	Nanogram
NL	Non-linear
OD	Optical density



OpCP	<i>Ornithodoros parkeri</i> lipoglycopheme-carrier protein
P	Pooled
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Potato dextrose broth
pl	Isoelectric point
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PMF	Peptide mass fingerprint
proPO	Prophenoloxidase
PO	Phenoloxidase
PPase	Peptidyl-prolyl <i>cis-trans</i> isomerise
PRPs	Pattern recognition proteins
Psh	Pershephone
RP-HPLC	Reverse phase high performance liquid chromatography
S2	Saline induced, 2 hours challenged
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SG	Salivary gland
SGE	Salivary gland extract



TAM	Tick α -microglobulin
TBB	Tick bleeding buffer
tBLASTn	Translate to nucleotide basic local alignment search tool
Tm-GRP	<i>Tenebrio molitor</i> β -1,3-glucan recognition protein
μ g	Microgram
μ l	Microliter
UN	Unchallenged hemolymph
V	Volts
Vg	Vitellogenin
Vn	Vitellin
W	Water
WSV	White spot virus
Y	Yeast with broth
YC1/2	Yeast control different concentrations
Y2	Yeast induced, 2 hours challenged
Y72	Yeast induced, 72 hours challenged

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Chapter 1

Literature overview

1. Introduction

1.1. Economical importance of ticks and their control

Ticks are believed to have originated 120 million years ago and approximately 900 tick species have been described worldwide (Barker and Murrell, 2004). They have a direct effect on the economy, as tick bite marks decrease the value of skins and hides, as well as a decrease in milk and meat production (Biswas, 2003). These organisms are important vectors of different pathogens (bacteria, viruses, fungi, rickettsia and protozoa) and surpass mosquitoes and other blood feeding insects in the variety of infectious microbes that they are able to transmit (Sonenshine and Azad, 1999). The pathogens that ticks transmit cause diseases affecting livestock, humans and other animals (Jongejan and Uilenberg, 2004).

Ticks are subdivided into two major taxonomic families: the *Ixodidae* (hard ticks), the *Argasidae* (soft ticks) and a third minor family that is represented by only one species namely, *Nutallielidae* (Sonenshine, 1991).

Hard ticks are primarily identified by the hard shield (scutum) on their dorsal surfaces and 13 genera have been identified (Sonenshine, 1991). Their mouthparts are visible from above and they are short-lived after feeding (Figure 1.1).



Figure 1.1: A schematic representation of the morphology of hard ticks, *Ixodidae* (Hardin MD/University of Iowa and CDC www.lib.uiowa.edu/Hardin/md/pictures22/cdc/5993_lores.jpg)

These ticks are mostly active during warm seasons and are found in open environments. They crawl up stems of grass or on edges of leaves with their front legs extended, in order to attach to their host when passing by. The hard ticks feed for long periods at a time and attach themselves to the host with cement produced by the salivary glands. *Boophilus microplus* are one host ticks, they remain on one host from the larval to the adult stages. After feeding the adults drop off and the females lay their eggs. *Rhipicephalus evertsi* require two hosts with the larvae and nymphae feeding on the first host and then dropping off. The adults then attach to a different host, feed and drop off to lay their eggs. Other ticks e.g., *Dermacentor andersoni* feed on a different host in all three of its life stages. After feeding, the ticks drop off and reattach to a new host until the adult females lay their eggs.

The hard ticks are major vectors of pathogens that cause diseases as they are attached to their host for long periods at a time while feeding. These diseases

include Rocky Mountain spotted fever, Lyme disease, monocytic and granulocytic ehrlichiosis in humans and theileriosis and babesiosis in cattle (Sauer *et al.*, 2000). These ticks can be collected by dragging a 1 m square piece of textured fabric attached to a rod handle across the surface of vegetation (Sonenshine, 1991).

Soft ticks are primarily identified by a soft wrinkled back (Figure 1.2) and 5 genera are known. Their mouthparts are not visible from above (Sonenshine, 1991).



Figure 1.2: A schematic representation of the morphology of soft ticks, *Argasidae* (Hardin MD/University of Iowa and CDC www.lib.uiowa.edu/Hardin/md/pictures22/cdc/5993_lores.jpg)

These ticks are mainly found in sheltered environments and they stay close to their hosts. The life cycle of these ticks cannot be readily distinguished as they can have up to 11 stages. Feeding takes place rapidly (varying from minutes to days) and they have the ability to survive for long periods without a blood meal. In the first life stage, the eggs hatch and six legged larva feed on a host until they reach the first nymphal

stage. The soft ticks have multiple nymphal stages, during which they increase in size until the adult stage is reached.

The soft ticks e.g., *Ornithodoros moubata* cause diseases that include West Nile virus, Q fever rickettsia, African relapsing fever, borrelia and African swine fever virus (Estrada-Pena and Jongejan, 1999). They can be collected with dry ice traps, by placing dry ice near the nesting areas of the hosts. The ticks are seen to run along the surface of the ground towards the trap, where they are collected into a container.

Ticks are able to feed undetected for long periods of time by injecting saliva into their hosts. Saliva contains a complex mixture of bioactive proteins and small molecules with anti-coagulating, anti-inflammatory and immuno-suppressive activities (Bowman *et al.*, 1997). Artificial feeding systems have been developed and can be used in the laboratory to eliminate the use of animals for tick feeding. The ticks can feed through membranes that are placed over blood that is heated to 37°C (Sonenshine, 1993). In more recent studies, artificial feeding systems have been used to infect ticks with microorganisms so as to study the effect of these invasive microbes on the immune system of ticks (Nakajima *et al.*, 2002; Matsuo *et al.*, 2004).

Biological control has become an increasingly attractive approach to use in tick management (reviewed in Samish *et al.*, 2004). Pathogens, bacteria, fungi, entomopathogenic nematodes, parasitoids and natural predators of ticks have all been used in this type of control. However, ticks do not have many natural enemies making their control difficult (de la Fuente and Kocan, 2006). Ticks are presently

controlled with chemical acaracides that are applied to the host through dipping or spraying. Acaracides have a limited efficacy in reducing tick infestations as they are impractical, due to the fact that ticks are widely distributed and reside on their hosts for short periods at a time. The use of acaracides are accompanied with serious drawbacks that include the increasing cost in the development of chemicals, the development of acaracide-resistant ticks, contamination of the environment and of milk and meat products (Graf *et al.*, 2004). A recombinant vaccine (BM86) was developed against the tick, *B. microplus*, based on a protein isolated from the gut wall (Rodríguez *et al.*, 1994, 1995). It was found that this vaccine decreased the population of tick larvae available for infestation. Anti-tick vaccines may represent a promising alternative to chemical control. These vaccines are target specific, environmentally safe and they do not pose a risk to human health. They are also easy to administer and cost affective (Willadsen, 2004). The use of vaccines also prevents the development of drug-resistant ticks that would have resulted from repeated acaracide use. Vaccine formulations can include multiple antigens, which are able to target a broad range of tick species. These vaccines are also likely to prevent the transmission of pathogens from the tick to their host.

Despite the importance of these ticks as vectors of disease, very little is known about their immune system and their ability to survive the vast amount of pathogens they are able to transmit. This makes it important to understand the mechanism by which ticks protect themselves against these pathogens in order to develop new tick control strategies, as well as prevent tick-borne pathogen transmission.

1.2. *Ornithodoros savignyi* as a model for tick investigations

The soft tick, *O. savignyi*, was used in this set of investigations, as they are relatively easy to obtain and require little care. Their hemolymph and midguts can be obtained easily and their salivary glands are of manageable size. These ticks were first identified by Audouin in 1827 and they are more commonly known as the sand tampan. They are distributed throughout the North-Western parts of southern Africa and are also found in Egypt, Ethiopia, Kenya and Zimbabwe (Paton and Evans, 1929). These ticks live in sandy regions, below the sand in areas which are likely to attract animals. In dry areas they commonly attack humans resting under shady trees and around wells where animals gather. The bites of these ticks cause irritation, blisters and bruising (Estrada-Pena and Jongejan, 1999). They cause large losses of livestock (especially young calves and lambs) in the areas where they occur and are of special relevance in Southern Africa. Pathogenesis associated with these ticks is caused by toxicoses; which was found to lead to cardiac failure (Mans *et al.*, 2004). The secreted toxin also induces a serious allergic reaction in humans (Howell *et al.*, 1975). No infectious diseases are transmitted by these ticks, although it has been reported that *O. savignyi* caused relapsing fever in humans (Paton and Evans, 1929).

A few studies in previous years investigated the role of cellular immunity in the tick's defence against infection. In recent years most research in this field entails the identification of antimicrobial peptides/ proteins in various hard tick species and in one soft tick, *Ornithodoros moubata*. To date no information on innate immunity has been published for *O. savignyi*. The present study focused on the anti-fungal response of this tick.

1.3. Fungi as disease causing agents

Yeasts are unicellular fungi that grow as single cells and produce daughter cells by budding or binary fission (www.doctorfungus.org). The cell wall accounts for about 30% of the dry weight of the fungal cells and it is used to protect the cell against damage and to keep the shape of the cell (Kliss, 1994). The fungal cell wall of e.g., *Candida albicans* (Figure 1.3) is composed of chitin, β -1,3-glucan, β -1,6-glucan, lipids and peptides that are embedded in a protein matrix (Selitrennikoff, 2001).

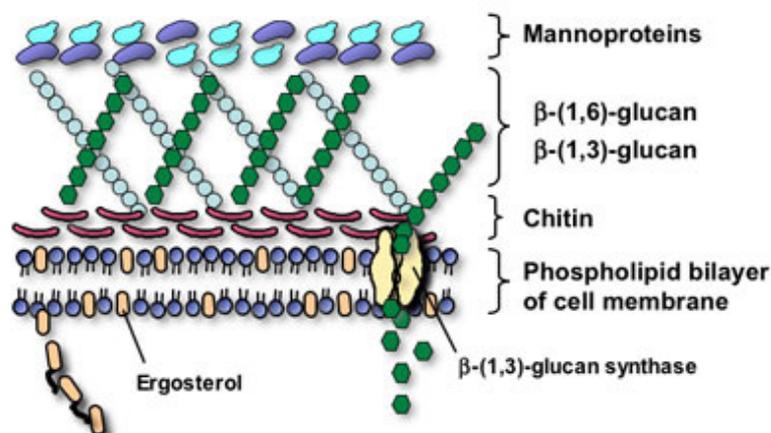


Figure 1.3: Schematic representation of the fungal cell wall (adapted from www.doctorfungus.org)

Differences between the cell wall composition of fungi and their plant or animal hosts provide an experimental target for antifungal antibiotics. β -glucans are a heterogeneous group of glucose polymers (Brown and Gordon, 2005). They consist of linear β -1,3-linked backbones with β -1,6-linked side chains. Recognition of β -glucan by the innate immune system of both vertebrates and invertebrates, initiate immune responses that control fungal pathogens.

C. albicans is classified as a dimorphic fungi, being able to alternate between a yeast phase (Figure 1.4A) and a hyphal phase (Figure 1.4B) during which they grow as thread-like hyphae (Deacon, 2006).

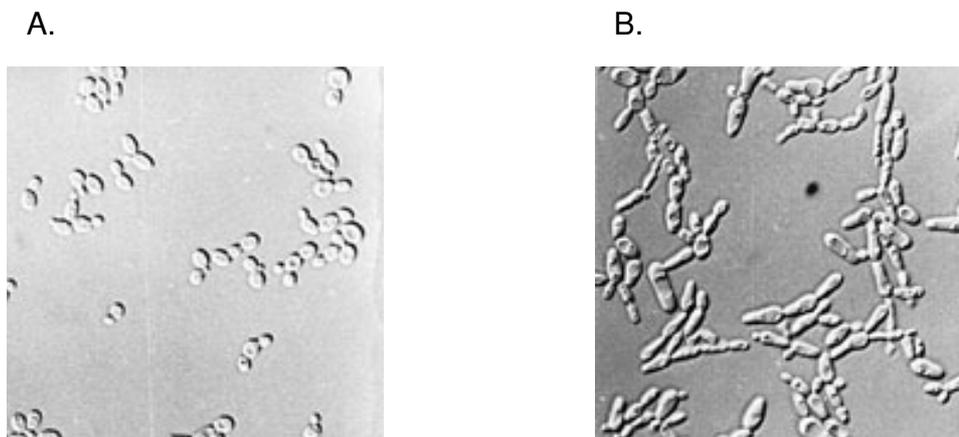


Figure 1.4: The dimorphic fungi *Candida albicans* (adapted from Stoldt *et al.*, 1997) A. Control strain forming buds, B. The pathogenic strain forming hyphae

The normal habitat of this fungus is the mucosal membranes of humans and other warm blooded animals where they grow causing little or no damage (Sudbery *et al.*, 2004). Various factors can cause these yeast populations to multiply and escape the resident bacteria, which keep the yeast population in check. These yeasts then go over to a hyphal growth stage and penetrate the mucosal membrane, which causes irritation and shedding of tissues.

Antifungal drugs are the most commonly used medication for the treatment of fungal infections (Mean *et al.*, 2008). These drugs exploit the differences that exist between mammalian and fungal cells to kill the fungal infection, without harming the host. Due to the fact that both yeast and mammals are eukaryotes the basic structure of fungal

and human cells are very similar, making it difficult to identify new targets for antifungal therapy. These drugs often have side-effects and can be life-threatening. Several classes of antifungal drugs have already been developed. These include polyenes, azoles and pyrimidine antifungal drugs (Dismukes *et al.*, 2000).

The polyenes, e.g., amphotericin B (AmpB), are the most commonly used antifungal drugs for the treatment of *C. albicans* (Dismukes, 2000). This treatment is associated with significant toxicity, chills, fever, headache, nausea and vomiting (Gallis *et al.*, 1990). Three different lipid formulations of AmpB: AmpB lipid complex, AmpB cholesteryl sulphate and liposomal AmpB have been developed (Hiemenz and Walsh, 1996). An increase in the daily dose of the parent drug, decrease in the infusion-associated side effects and a decrease in the nephrotoxicity are some of the advantages that were seen using these formulations (Wong-Beringer *et al.*, 1998). The high cost of these lipid formulations of AmpB explains why these are usually only second-line drugs in patients with candidiasis. Triazoles e.g., fluconazole were found to be just as effective, but better tolerated than AmpB in patients with candidiasis that are non-neutropenic. Various cases of resistance to the azole drugs have been noted world wide, which limits the use of fluconazole in patients before the identification and susceptibility of the antifungal drugs in the patients are known. Echinocandins are a new class of fungicidal drugs with the ability to inhibit the synthesis of β -1,3-glucan in the fungal cell wall (Denning, 2003). Three types are currently available for clinical use: caspofungin, micafungin and anidulafungin. Studies have shown that these

drugs are effective and safe thus, making them important antifungal drugs to be used for first-line therapy in patients with invasive candidiasis.

With the widespread and incorrect use of antifungal agents, drug resistance has emerged in several pathogenic fungi and a need for novel antifungal drugs has arisen. For this reason it is important to understand the immune response of ticks to fungal infection, as they may serve as a potential source of molecules that may be developed further as novel antifungal agents.

1.4. Innate immunity of invertebrates

The immune system of *Drosophila melanogaster* has been studied extensively and is used as a model for studying the immune system of other organisms. The innate immune system is the first-line of defence in invertebrates, as they do not have an adaptive immune system. An immune response usually involves the recognition of a pathogen and the activation of both cellular and humoral responses to kill and eliminate these pathogens (Imler and Hoffmann, 2000). Cellular responses include phagocytosis and encapsulation, while humoral responses lead to the secretion of antimicrobial peptides or proteins.

The most important function of the immune system is its ability to distinguish self from non-self pathogenic microorganisms (Steiner, 2004). Microorganisms that cross epithelial barriers are detected by a limited number of receptors that are encoded by germ-line DNA. Recognition takes place by pattern recognition proteins (PRPs) that bind to microbial motifs called the pathogen associated molecular patterns (PAMPs).

The latter are present on the cell walls of microorganisms and not in host cells (Girardin and Philpott, 2004). β -1,3-glucan, lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan (PGN) have been identified as PAMPs. The two most important PRPs that have been identified from invertebrates are the peptidoglycan recognition proteins (PGRPs) and the β -1,3-glucan recognition proteins (β GRPs) (Fabrick *et al.*, 2003). As the antifungal response in ticks is investigated only the β GRP will be discussed in this chapter.

1.4.1. β -1,3-glucan recognition proteins (β GRPs)

β GRPs have been isolated from various invertebrate sources (Table 1.1) and were found to have a strong and specific binding affinity for β -1,3-glucan from fungi cell walls and to a lesser extent, bacterial LPS (Fabrick *et al.*, 2004). These receptors have conserved regions with high sequence similarity to β -1,3-glucanases but lack glucanase activity, because of amino acid substitutions in key residues of their catalytic sites. β -glucan recognition is usually mediated by the 100 amino acid N-terminal, which can also modulate immunity.

Table 1.1: β -1,3-glucan recognition proteins (β GRP) from invertebrates

Source	PRR	MM (kDa)	PAMP	Response activated	Reference
Silkworm (<i>Bombyx mori</i>)	β GRP	-	β -1,3-glucan	proPO	Ochiai and Ashida, 2000
Tobacco hornworm (<i>Manduca sexta</i>)	β GRP-1	53	β -1,3-glucan	proPO	Ma and Kanost, 2000
Freshwater crayfish (<i>Pacifastacus leniusculus</i>)	LGBP	36	β -1,3-glucan and LPS	proPO	Lee <i>et al.</i> , 2000
Shrimps (<i>Penaeus stylirostris</i>)	LGBP	-	β -1,3-glucan and LPS	proPO	Roux <i>et al.</i> , 2002
Mealworm (<i>Tenebrio molitor</i>)	Tm-GRP	-	β -1,3-glucan	proPO	Zhang <i>et al.</i> , 2003
Indian meal moth (<i>Plodia interpunctella</i>)	β GRP	53	β -1,3-glucan	proPO	Fabrick <i>et al.</i> , 2003
Tobacco hornworm (<i>Madunca sexta</i>)	β GRP-2	52	β -1,3-glucan and LPS	proPO	Jiang <i>et al.</i> , 2004
Mosquito (<i>Armigeres sublabatus</i>)	-	-	β -1,3-glucan	proPO	Wang <i>et al.</i> , 2005
Fruit fly (<i>Drosophila melanogaster</i>)	GGBP-3	-	β -1,3-glucan	Toll pathway	Gottar <i>et al.</i> , 2006
Fleshy prawn (<i>Fenneropenaeus chinensis</i>)	LGBP	46	β -1,3-glucan and LPS	proPO	Du <i>et al.</i> , 2007

The first β -1,3-glucan recognition protein was purified from the silkworm (*Bombyx mori*) by Ochiai and Ashida in 1988. It was found that β GRP has a strong affinity for β -1,3-glucan and upon interaction the proPO cascade is activated (Ochiai and Ashida, 2000). The cDNA of β GRP was cloned and it was found that the mRNA is expressed in the hemocytes, fat body and epithelial cells. Upon challenge with either bacteria or yeast the transcription of the β GRP mRNA was increased. Upon sequence analysis it was found that the region 264Thr-386Pro is very similar to the catalytic regions of bacterial β -1,3-glucanases, but has higher similarity to the

glucanase-like region of Gram-negative binding proteins. It was also determined that the binding domain of β -1,3-glucan is located on the N-terminal region.

A 53 kDa β GRP-1 that is expressed in the fat body and secreted in the hemolymph of the tobacco hornworm, *Manduca sexta* was purified and cloned (Ma and Kanost, 2000). This protein binds very tightly to β -1,3-glucan on the surface of fungal cell walls and interacts with a serine protease and also activates the proPO cascade. Binding to the cell wall polysaccharides produces a conformational change in β GRP. The β GRP then interacts with a proteinase zymogen, which leads to the auto-activation of the proteinase.

A pattern recognition protein that binds to lipopolysaccharide and β -1,3-glucan (LGBP) was isolated from the hemocytes of freshwater crayfish, *Pacifastacus leniusculus* (Lee *et al.*, 2000). This single polypeptide protein with a MW of 36 kDa (reducing conditions) activates the proPO cascade. A β GRP from the plasma of the crayfish was also purified and characterized (Duvic and Söderhall, 1990). Two glucan binding pattern recognition proteins were found to be required for the complete activation of the proPO cascade in crayfish. The proPO system is present in the hemocytes and the plasma of the crayfish. β GRP recognizes β -1,3-glucan and binds to a specific hemocyte membrane receptor. ProPO and LGBP are released from hemocytes. LGBP recognizes β -1,3-glucan and the proPO cascade is activated.

Shrimps (*Penaeus stylirostris*) that were infected with white spot virus (WSV) showed an up-regulation of a LPS and β -1,3-glucan binding protein gene (Roux *et al.*, 2002). Binding of LGBP to LPS or β -1,3-glucan were found to activate the proPO cascade. Expression of LGBP and proPO was measured by real-time quantitative reverse transcription-PCR and it was found that the LGBP gene expression was up-regulated upon WSV infection. The proPO activity was also up-regulated after initial infection, but it was found to be down-regulated as the viral infection progressed. The authors concluded that LGBP is an inducible acute-phase protein, which plays an important role in the regulation of the proPO pathway after a viral infection in invertebrates.

A β -1,3-glucan recognition protein that is involved in the proPO cascade of the mealworm *Tenebrio molitor*, Tm-GRP was isolated from the hemolymph (Zhang *et al.*, 2003). Tm-GRP was also found to be specifically degraded by serine proteases during the activation of proPO, suggesting that it plays a regulatory role in the activation of the proPO system. Tm-GRP alone is not sufficient for the induction of the β -1,3-glucan dependent proPO activation. Additional proteins are required for this activation.

A soluble 53 kDa protein with strong affinity for β -1,3-glucan was purified and cloned from the Indian meal moth, *Plodia interpunctella* (Fabrick *et al.*, 2003). It was found that β GRP interacts with β -1,3-glucan and forms a complex that activates the β PRP, which in turn activates the proPO cascade.

Jiang *et al.* (2004) isolated and characterized a 52 kDa β GRP-2 from the cuticle and hemolymph of the tobacco hornworm, *Manduca sexta*. It shares 57% sequence identity with the previously identified β GRP-1 and is synthesized mainly in the fat body. The gene expression was found to be up-regulated upon challenge with yeast or bacteria. It was also found that this recognition protein leads to aggregation of microorganisms to limit the spread of infection. β GRP-2 binds to both β -1,3-glucan and lipoteichoic acid and activates the proPO cascade.

A PRP has been identified and its function was determined in the mosquito, *Armigeres subalbatus* (Wang *et al.*, 2005). This protein recognizes β -1,3-glucan and initiates the activation of the proPO cascade leading to melanotic encapsulation of the microorganisms. It is expressed in the hemocytes and rapidly secreted into the hemolymph and it is up-regulated upon challenge.

In *Drosophila*, Gottar *et al.* (2006), reported that GGBP-3 (Gram-negative binding protein) recognizes β -1,3-glucan and that it is required for Toll activation. It was also found to be involved in a second, but complementary pathway that acts in parallel to the Toll pathway. It was suggested that some pathogens have the ability to inactivate GGBPs and a counter-strategy was then developed to fight fungal infections. This strategy leads to the activation of an alternative pathway involving the detection of virulence factors by Persephone (protease). Recognition of these virulence factors leads to the activation of the Toll pathway. These virulence factors (chitinases and proteases) are secreted when fungi penetrate the physical barrier of their host.

Du *et al.* (2007) identified a LGBP that binds to LPS and β -1,3-glucan from the fleshy prawn (*Fenneropenaeus chinensis*). The gene encoding this LGBP was cloned using homology cloning with rapid amplification of 3' or 5' complementary DNA ends. The mRNA is mainly synthesized in the hemocytes and its expression was found to be down-regulated 24 hours after bacterial challenge. This 46 kDa protein was found to have strong Gram-negative binding activity, but little affinity for Gram-positive bacteria or yeast.

1.4.2. Responses elicited by recognition

Following the recognition of pathogens by the PRPs, intracellular signalling pathways are activated which in turn lead to the activation of the proPO and the Toll or Imd pathways, which are well characterized for *Drosophila* (Nappi and Vass, 2001). Recognition of microorganisms can also lead to encapsulation, melanisation, phagocytosis and coagulation. The immune response of *Drosophila* has been studied extensively and has been reviewed by Lemaitre and Hoffmann (2007). Figure 1.5 gives a summary of the cellular and humoral responses that have been identified in *Drosophila*.

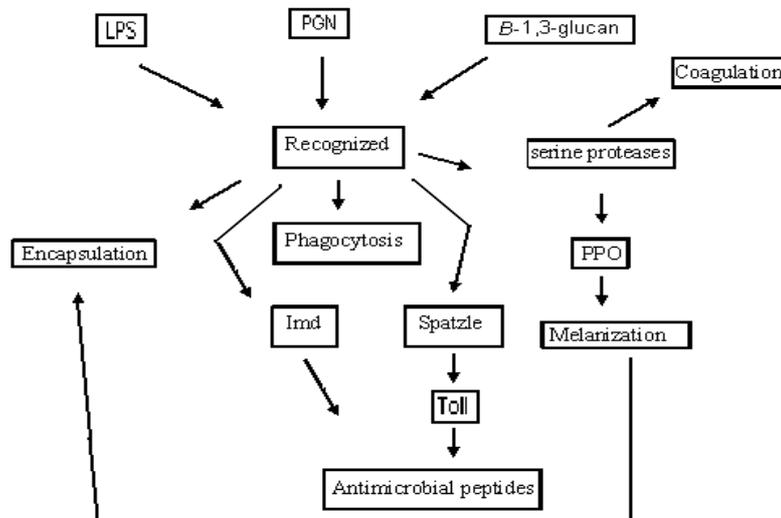


Figure 1.5: The innate immune response of *Drosophila melanogaster*

1.4.3. Cell-mediated immune responses in invertebrates

The cellular responses are mediated by hemocytes in the hemolymph that act against injury as well as invading microorganisms (Sonenshine and Hynes, 2008).

1.4.3.1. Phagocytosis

One of the most important innate immune responses is phagocytosis, which involves the engulfment of small particles such as bacteria. The plasmatocytes from *Drosophila* are the main class of hemocytes and are involved in phagocytosis (Govind and Nehm, 2004).

Three different types of hemocytes (Figure 1.6) were found to be involved in tick phagocytosis, the prohemocyte, the plasmatocytes with a low number of granula and the granulocytes (Inoue *et al.*, 2001; Kuhn and Haug, 1994). Phagocytosis is a

specific process and the hemocytes must therefore have the ability to identify pathogens. Several studies have shown that bacteria are phagocytosed from tick hemolymph (Machel, 1977, Podboronov *et al.*, 1982; Gupta, 1991; Johns *et al.*, 1998, 2000). In one study it was shown that the hemocytes can also phagocytize pathogenic yeast cells (Loosova *et al.*, 2001).

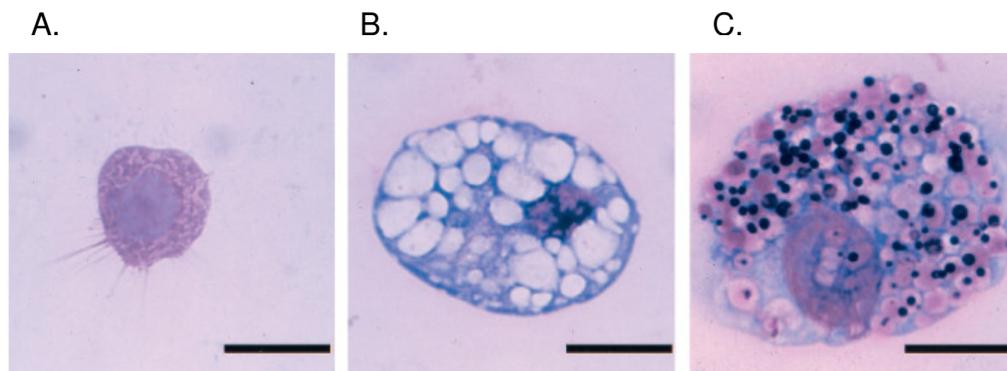


Figure 1.6: Prohemocyte (A), plasmatocyte (B) and granulocyte (C) of *Ornithodoros moubata* (Adapted from Inoue *et al.*, 2001)

1.4.3.2. Nodulation and encapsulation

Nodulation is defined by the multi-cellular hemocytic aggregates which are formed around a large number of bacteria. These nodules aggregate and adhere to tissues, forming larger nodules that may later be encapsulated (Lackie, 1988). This process of nodulation is similar to the melanotic encapsulation in insects, but without melanin (Ceraul *et al.*, 2002). Encapsulation is the process in which hemocytes bind to larger targets. These are processes in which a multilayered hemocyte sheath is formed around the invading microorganism. This process is then accompanied with melanisation eliminating the pathogen within the capsule (Gillespie *et al.*, 1997).

The lamellocytes of *Drosophila* are large flat cells that are produced by the proliferation and differentiation of hemocytes upon infection. They form cellular capsules around the parasites that are too big for phagocytosis, encapsulating them (Govind and Nehm, 2004).

Few studies have been performed on nodule formation and encapsulation in ticks. Nodulation begins with the entrapment of a microorganism by the micro-aggregation of hemocytes 1 hour after inoculation with *E. coli* (Ceraul *et al.*, 2002). The micro-aggregation grows larger as more hemocytes are recruited to the microorganism. This leads to melanization and then the formation of darkened nodules that are attached to the body wall or to various internal organs. This process is similar to that in other organisms. This response was found to be fast and efficient as *E. coli* could not be cultured 6 hours post inoculation.

1.4.4. Humoral mediated immune responses in invertebrates

The expression of the molecules that act directly on pathogens is up-regulated in response to microbial invasion. These molecules are secreted into the hemolymph plasma and they are termed humoral factors (Sonenshine and Hynes, 2008). Arthropods have strong humoral responses that work with cellular responses to fight foreign molecules.

1.4.4.1. Humoral encapsulation

Humoral encapsulation involves the formation of loose aggregates of melanotic material that accumulates around the invader (Hultmark, 2003). These aggregates

become denser until the invader is completely trapped. Encapsulation usually involves the activation of the proPO pathway. The melanotic capsules that are formed around microorganisms consist of the plasmatocytes and the lamellocytes. It is proposed that the crystal cells lyse or release their inclusions that contain phenoloxidase and initiate the prophenoloxidase-mediated melanization cascade (Nappi *et al.* 1995, 2000; Nappi and Vass, 1998, 2001; Carton and Nappi, 2001).

In *Drosophila*, the proPO pathway (Figure 1.7) is activated after β -1,3-glucan, lipopolysaccharide (LPS) or PGN binds to a PGRP or GNBPs.

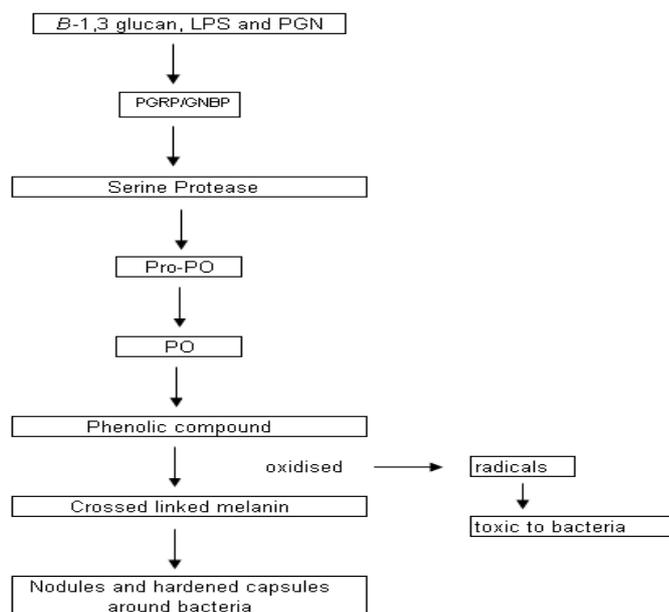


Figure 1.7: The activation of the proPO cascade in *Drosophila melanogaster*

Recognition of these microorganisms by PRPs leads to the activation of proPO by proteolytic cleavage through a native serine proteinase. The active phenoloxidase

(PO) which is formed catalyses the conversion of phenols to quinones. These quinones are toxic to bacteria and can form crossed linked melanin around pathogens. The melanization of these pathogens constricts their movements, killing the pathogens (Ashida and Brey, 1998).

Only two studies have been performed on the prophenoloxidase activating system in ticks. Zhioua *et al.* (1997) was unable to detect phenoloxidase activity in various tick species, but detected it in *Galleria mellonella*. Kadota *et al.* (2002) detected phenoloxidase activity in nymphs of *Ornithodoros moubata*. As these studies contradict each other, more studies need to be performed on the involvement of the prophenoloxidase activation in the hemolymph of ticks.

1.4.4.2. Humoral nodulation

Lectins play a role in aggregation and opsonization of microorganisms by binding to specific carbohydrates. These molecules have been isolated from hemolymph plasma, hemocytes, gut and salivary glands of tick species (reviewed by Sonenshine and Hynes, 2008). Lectins have been found to play an important role in non-self recognition in tick hemocytes (Grubhoffer *et al.*, 1991). Dorin M was identified from the plasma of the soft tick, *O. moubata*, and was found to recognize foreign molecules (bacteria, fungi, etc.) through a lectin-carbohydrate chain interaction. This lectin is synthesized in the hemocytes and contains a fibrinogen-related domain that functions in non-self pattern recognition and pathogen transmission (Grubhoffer and Kover, 1998).

1.4.4.3. Antimicrobial peptides (AMPs)

In *Drosophila*, two pathways involved in the production of AMPs have been well characterized. No information on signalling pathways involved in AMP production in ticks is known to date. These pathways include the Toll pathway (activated by Gram-positive bacteria, PGN and fungi) and the immunodeficiency (Imd) pathway (activated by Gram-negative bacteria; Dziarski, 2004). As our studies focus on the antifungal response of ticks only the Toll pathway will be described.

In the Toll pathway of *Drosophila* (Figure 1.8) it was discovered that AMP gene transcription is not activated by the direct interaction of microorganisms with membrane bound Toll receptors (Brown and Gordon, 2005).

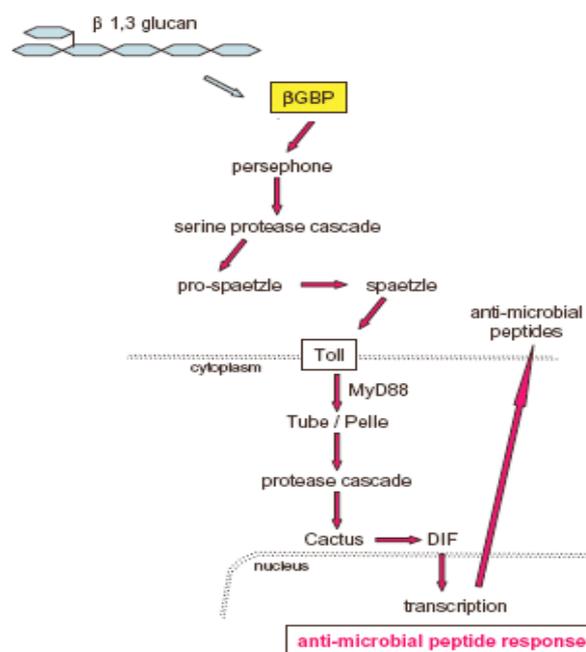


Figure 1.8: Activation of the Toll pathway after fungal infection (Brown and Gordon, 2005)

Fungi, Gram-positive bacteria or PGN trigger the activation of the pathway. During a fungal infection, the fungi will be recognized by a β GRP. Recognition leads to the activation of Spaetzle when proSpaetzle is cleaved into an active form and binds to the Toll receptor, activating it. A complex consisting of myeloid-differentiation factor 88 (MyD88), Tube and kinase Pelle is formed, which induces the phosphorylation of Cactus (Ik-B-like inhibitor). This phosphorylated Cactus is then degraded by proteasomes and it dissociates from the transcription factor dorsal-related immunity-factor (DIF). Dif can then translocate into the nucleus and AMP genes are transcribed (e.g., Drosomycin), producing AMPs active against fungi (Weber *et al.*, 2003).

In insects AMPs were found to be mainly synthesized in the fat body and their transcription was found to be induced after injury and/or infection (Bulet *et al.*, 2002). In contrast, the syntheses of AMPs were found to be constitutively expressed in chelirates and were found to occur mostly in the hemocytes (Iwanaga, 2002). These AMPs were found to be stored in granules, before they were released into the hemolymph after microbial challenge. The site and regulation of synthesis of AMPs were found to differ among arthropod groups (Fogaca *et al.*, 2004). The largest group of AMPs are the cationic molecules and more than 400 peptides have been characterized of which half have been isolated from insects. AMPs are divided into three classes based on their structural features: 1) linear peptides with α -helical structures, 2) cysteine rich open-ended peptides with one or more disulfide bridges and 3) molecules that are rich in proline, glycine or histidine (Zasloff, 2002).

These membrane acting proteins are active against a wide range of microorganisms, which include Gram-positive, Gram-negative bacteria and fungi. AMPs form pores in the membranes of the microorganisms leading to the leakage of intracellular fluid from the cells (Figure 1.9). These molecules can lead to the formation of a toroidal pore, barrel-stave pore or it can lead to total membrane disruption (Theis and Stahl, 2004).

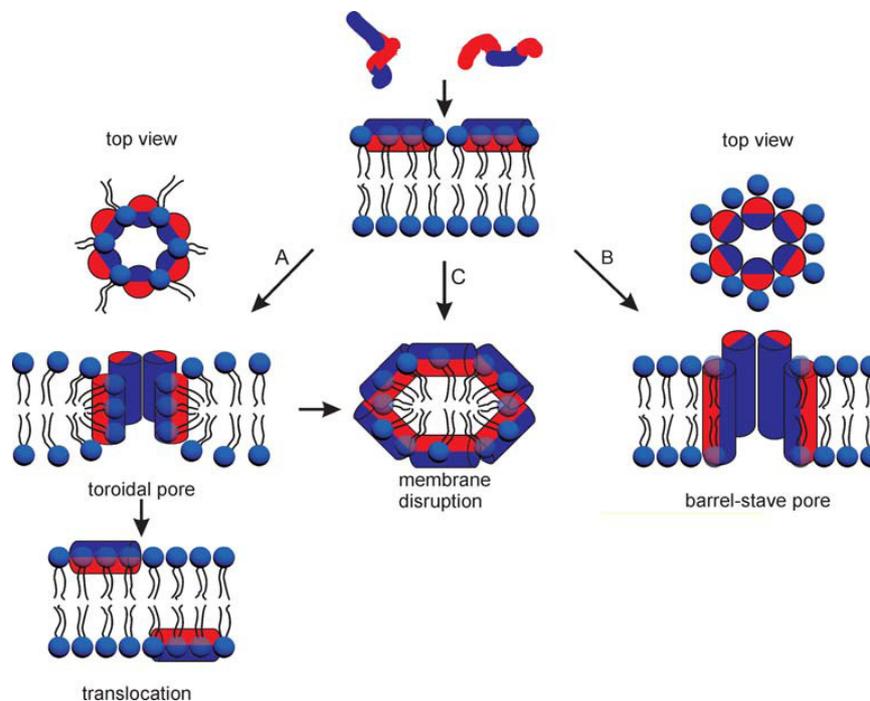


Figure 1.9: Shai-Matsuzaki Huang model of membrane permeabilisation with AMPs (Matsuzaki, 1999). Membrane phospholipids (blue: hydrophilic head group, black: hydrophobic acyl sidechains), α -helical conformation (blue part: hydrophilic side; red part: hydrophobic part).

The toroidal pore model is formed when the hydrophobic core of the protein interacts with the phospholipid head of the membrane. These proteins bind to the membrane

and cover it like a carpet, pores are then formed in the membrane when the protein: lipid ratio is very high. The barrel-stave model is used to describe how amphipathic α -helices insert into the hydrophobic core of the membrane and transmembrane pores are formed. This interaction is largely due to the hydrophobic interactions between the membrane and the AMPs (Pouny *et al.*, 1992).

Far less is known about the AMPs of ticks in comparison to insects. The only known humoral AMPs in ticks are defensins and lysozymes (Sonenshine and Hynes, 2008). The defensins have been mostly described in ticks and 20 different defensins have been identified in the midgut, salivary glands and the hemolymph of soft and hard ticks. Tick defensins primarily target Gram positive bacteria. Only a few have been found to be active against Gram negative bacteria as well as fungi.

Lysozymes are small proteins that play a role as digestive enzymes as well as antimicrobials. They have the ability to disrupt the integrity of cell walls, killing microorganisms. In most reports it was shown that lysozyme acts on the cell walls of Gram positive bacteria and they were isolated from soft and hard ticks. It was found (Simser *et al.*, 2004) in the hard tick, *Dermacentor variabilis*, that transcript levels increased 17-fold in the hemolymph 72 hours after challenge with *E. coli*. In contrast to hard ticks, it was found that in the soft tick, *O. moubata* (Grunclova *et al.*, 2003) the expression of the lysozyme gene was increased after blood feeding, but not after bacterial challenge.

1.4.5. Coagulation

Hemolymph coagulation is triggered in response to injury and immobilizes microbes, preventing them from entering invertebrates. The clotting mechanism for insects and other arthropods is poorly understood (Scherfer *et al.*, 2006). As insects do not have a closed circulatory system mechanisms exist that can quickly prevent blood loss and trap microorganisms, preventing them from spreading throughout the hemocoel. Hemolymph clotting plays a very important role in the innate immune system of invertebrates and has been characterized for *Drosophila* in response to fungi.

In *Drosophila*, Factor G functions (Figure 1.10) as a biosensor for β -1,3-glucan and triggers the activation of coagulation factors, which leads to the conversion of coagulogen to coagulin (Theopold *et al.*, 2002). The coagulins then interact with each other forming a homopolymer. It was found to be a critical response in preventing loss of hemolymph and inhibiting pathogens from entering the body cavity. This response is also involved in wound healing in insects and invertebrates.

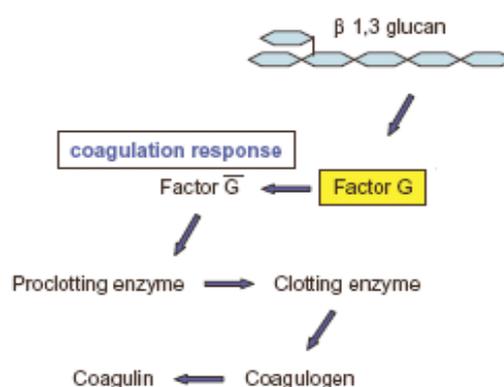


Figure 1.10: β -Glucan induced coagulation response in invertebrates (adapted from Brown and Gordon, 2005) (β -Glucan receptors are highlighted in yellow)

A clotting process that is similar to that of the insects is most probably present in ticks. Hemolymph clots at the site of injury, limiting further loss and prevent microbial infection. However, the molecular basis of the clotting mechanism in ticks remains unknown (Sonenshine and Hynes, 2008).

1.5. Aims of this study

The overall aim of this study was to investigate the antifungal response of the soft tick, *Ornithodoros savignyi*.

Chapter 2 investigated whether antifungal activity is present in the hemolymph, salivary glands or midguts of these ticks. In chapter 3, proteomics was employed to determine which hemolymph proteins are differentially expressed after a fungal challenge, while chapter 4 was aimed at identifying hemolymph proteins with a putative fungi-recognition role.

Chapter 2

Investigation into the antifungal activity in crude tick tissue extracts

2.1. Introduction

2.1.1. Antimicrobial peptides from ticks

Over the past few years an increase in the number of fungal infections in patients has been observed. Fungal infections mainly due to *Candida albicans* in immunocompromised patients have become more common (Sudbery *et al.*, 2004). Some antifungal drugs with fungistatic properties have been shown not to eradicate the fungal populations they were used on, but to increase the number of resistant species (Lupetti *et al.*, 2002). For this reason the search for alternatives to antifungal drugs is urgently required. Various cationic and non-cationic antimicrobial peptides (AMPs) have been discovered in vertebrates, invertebrates and bacterial species (Luders *et al.*, 2003). These natural antibiotics are an attractive alternative for the treatment of bacterial and fungal infections (Hoffmann and Reichart, 2002). Only a few AMPs have been isolated from arachnids and very few from ticks. The antifungal activity of *O. savignyi* ticks is to be investigated in this chapter. A summary of the AMPs that have antifungal activity in ticks is given in Table 2.1. These AMPs have been isolated from hard ticks and none to date from soft ticks.

Table 2.1: Tick AMPs with anti-fungal activity

Tick species	Name of AMP	Molecular weight (kDa)	Source	Specificity	Reference
<i>Boophilus microplus</i>	Bovine α -hemoglobin fragment	3.2	Midgut	Gram-positive bacteria and fungi	Fogaça <i>et al.</i> , 1999
<i>Boophilus microplus</i>	Microplusin	10.2	Hemolymph plasma	Gram-positive bacteria and fungi	Fogaça <i>et al.</i> , 2004
<i>Amblyomma heraeum</i>	Hebraein	11.4	Hemolymph plasma	Gram-positive bacteria, Gram-negative bacteria and fungi	Takeuchi <i>et al.</i> , 2004
<i>Ixodes sinensis</i>	Ixosin	2.8	Salivary Glands	Gram-positive bacteria, Gram-negative bacteria and fungi	Yu <i>et al.</i> , 2005
<i>Ixodes sinensis</i>	Ixosin-B	3.8	Salivary Glands	Gram-positive bacteria, Gram-negative bacteria and fungi	Liu <i>et al.</i> , 2007

Fogaça *et al.* (1999) used the inhibition zone assay to test whether non-infected, partially engorged *B. microplus* ticks possess antibacterial or antifungal activity. The authors found that the hemolymph and gut contents of these ticks had activity against Gram-positive bacteria and fungi. After challenging ticks for 2, 24, 48 and 72 h with bacteria (*Micrococcus luteus*, *Enterobacter cloacae* and *Escherichia coli*) they found that the activity was not increased in the hemolymph. A peptide with antibacterial activity was then isolated from the midgut of uninfected, partially engorged ticks. This peptide was purified to homogeneity with reversed-phase chromatography (RP-HPLC). The molecular mass of the purified peptide was determined with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and was found to be 3.2 kDa. Edman degradation was performed to obtain the N-terminal amino acid sequence of the peptide, yielding a peptide sequence of 29 amino acids. This

fragment was found to be identical to a bovine α -hemoglobin fragment. A peptide was then synthesized based on the sequence data that was obtained. The mass of the synthetic peptide was confirmed by MS and amino acid analysis. The antimicrobial activity of this synthetic peptide was determined using a liquid broth inhibition assay and it was found to be active against Gram-positive bacteria (*Micrococcus luteus* and *Staphylococcus epidermidis*) and fungi (*Candida albicans*, *Saccharomyces cerevisiae* and *Aspergillus nidulans*) in micromolar concentrations. This antimicrobial activity was not due to the presence of a tick-derived peptide, but was as a result of a fragment generated by enzymatic digestion of host hemoglobin. Whole hemoglobin did not exhibit any antimicrobial activity.

Microplusin, a cysteine-rich AMP, was isolated from cell-free hemolymph of fully engorged *B. microplus* ticks (Fogaça *et al.*, 2004). Cell-free hemolymph was subjected to RP-HPLC and the active fraction was further purified with an analytical C₁₈ column. The N-terminal amino acid sequence was determined with Edman degradation, yielding a partial sequence of 78 residues. The full amino acid sequence of the polypeptide was also determined by reducing the polypeptide and subjecting it to carboxyamidomethylation and desalting. The carboxyamidomethylation indicated the presence of six cysteine residues with 3 disulfide bonds. It was also found that it contained four histidine residues near the C-terminus. The mature peptide consisted of 90 amino acid residues with a molecular weight of 10.2 kDa. The antibacterial and antifungal activity of microplusin was determined using a liquid broth inhibition assay. It was found that microplusin had marked activity

against Gram-positive bacteria (*M. luteus*, MIC between 0.38-0.76 μM) and against yeast (*C. albicans*, MIC up to 25 μM).

Differentially expressed cDNA clones in the synganglia of fed and unfed *A. hebraeum* ticks were analyzed by Takeuchi *et al.* (2004). A cDNA clone encoding a protein with six cysteine residues and 9 histidine residues in its C-terminal was found after analyzing the differentially expressed cDNAs. This protein matched the cDNA sequence of microplusin from the hard tick, *B. microplus* (Fogaça *et al.*, 2004), but has only 4 histidine residues near the C-terminus. This mature peptide was named hebraein and its secondary structure was found to be all α -helical, with the helices being distributed evenly throughout the whole peptide. Hebraein was then isolated from the hemolymph of fully engorged ticks, using a histidine-tag affinity column and the peptide was purified with RP-HPLC. The N-terminal sequence was obtained with Edman degradation and the molecular mass of the purified peptide was determined to be 11.4 kDa. Both the naïve and recombinant forms of hebraein possessed antibacterial (*E. coli*, *S. aureus*) and antifungal (*C. albicans* and *C. glabrato*) activities. It was found that the histidine residues play an important role in the antifungal activity, as the histidine poor mutant had weak activity. Additionally, it was observed that hebraein exerts its activity in a pH dependent manner, as the positive charge on the antimicrobial peptide interacts with the anionic microbial components.

The antimicrobial peptide, ixosin, was isolated from the salivary gland extract of the hard tick, *Ixodes sinensis* (Yu *et al.*, 2005) by gel filtration, ion exchange

chromatography and RP-HPLC. The complete amino acid sequence of the purified peptide was determined by Edman degradation (GLHKVMREVLGYERN SYKKFFLR) and its molecular weight (2.8 kDa) was determined by fast atom bombardment (FAB) MS. The ixosin amino acid sequence was searched with BLAST and no similarity was found in the databases. Ixosin differs from other antimicrobial peptides in that it is a linear peptide composed of 23 amino acids without cysteine residues. Antimicrobial activity with purified ixosin was determined against Gram-negative bacteria (*E.coli*, MIC of 30 μ M), Gram-positive bacteria (*S. aureus*, MIC of 3.7 μ M) and fungi (*C. albicans*, MIC of 7.5 μ M) using a radial diffusion assay.

Liu *et al.* (2007) isolated another antimicrobial peptide, ixosin-B, from the salivary glands of *I. sinensis*. This antimicrobial peptide was isolated using the same strategy as for ixosin. The amino acid sequence was determined by Edman degradation (QLKDVLWGTRSGIQPEQHSSGKSDVRRWRSRY) and the molecular mass was determined by FAB MS (3.8 kDa). The cDNA encoding ixosin-B was cloned by cDNA library screening. Matches to the amino acid sequence were searched for with BLAST, but no similarity was found in the databases. It is a novel, linear antimicrobial peptide containing 32 amino acids, without any cysteine residues. Antimicrobial activity against bacteria and fungi was detected with the purified peptide using a radial diffusion assay. The MIC of ixosin-B for Gram-negative bacteria (*E. coli*) being 15 μ M, Gram-positive bacteria (*S. aureus*) being 2.5 μ M and for fungi (*C. albicans*) being 7.5 μ M.

2.1.2. Antimicrobial assays for the determination of antimicrobial activity

With the discovery of antibiotics and the increasing number of antimicrobial peptides, methods were needed to test these compounds for antimicrobial activity (du Toit and Rautenbach, 2000). Various antimicrobial assays have been developed for pathogenic bacteria and yeast, because of their clinical importance (Rex *et al.*, 1995). The first assays consisted of incubating bacteria with antimicrobial compounds, streaking the bacterial suspension out onto agar plates and counting the bacterial colonies that formed. These methods were found to be time and material consuming and more efficient methods were sought (du Toit and Rautenbach, 2000). One of the most successful methods entailed the formation of inhibition zones by an antibiotic in a lawn of bacteria that was grown on agar and was called the agar radial diffusion assay (Barry, 1980). This method was favored by researchers as little test material was required and it was relatively simple to perform. It was found that the agar was unsuitable for testing certain antimicrobial agents, since the agar inactivated the peptides (Kunin and Edmondson, 1968). It was also observed that these assays lead to false positive or false negative results when the extract that was used contained unknown components (Eloff, 1998). This led to the replacement of agar with high quality agarose in the assays and the development of micro-gel assays (du Toit and Rautenbach, 2000). These assays are performed in 96-well microtiter plates, making it more economical. Small amounts of test material are required and results can be obtained spectrophotometrically, making it both sensitive and accurate.

An alternative method that is generally used in microbial assays is the macro-broth dilution assay (Barchiesi *et al.*, 1994). These assays use a number of extracts that

are added to the test organism in a test tube, the minimum inhibitory concentration (MIC) is then determined by using turbidity as an indication of growth. A major drawback of this technique is that it requires large quantities of extracts and it is not useful in bioassays that are used in isolation of antimicrobial compounds.

The micro- broth dilution assay was then developed (Broekeart *et al.*, 1990; Barchiesi *et al.*, 1994). It was found to be less labor intensive and it is also ideal for screening large numbers of test samples. The liquid broth used in this type of assay allows for almost immediate antibiotic-cell interaction, whereas the assays using agar or agarose interaction depends on the diffusion rate. Advantages of using a microtiter-based system include increased sampling rates and lower concentrations of antibiotics that are used. As the turbidity of the samples can be read at 600_{nm} it is possible to analytically quantify the antifungal activity (Vlok and Rautenbach, 2000).

In the present study a micro-broth dilution assay (Masako *et al.*, 2007) was used to determine whether antifungal activity is present in the various tick tissue extracts. This assay was initially developed by Eloff (1998) to determine whether plant extracts have antibacterial activity. Different tetrazolium salts were used to indicate bacterial growth and *p*-iodonitrotetrazolium violet (INT) was found to be very stable. The method was found to be quick, sensitive and reproducible with plant extract experiments.

INT (colorless) is an electron acceptor and it is reduced to a formazan (red) product to indicate yeast growth (Figure 2.1). Thus, in the presence of yeast growth a red color

will be present and if less/no growth is present the less red the color will be. This difference in color can then be used to determine which concentration of tick tissue extract inhibits the yeast growth the best. If the tick tissue extracts kill fungi or inhibit their growth, the active compound can be isolated in future studies. The isolated component could be developed into a novel antifungal drug.

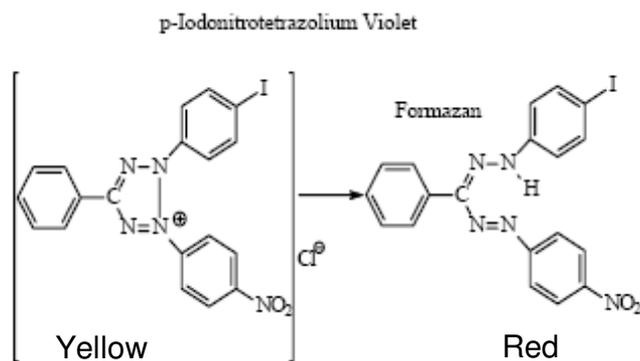


Figure 2.1: The conversion of INT to formazan (SIGMA)

In this study amphotericin B (AmpB) was used as the reference antibiotic (positive control) to inhibit fungal growth. AmpB is a fungizone (Figure 2.2) that is used in the treatment of many invasive or life-threatening fungal infections (Dismukes, 2000). It is an amphipathic polyene antifungal drug that is a natural product derived from *Streptomyces nodosus*. It associates with the ergosterol-containing membrane of the fungi, causing pore formation (Hartsel and Bolard, 1996). This leads to the leakage of ions and other small molecules from the cell resulting in damage or cell death. Higher doses of AmpB are fungicidal, while lower doses of AmpB are fungistatic.

2.1.3. Hypothesis

Antifungal activity is present in the hemolymph, salivary gland and midgut extracts of the soft tick.

2.1.4. Aims

- To determine the optimum concentration of amphotericin B to be used in the assays as the positive control
- To determine whether tissue extracts from ticks that were not challenged in the laboratory have antifungal activity
- To determine whether tissue extracts from ticks that were challenged with *Candida albicans* in the laboratory have increased antifungal activity

2.2. Materials and Methods

2.2.1. Materials

All reagents used were of analytical grade and for all experiments, double distilled deionised water was used (Millipore system Q, Millipore, USA).

2.2.2. Ticks

Ticks were collected from the Upington region (Northern Cape, South Africa), by sifting of sand. They were placed in plastic containers containing sterile sand. The ticks were kept at room temperature and female ticks were used for the determination of antifungal activity.

2.2.3. Culturing of microorganisms

C. albicans in pellet form was obtained from the American type culture collection (ATCC catalog no. 90028TM, USA). The pellet was suspended in 1 ml of sterile water and then added to a test tube containing 5 ml of sterile water and left at room temperature for 2 hours without agitation. Agar plates (2% agar) were prepared with 3 g bacto-yeast extract, 3 g bacto-malt extract, 5 g bacto-peptone, 10 g bacto-dextrose and 20 g bacto-agar in 1 l of water (YP agar) and autoclaved at 121°C for 15 minutes. Agar was poured into Petri dishes and left at room temperature to solidify. The yeast suspension was then streaked onto the agar plates and the plates were incubated overnight at 30°C. Potato dextrose broth (PDB, Sigma, Germany) was prepared by suspending 24 g of PDB in 1 l of water. The suspension was heated with frequent agitation to dissolve completely and it was autoclaved at 121°C for 15 minutes before use. A single colony was picked from the agar plate and placed into

20 ml of PDB. The yeast was grown overnight at 30°C with agitation until it reached an OD_{600nm} higher than 1.5. For long term storage, the 20 ml yeast culture was aliquoted into Eppendorf tubes (500 µl of yeast each) and 500 µl of 25% glycerol was added to the yeast cultures. The tubes were stored at -70°C.

For the antifungal assays, an aliquot of the yeast culture was thawed at room temperature and 500 µl of the culture was added to 9.5 ml of PDB. The yeast was grown overnight at 30°C with agitation and was then diluted with PDB to obtain a 1% solution (OD_{600nm}= 0.005).

For the challenge of ticks with *C. albicans*, an aliquot of the yeast culture was thawed at room temperature and 500 µl of the culture was added to 9.5 ml of PDB and grown overnight at 30°C in a shaking incubator. The culture (1 ml) was then centrifuged at 8200 x g for 15 minutes, the supernatant was removed and the pellet was reconstituted in 1 ml of sterile saline (0.9% NaCl) to obtain an OD_{600nm}= 0.25. The suspension was inactivated by boiling at 95°C for 5 minutes (heat killed).

2.2.4. Challenge of ticks

Ticks were immobilized onto Petri dishes with double sided tape with their ventral sides up (Figure 2.3). Before inoculation the ticks were sterilized by wiping the integument with a cotton bud that was dipped in ethanol. The excess ethanol was removed with a paper towel and the ethanol was allowed to evaporate. Ticks (50) were injected into their idiosoma between their second and third coxa with 1 µl of the

inactivated yeast suspension using a 10 μ l Hamilton micro syringe (Hamilton Co., Reno, Nevada) and 30 G1/2 needle (Becton Dickinson & Co.).



Figure 2.3: Inoculation of ticks with various stimulants using a Hamilton micro syringe

2.2.5. Hemolymph collection from ticks

In one set of investigations, hemolymph (HL) was directly collected from ticks that were not challenged in the laboratory. For this experiment hemolymph from 50 ticks was collected until a total volume of 500 μ l was obtained.

For a second set of investigations, 50 ticks were challenged with the heat killed yeast suspension for 2 hours and 500 μ l of hemolymph was collected.

For the extraction of hemolymph, ticks were stuck onto Petri dishes with double sided tape with their ventral sides up (Figure 2.4). The hemolymph was collected by pricking their legs with a needle and extracting the hemolymph with a PCR micropipette (1-5 μ l, Drummond Scientific Company, USA). The hemolymph was collected into Eppendorf tubes that were kept on ice.



Figure 2.4: Extraction of hemolymph from ticks using a PCR micropipette

2.2.6. Tissue collection

The same ticks from which the hemolymph was extracted were used for the collection of salivary glands and midguts. These ticks were fixed onto wax plates with their dorsal side facing up. The ticks were then cut open with a sterile scalpel along their dorsal-lateral margin, the dorsal integument removed and the midguts (MG) collected into sterile saline. The MGs were washed several times with saline to remove ingested blood before they were placed in 100 μ l of sterile water. The MG extracts were prepared by sonification with a Branson sonicator (Branson Sonic Power Company, USA) using a 30% duty cycle and an output control of 6, followed by centrifugation at 8200 x g for 15 minutes. The supernatants were collected and stored at -70°C until needed.

The ticks were then washed out carefully with sterile saline as to expose the salivary glands (SG). The SGs were collected in sterile water, sonified and extracts were prepared as described above.

2.2.7. Protein concentration determination

The Bradford assay was performed using Quick start™ Bradford dye (Bio-Rad) to determine the protein concentration of the samples (Bradford, 1976). Bovine serum albumin (BSA, Bio-Rad) was used as the standard reference protein. A dilution series of the 100 µg/µl BSA standard was made in quadruplicate. The tick samples were diluted a thousand times and 100 µl of each sample was placed in duplicate in a well. To each well 100 µl of Bradford dye was added and the plate was left for 15 minutes. The absorbance of the plate was then read at 595 nm with a Multiskan Ascent spectrophotometer (Thermo Labsystems). The BSA standard curve was used to determine the protein concentrations of each tick sample.

2.2.8. Testing of the antifungal assay with AmpB as positive control

The assay was performed in a 96-well plate and the end volume in each well was 240 µl. A stock solution of 10 mg/ml amphotericin B from *Streptomyces* (AmpB, Fluka, Sigma-Aldrich) was made up in dimethylformamide and 1 M hydrochloric acid (3 DMF:1 HCl). The stock solution was diluted a 1000 fold to obtain a concentration of 10 µg/ml. A dilution series was prepared from the 10 µg/ml AmpB in DMF/HCl ranging from 4-0.004 µg/ml with water. This was done by adding 200 µl of the 10 µg/ml AmpB to the first row and transferring 100 µl of the AmpB to the second row containing 100 µl of water and repeating this 10 fold. A dilution series was made of the DMF/HCl corresponding to equivalent amounts present in the AmpB in DMF/HCl dilution series. The yeast was cultured as described in 2.2.3. and a stock solution of 0.2 mg/ml of iodinitrotetrazolium chloride (INT, Sigma-Aldrich, Germany) prepared. As a negative control 100 µl of yeast with no AmpB or DMF/HCl was also included in

the assay. To each well 100 μ l of the 1 % yeast solution and 40 μ l INT was added, the final volume in each well was 240 μ l. This assay was performed in duplicate.

The absorbance of the plate was read at 620 nm with an ELISA plate reader before incubation (T0). The Parafilm™ sealed plate was then incubated with agitation at 35 °C and the absorbance of the plate was read every 2 hours for 8 hours (T2, T4, T6 and T8) and then after 24 hours (T24) of incubation.

2.2.8. Determination of antifungal activity in hemolymph, salivary gland and midgut extracts

The tick tissue samples were freeze dried overnight and reconstituted with water to obtain a stock solution of 40 μ g/ μ l. The stock solution was then diluted 8 and 16 times respectively, to obtain a 5 and 2.5 μ g/ μ l solutions. For the assay, 100 μ l of the two different concentrations of tick tissue samples were added to the wells of a 96-well plate, in duplicate. Appropriate solvent blanks and controls were included in the assay. To each well 100 μ l of a 1% yeast solution (as described in 2.2.3.) and 40 μ l of 0.2 mg/ml INT were added. The absorbance was read at 620 nm before incubation (T0). The Parafilm™ sealed plate was then incubated at 35°C and the absorbance was read every 2 hours for 8 hours (T2, T4, T6 and T8) and after 24 hours of incubation (T24).

This assay was first done with tissue extracts from ticks that were not challenged in the laboratory and it was then repeated with tissue extracts from ticks that were challenged with heat killed *C. albicans* for 2 hours.

2.3. Results and Discussion

2.3.1. Antifungal assay with AmpB as positive control

A micro-broth dilution assay was used to determine the lowest concentration of AmpB that will inhibit *C. albicans* growth. Due to the low solubility of AmpB in water it was dissolved in DMF/HCl and the effect of this solvent on yeast growth was also determined. Table 2.2 indicates the inhibition obtained with AmpB and the corresponding DMF/HCl control.

Table 2.2: The effect of AmpB on yeast growth

Concentration AmpB in DMF/HCl ($\mu\text{g/ml}$)	Percentage inhibition	
	AmpB in DMF/HCl	DMF/HCl alone
0.004	0	0
0.008	0	0
0.016	0	0
0.032	0	0
0.064	53	0
0.125	58	0
0.25	77	0
0.5	80	27
1	88	64
2	90	82
4	92	84

AmpB – Amphotericin B, DMF – Dimethylformamide, HCl – Hydrochloric acid

A stock solution of 10 $\mu\text{g/ml}$ AmpB was prepared in DMF/HCl and from this a dilution series of AmpB (0.004-0.4 $\mu\text{g/ml}$) was made in water. Corresponding DMF/HCl controls and appropriate solvent blanks were also included in the assay. To each dilution 100 μl of a 1% yeast solution and 40 μl of a 2 mg/ml INT solution was added. The absorbance of the plate was read and the % Inhibition was determined after 6 hours of incubation. The % Inhibition was calculated by subtracting the absorbance at T2 with the absorbance at T0, this was done for all of the time points. The absorbance value of the sample at each time point was then divided with the absorbance value of the yeast at that time point; this value was multiplied with 100 to obtain the percentage growth. The percentage inhibition obtained from the different samples was then determined by subtracting the percentage growth from 100.

The maximum inhibition observed for AmpB was 92% at a concentration of 4 $\mu\text{g/ml}$.

However, as can be seen from Table 2.2 at concentrations higher than 0.25 $\mu\text{g/ml}$, the inhibition caused by the solvent alone was significant. The concentration of

AmpB at which the solvent had no effect was 0.25 µg/ml and lower. At this concentration an inhibition of 77% was obtained. In subsequent experiments 0.064 µg/ml AmpB was used as a positive control as this is the lowest concentration of AmpB that inhibited yeast growth. The MIC of AmpB on *C.albicans* as determined by Rifai *et al.* (2004) was between 0.06-0.12 µg/ml. Even though different antimicrobial assays were used, the value obtained in our study was in the same range as the value obtained by the above mentioned authors.

2.3.2. Antifungal activity of tick tissue extracts

The overall objective of this study was to determine whether antifungal activity was present in different tick tissue extracts and which tissue had the most activity. It was also necessary to determine whether challenge would increase the observed activity. Table 2.3 indicates the effect of the different tissue extracts prepared from unchallenged and challenged ticks on fungal growth.

Table 2.3: The effect of tick tissue extracts on fungal growth

Tick tissue extract (concentration)	Unchallenged			Challenged		
	Percentage inhibition			Percentage inhibition		
(µg/µl)	SG	MG	HL	SG	MG	HL
1	57	80	17	29	68	21
2	64	100	35	37	90	45

SG – salivary glands, MG – midguts, HL – hemolymph

A stock solution (40 µg/µl) was made of each tick tissue sample. The antifungal activity of the 1 and 2 µg/µl SG, MG and HL were determined. To each well 100 µl of the tissue sample, 100 µl of yeast and 40 µl of INT was added. The antifungal activity (Percentage inhibition) was then determined from the absorbance values that were obtained.

Antifungal activity was present in all three tissue extracts prepared from unchallenged ticks at 1 $\mu\text{g}/\mu\text{l}$. The highest activity was observed for MG extracts (80%), followed by the SG (57%) and the lowest activity for HL (17%). When 2 $\mu\text{g}/\mu\text{l}$ of tissue extracts were used, the MG gave the highest inhibition (100%), followed by the SG (64%) and the least inhibition was observed for HL (35%). Since large numbers of ticks were required for the assay (100 ticks per assay), the assay was performed once only, but each sample was done in duplicate.

For ticks that were challenged with heat killed yeast for 2 hours the antifungal activity observed in the MG and HL was similar to that observed in these tissues without challenge. However, challenge decreased the activity observed in the SG. Whether this decrease in the SG is of significance is uncertain at this time as this assay was only performed once. It was seen that the inhibition for each tick tissue extract increased as the protein concentration of the samples was doubled. This was seen in the unchallenged and challenged tick tissue extracts.

The results obtained from unchallenged ticks indicated that the MG gave the highest antifungal activity, followed by the SG and then the HL. Multiple defenses play a role in limiting the opportunities for infection by invading microorganisms (Sonenshine and Hynes, 2008). When the normal route of infection is followed, microbes or foreign organisms enter the tick through an accidental puncture of the integument or by the ingestion of a blood meal (Munderloh and Kurtti, 1995). With a blood meal the MG and SG are the first to come into contact with pathogenic microorganisms. During blood feeding the immune response was found to be either constitutively expressed

or up-regulated. The AMPs are usually stored in granules and are then released upon infection (Fogaca *et al.*, 2004). As the feeding or immune status of these ticks is not known in this study, blood feeding might not up-regulate these molecules. It was found in our studies that antifungal activity was not increased 2 hours following challenge with yeast. However, at this stage it is not possible to confirm that challenge of ticks is not required to increase the antifungal activity. Collection of HL from challenged ticks at time intervals greater than 2 hours may yield different results.

Chapter 3

Proteomic profiling of hemolymph proteins in response to fungal challenge

3.1. Introduction

3.1.1. Proteomic analysis

Proteomics is defined as the study of a complex mixture of proteins that is transcribed by the genetic material in the cell (Barret *et al.*, 2000). This leads to the qualitative, quantitative and functional analysis of all the proteins in one sample. The growth of proteomics has been directly related to the advances that have been made in large-scale nucleotide sequencing of expressed sequence tags (ESTs) and genomic DNA (Graves and Haystead, 2002). In order to determine the dynamics and functions of these genes and their products it is necessary to analyze them further. The identification of proteins relies on the presence of a sequence database for a given organism. Neither the genomic DNA nor the mRNA levels expressed in cells correlate with the protein content in the cell (Lubec *et al.*, 1999).

Methods have been developed to resolve protein mixtures into their individual components. The predominant technology that is used for the separation and quantification of protein species from complex mixtures is two-dimensional electrophoresis (2DE, Gorg *et al.*, 2000). One-dimensional electrophoresis (1DE) does not have the same resolving power, but is a useful technique when used in conjunction with 2DE. This is due to the fact that the higher molecular weight proteins

do not always enter into the first dimension and the lower molecular weight proteins usually run off at the bottom of the gel.

3.1.2. 2DE

Due to its sensitivity and resolution power, two-dimensional gel electrophoresis is used to separate complex protein mixtures into many more components, due to its resolution and sensitivity. Proteins are first separated according to their charge (first dimension) and then according to their molecular weights (second dimension). Better separation of proteins is achieved with 2DE as compared to 1DE.

In the first dimension, isoelectric focusing (IEF) of proteins is performed to separate the proteins through a pH gradient until proteins reach a pH equal to their respective isoelectric points (pI, the pH at which the charge is zero). Proteins have a net charge which is equal to the sum of all the positive and negative charges on the protein at a specific pH. Previously, carrier ampholytes were prepared into a gel tube, but this created various problems with the first dimension. The limitations of these carrier ampholytes include: batch-to-batch variation that reduces the reproducibility, the pH gradients of these ampholytes are unstable and they drift towards the cathode over time, the soft polyacrylamide tube gels are not stable and can break easily. Because of these limitations immobilized pH gradients (IPG) were developed by Gorg *et al* (1988). The IPG gel was placed on a plastic support, allowing higher throughput of samples. These strips are easier to use and more reproducible (Gorg, 2000). Before focusing, carrier ampholytes migrate to their pI point through an electric field establishing a pH gradient on the IEF strip. The protein sample is then applied to the

strip and each protein migrates to a pH region that is equal to its pI when an electrical field is applied.

The second dimension, SDS polyacrylamide gel electrophoresis (SDS-PAGE), separates polypeptides not by electrical charge, but by their molecular weights (Campbell and Farrell, 2003). Sodium dodecylsulfate (SDS) is an anionic detergent and is used to denature proteins. The proteins also obtain a net negative charge that is proportional to the length of the polypeptide. When separating the protein mixture the protein bands will be separated based on their size and not charge or shape.

Two types of buffer systems are used in electrophoresis, continuous and discontinuous. A continuous system uses one separating gel and has the same buffer in the tanks and in the gel. A discontinuous system uses a stacking gel on top of a separating gel. The two gel layers are made up using different buffers and the tank buffer is also different from the gel buffers. The discontinuous system gives better sample resolution and is the preferred system to use. The reason for this increased resolution is that the proteins first concentrate into a thin zone in the stacking gel and then move into the next gel to be separated based on their size. The most widely used electrophoresis system that is still used today is a discontinuous SDS system that was developed by Laemmli (1970).

3.1.3. Detection of proteins

Different stains are available for the detection of proteins and the amount of protein in the gel is usually the determining factor as to which stain will be used. Coomassie

stains were first reported by Fazekas de St. Groth (1963) and are very popular due to their ease of use and reasonable sensitivity. Two types of Coomassie stains are used: Coomassie Blue R-250 and Coomassie Blue G-250, both of which can detect approximately 1-2 ug of protein. The high sensitivity of these dyes is due to the dye-dye interaction of Coomassie as well as the ionic interactions or hydrophobic interactions with the protein molecules. Basic amino acids (Lys, His and Arg) are important for interaction with the sulfonated Coomassie moieties (Candiano *et al.*, 2004).

Silver staining is a very sensitive staining method as it can detect down to 1ng of protein. A polyacrylamide gel is soaked in a solution containing soluble silver ion (Ag^+) and then developed by adding a reductant (formaldehyde). The protein molecules that are present in the gel promote the reduction of Ag^+ to the metallic silver (Ag^0). The Ag^0 is insoluble and visible; while Ag^0 promotes the deposition of other Ag^0 in an autocatalytic process, which results in the high sensitivity of the silver stain (Yan *et al.*, 2000). The major disadvantages of the stain are: 1.) that it is a very labour intensive staining method, 2.) it has a limited linear range (8-60 ng, Lopez *et al.*, 2000) and 3.) it also has very poor reproducibility, making quantitative analysis difficult. An MS compatible silver stain was first developed by Shevchenko *et al.* (1996), but it reduced the sensitivity of the stain as it did not contain gluteraldehyde. Yan *et al.* (2000) developed a modified silver staining method that is compatible with in-gel digestion, MALDI analysis. The protocol omits gluteraldehyde in the sensitization step and uses formaldehyde in the development step only, increasing the sensitivity of the stain.

With the recent development of fluorescent stains, the sensitivity of in-gel protein detection has dramatically been improved (Harris *et al.*, 2007). This makes protein quantification more accurate, as these stains have a broader linear range of detection. Sypro ruby protein stain (linear range of 1 ng - 1 µg) is able to quantify differences in protein expression of low abundance proteins in 2D-gels, making it a better stain than silver stain. This makes Sypro ruby very useful to determine differentially expressed proteins in studies where a small increase or decrease in the abundance of proteins plays an important role. This stain is composed of ruthenium-ions, which interact permanently with proteins. The fluorescent staining methods are also less labour intensive and quantitative information is more readily obtained. These stains are also more compatible with mass spectrometry as peptides are recovered more effectively after in-gel digestion (Lopez *et al.*, 2000). The major drawback is the high cost of these stains.

Flamingo, another fluorescent dye, fluoresces minimally at low pH in the absence of protein, but acquires a strong fluorescence in the presence of denatured proteins (Berkelman, 2007). As proteins that are separated on polyacrylamide gels are usually denatured and then fixed in an acidic solution, this feature of flamingo makes it useful to stain protein gels. Flamingo dye can detect down to 30 pg of protein in a gel. Gels stained with flamingo are more reproducible, but spot intensities were found to be lower than in gels stained with Sypro ruby. This stain is not as expensive as Sypro ruby stains and Flamingo does not appear to oxidize tryptophan residues as was seen by other stains.

3.1.4. Identification of proteins

Tandem mass spectrometry (MS/MS) plays an important role in protein identification, due to its high throughput and sensitivity. For protein identification, all proteins are first in-gel digested with trypsin producing smaller peptides. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-MS) separates these peptides according to their different mass-to-charge ratios. This is also known as a peptide mass fingerprint (PMF) which is compared to theoretical tryptic digested peptides generated *in silico* and stored in databases (Shevchenko *et al.*, 2001). MASCOT is a powerful search engine that is used to search databases for matches using the mass spectra obtained. If a match is not obtained using this approach, tandem mass spectrometry (MS/MS) is used to increase the probability of getting a database match. PMF only contains information about the molecular masses of the peptides and MS/MS is then used to give a spectrum of fragment ions that contains information about the amino-acid sequence. This additional information allows MASCOT to increase the statistical significance of a match. If no genomic or EST database is available for an organism, MS-BLAST (basic local alignment search tool) searching is performed. If the protein is still unidentifiable, *de novo* sequencing is necessary. This method identifies a protein using the sequence data determined in tandem MS analysis. This is an automated MS/MS method that provides a complete sequence of the protein spectra and can further be interpreted with PredictSequence software. These predicted sequences are then used to search databases with BLAST to identify the protein or confirm the vague hit from MASCOT (Altschul *et al.*, 1990). These results are then used to compare with the results obtained from MASCOT and the protein can then be identified (Shevchenko *et al.*, 2001).

3.1.5. Using proteomics to identify immune-related proteins

The proteins in the hemolymph of *Drosophila melanogaster* have been studied extensively. The first 2D-map of hemolymph proteins of *Drosophila* (Vierstraete *et al.*, 2003) was constructed to serve as a reference database for researchers investigating the changes that occur at protein level in different developmental stages, physiological conditions or after infection. The 2-D database was then updated by de Morais Guedes *et al.* (2003).

Levy *et al.* (2003) were the first to report on the differential proteomic analysis to identify proteins involved in the immune response of *Drosophila*. A combination of 2DE, MS and database searches were used to carry out protein profiling of the systemic immune response proteins. The proteins that were present in the hemolymph of *Drosophila* were compared before and after challenge with bacteria (Gram- positive: *Micrococcus luteus*, Gram-negative: *Escherichia coli*) and fungi (*Beauveria bassiana*). It was found that at least 70 of the 160 proteins were up- or down regulated 5-fold or more after challenge. These proteins were identified as proteases, serpins, enzymes involved in energy metabolism or molecules involved in iron metabolism.

The instantly released hemolymph proteins of *Drosophila* were analyzed by comparing the proteome of infected and non-infected samples by using a single 2D difference gel electrophoresis (DIGE) gel (Vierstraete *et al.*, 2004). *Drosophila* was inoculated with Gram-positive bacteria (*M. luteus*) or yeast (*Saccharomyces cerevisiae*) and their hemolymph was collected after 25 minutes of challenge. The

hemolymph samples were stained with propyl-Cy3 and methyl-Cy5 and the images were overlaid. The spots that coloured red or green, indicated proteins that were differentially expressed. From these experiments 20 and 19 spots were found to be up-regulated after bacterial and fungal challenge, respectively. These spots were then identified by scanning the *Drosophila* genome database with the MS data obtained. These proteins were predicted to play a role in pathogen recognition, phagocytosis, storage and transport.

3.1.6. Proteomic analysis of tick proteins

A few proteomic studies have been performed on the tick salivary gland proteins. The tick salivary glands contain a complex mixture of bioactive molecules and small molecules that have anti-coagulant, anti-inflammatory and immuno-suppressive activities. These molecules are usually released while ticks feed on their hosts (Sauer *et al.*, 2000).

A proteomic approach was used to characterize the protein profiles of saliva from two tick species, *Amblyomma americanum* and *Amblyomma maculatum*. Madden *et al.* (2002) found that separating saliva proteins with 2-DE was more complex than initially thought to be. Saliva has a high ionic strength and had to be desalted before analysis. The low abundance of proteins in saliva also made 2-DE more complex as a concentration step had to be included before analysis. It was also determined that not all of the proteins in the salivary glands were resolved on the 2D-gel and 1DE needed to be performed to analyze predominant proteins of molecular weights higher than 95 kDa. Analysis revealed that the majority of proteins were of host origin.

These proteins included albumin, hemoglobin, IgG and other unknown proteins from the host blood. The presence of host proteins makes it very difficult to use proteomic methods to characterize proteins of tick origin. The separation of host and tick proteins before separation is thus crucial.

Proteins that were abundantly expressed in unfed and non-challenged *Boophilus microplus* ticks were analyzed to assemble a 2D database of expressed larval proteins (Untalan *et al.*, 2005). Soluble and insoluble larval proteins were resolved by 2DE and it was found that 20 proteins were abundantly expressed. These proteins were identified with MALDI-TOF MS/MS. One of these proteins was identified as tropomyosin, 10 proteins were assigned putative identities, 8 proteins were identified by searching the EST database of *B. microplus* and one protein could not be identified.

Differences in protein expression in ovarian and midgut tissues from *Babesia bovis*-infected and uninfected *B. microplus* ticks were investigated with 2DE (Ranchinsky *et al.*, 2007, 2008). Soluble and membrane proteins were extracted from the tissues and the proteins that were up- or down-regulated after infection were further analyzed with capillary-HPLC-electrospray tandem MS (HPLC-ESI-MS/MS). Up-regulated proteins in the ovaries of infected ticks included calreticulin, two myosin subunits, an endoplasmic reticulum protein, peptidyl-prolyl *cis-trans* isomerase (PPIase), cytochrome c oxidase subunit, glutamine synthetase and a family of Kunitz-type serine protease inhibitors. The down-regulated proteins in the ovaries were another PPIase, a hemoglobin subunit and a lysozyme. Up-regulated proteins in the midgut

tissue included 5 metabolic enzymes and the down-regulated proteins included a molecular chaperone, a cytoskeletal protein and a multifunctional protein of the prohibitin family. This first study was used to contribute to the proteome database of ticks that can be used to investigate specific proteins involved in pathogen transmission. The identification of these proteins in the midgut could lead to the identification of anti-tick and transmission-blocking vaccine candidates.

In the present study a proteomic approach was used to determine which proteins are differentially expressed in the hemolymph of the soft tick, *O. savignyi*, shortly after infection with fungi (*C. albicans* and β -1,3-glucan). This differential proteomic analysis of the proteins involved in the immune response of the tick can be used to identify the proteins that play an important role in the defence against a fungal infection.

3.1.7. Hypothesis

Modulation of hemolymph proteins occurs in response to challenge of ticks with β -1,3-glucan or *Candida albicans*.

3.1.8. Aims

- To determine the reproducibility of 1DE of hemolymph samples obtained from unchallenged ticks
- To determine the differences between hemolymph proteins from ticks that were either challenged or not challenged using 1DE
- To determine the reproducibility of 2DE of hemolymph samples obtained from unchallenged ticks
- To determine whether proteins are differentially expressed in the hemolymph of ticks after fungal challenge with 2DE

3.2. Materials and Methods

3.2.1. Materials

All reagents used were of analytical grade and for all experiments double distilled deionised water was used (Millipore system Q, Millipore). Ticks were collected and treated as described in 2.2.2. β -1,3-glucan (Fluka, Sigma-Aldrich) was made up to a concentration of 5.4 mg/ml in sterile saline (9 g NaCl /l of dddH₂O). *Candida albicans* (ATCC 90028) was cultured as described in 2.2.3.

3.2.2. Challenge of ticks

For 1DE, ticks were challenged with 1 μ l of sterile saline (injury response) or 1 μ l of 5.4 mg/ml β -1,3-glucan (5.4 μ g per tick) for 2, 24 or 72 hours. For 2DE, ticks were challenged for 2 hours with 1 μ l of sterile saline or 5.4 mg β -1,3-glucan (fungal cell wall determinant). Challenge with heat-killed *C. albicans* was performed for 2 and 72 hours, as described in 2.2.4.

3.2.3. Hemolymph collection

Hemolymph was collected from ticks as described in 2.2.5. As a control, hemolymph was collected from ticks that were not inoculated in the laboratory (UN). Hemolymph was obtained from ticks 2 hours after injection with either sterile saline (S2), β -1,3-glucan (B2) or heat killed *C. albicans* (Y2). Hemolymph was also collected from ticks that were challenged for 72 hours with heat killed *C. albicans* (Y72). The hemolymph samples were centrifuged at 8200 x g for 15 minutes. The plasma samples were

collected into sterile tubes and stored at -70°C until required. Hemocyte-free plasma was used throughout this study.

3.2.4. Sample preparation

For the first 1DE experiment, four separate hemolymph samples were collected from 5 UN ticks each. These hemolymph samples were collected into 20 μl of buffer (0.0625 M Tris-HCl, pH 6.8). For the fifth sample hemolymph from 25 UN ticks was collected into 20 μl of buffer. For the second 1DE experiment, hemolymph from 5 ticks each, was collected from UN and challenged ticks. The ticks were injected with saline or β -1,3-glucan for 2, 24 and 72 hours. The hemolymph samples were centrifuged for 15 minutes at 8200 x g. The supernatants was collected and stored at -70°C until required.

For the first set of 2DE experiments, hemolymph from unchallenged and challenged ticks was collected. The hemolymph from 5 ticks each was collected into 20 μl of buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer and 50% protease inhibitors). The protease inhibitor cocktail (Complete, Roche) is active against pancreas extract, pronase, thermolysin, chymotrypsin, trypsin and papain. For the second set of 2DE experiments, hemolymph from 25 ticks each was collected from unchallenged and challenged ticks. The hemolymph samples were collected into a second buffer (8 M urea, 0.5% CHAPS in 10 ml and 1 ml of 10x Protease inhibitor stock solution). The samples were centrifuged for 15 minutes at 8200 x g and the supernatants were stored at -70°C until required.

3.2.5. Protein concentration determination

The protein concentrations of all of the hemolymph samples were determined as described in 2.2.7. For 1DE, 10 µg of protein was loaded per lane and for 2DE, 60 µg of protein was analyzed.

3.2.6. 1DE

The low molecular mass markers (LMM) obtained from Amersham Biosciences were prepared by adding 100 µl of reducing buffer to the protein mixture and boiling it for 5 minutes at 95°C. Before use the LMM solution was diluted 10 times with reducing buffer and 10 µl was loaded onto the gel. The LMM consisted of phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α -lactalbumin (14 kDa).

Electrophoresis was carried out according to the method described by Laemmli, (1970). A stock solution of 30.8% T, 2.6% C acrylamide (30% acrylamide, 0.8% N',N'methylene bisacrylamide) was used to prepare a 12.5% T, 2.6% C separating gel (0.375M Tris-HCl, 0.1% SDS, pH 8.8) and a 4% T, 2.6 % C stacking gel (0.125 M Tris-HCl, 0.1% SDS, pH 6.8). Both solutions were degassed for 15 minutes under vacuum and then polymerized with the addition of 0.05% ammonium persulphate and 0.033% TEMED. A Hoefer miniVE vertical electrophoresis system (Amersham Pharmacia Biotech, USA) with dimensions: 1.5 cm x 10 cm x 10 cm was used.

Five different samples were used; four samples each containing hemolymph from 5 ticks and one sample containing hemolymph from 25 ticks. Each hemolymph sample was diluted with reducing buffer (0.06 M Tris, pH 6.8, 2% SDS, 0.1% glycerol, 0.05% β -mercapto ethanol and 0.025% bromophenol blue) to contain 10 μ g of protein. The samples (final volume 10 μ l) were boiled for 5 minutes at 95°C, before loading. The electrophoresis was carried out by using a SDS running buffer (0.25 M Tris-HCl, pH 8.3, 0.1% SDS and 192 mM glycine) with an initial voltage of 60 V for 30 minutes and then the voltage was increased to 120 V until the bromophenol blue front reached the bottom of the gel. After separation the gels were either placed in a stain or in a fixing solution, depending on the staining method that was used.

3.2.7. 2DE

3.2.7.1. IEF

Sample preparation is the most important step to obtain reproducible 2-DE results; this must include complete solubilisation, disaggregation, denaturation and the reduction of all the proteins in a sample.

The hemolymph samples from ticks were solubilised in a rehydration solution (8M urea, 0.5% CHAPS, 0.002% bromophenol blue in 10 ml, 12.5 μ l IPG buffer and 2.8 mg DTT to 1 ml added before use). The final volume of the mixture was 125 μ l containing 60 μ g of protein. The solubilised protein samples were then added into the strip holder. The samples were then loaded onto immobilized non-linear (NL) pH

gradient/ IPG strips (GE Healthcare, Bio-Sciences, pH 3-10 NL, 7 cm) using an Ettan IPGphor II system (Amersham Bioscience) for 12 hours at 50 V.

In a separate experiment, 60 µg of the 2D protein standards (Bio-Rad) was loaded onto an IPG strip. These standards consist of conalbumin (MW= 76 kDa, pH 6.6), albumin (MW= 66 kDa, pH 5.5), actin (MW= 43 kDa, pH 5.1), GAPDH (MW= 36 kDa, pH 8.8), carbonic anhydrase (MW= 31 kDa, pH 5.9), trypsin inhibitor (MW= 21.5 kDa, pH 4.5) and myoglobin (MW= 17.5 kDa, pH 7.0).

After the samples were loaded onto the strips, IEF was performed with an Ettan IPGphor II system (Amersham Bioscience) at 20°C and 50 µA/ IPG strip as follows: 100 V for 30 minutes, 500 V for 30 minutes, 1 000 V for 30 minutes and 5 000 V for 1 hour 40 minutes. The strips were then stored at -70°C until required.

3.2.7.2. SDS-PAGE

Focused IPG strips were equilibrated: first with 10 ml of equilibration buffer (50 mM Tris-HCl pH8.8, 6 M Urea, 30% SDS and 0.002% bromophenol blue in 200 ml) containing 1% of 1,4-dithiothreitol (DTT) for 15 minutes and secondly with 10 ml of equilibration buffer containing 2.5% of iodoacetamide (IAA) for 15 minutes. The equilibrated IPG strips were then placed on top of a 1.5mm thick SDS-polyacrylamide gel (12.5%), covered with a 1% agarose (50 mM Tris-HCl) solution and run in a Hoefer miniVE. Electrophoresis was carried out at 60 V for 30 minutes and then 120 V until the bromophenol-blue front reached the bottom of the gel. After separation the gels were placed into a fixing solution, before staining.

3.2.8. Visualization of proteins

3.2.8.1. Colloidal Coomassie staining

The proteins were visualized by staining the gel overnight with 0.1% Coomassie Brilliant Blue G-250 (Bio-Rad, USA). After staining the gels were washed quickly with 25% methanol and 10% acetic acid. Gels were destained with a destaining solution of 25% methanol (3-4 hours), until the protein bands were clearly visible.

3.2.8.2. Non-MS Compatible silver staining

A non-MS compatible silver staining method was first used. This method was adapted from Nesterenko *et al.* (1994), as this method is more sensitive. The gels were placed in a fixing solution (30% ethanol, 10% acetic acid/ 100 ml dddH₂O) for 30 minutes. Thereafter, a sensitizing solution of 30% ethanol, anhydrous 0.5 M sodium acetate (CH₃COONa), 0.5% gluteraldehyde, 0.2% sodium thiosulfate (Na₂S₂O₃) /100 ml H₂O was added for 30 minutes. The gels were washed three times with water for 10 minutes and placed in a silver solution (0.1% silver nitrate (AgNO₃), 0.25% formaldehyde/ 100 ml H₂O) for 30 minutes. The gels were then washed briefly with water twice and placed into a developing solution (2.5% sodium carbonate, 0.01% formaldehyde/ 100 ml dddH₂O). The reaction was terminated by placing the gels in 0.05 M EDTA.

3.2.8.3. MS compatible silver staining

A MS compatible protocol was adapted from Yan *et al.* (2000) for the silver staining. Slow agitation was carried out through-out the entire staining procedure and 100 ml of each solution was added to each gel. The gel was fixed overnight in a 50%

methanol, 10% acetic acid solution. A second fixing solution (5% methanol, 1% acetic acid) was then added to the gel for 15 minutes. The gel was then washed three times with water (10 minutes each). A solution of sodium thiosulfate pentahydrate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$, 20 mg/100 ml) was added to sensitize the gel. It was then quickly rinsed three times with water. AgNO_3 (200 mg/100 ml) was used for 30 minutes to silver stain the gel. The gel was again washed three times for 60 seconds. A mixture of sodium carbonate (NaCO_3 , 6 g/100 ml), 37% formaldehyde and $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (50 μl /100 ml) was then used to develop the gel. The developing step was stopped with 14 g/l EDTA. The gels were placed in water and stored at 4°C.

3.2.8.4. Flamingo fluorescent staining

The gels were placed into a fixing solution of 40% ethanol, 10% acetic acid for 24 hours. A 1x Flamingo staining solution (Bio-Rad) of 50 ml per gel was made up by diluting the stain ten times (1 part stain: 9 parts filtered deionised water). The gels were stained for at least 3 hours. The staining solution was decanted and water was added to the gels.

3.2.9. Image analysis

The 1D-gels were scanned with an image scanner (Amersham Pharmacia Biotech). The 2-D SDS-PAGE gels were scanned with a Pharos FX Plus Molecular imager (Bio-Rad) and using software from Quantity One. For each experimental condition (UN, S2, B2, Y2 and Y72), a master image was prepared for each from 4 separate gels (4 biological repeats) using PDQuestTM software. This software uses all the gels from one sample set (e.g., the four gels from UN: 1 sample set) and compares these

gels to each other to determine which spots are present in more than two of the gels. These spots are then placed on the master image and this is then used as a representative gel for the four gels of one sample set. To determine which proteins are differentially expressed in the hemolymph, the master image obtained from the unchallenged hemolymph samples was compared to the master image obtained from saline injected, β -1,3-glucan induced and heat killed yeast induced ticks, respectively. Differentially expressed spots that are statistically significant ($p < 0.05$) were then determined using the Student t-test. All of the gels were analyzed with PDQuestTM software from BioRad.

3.2.10. MALDI-quadrupole time-of-flight mass spectrometry (MALDI-QToF MS)

The spots of interest were cut from the gel and placed into 200 μ l of dddH₂O for analysis at the University of York (UK). The gel pieces were washed twice with 50% (v:v) aqueous acetonitrile containing 25 mM ammonium bicarbonate, then once with acetonitrile and dried in a vacuum concentrator for 20 min. Sequencing-grade, modified porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold by adding 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.01 μ g/ μ l. The gel pieces were rehydrated with 10 μ l of trypsin solution and covered with 25 mM ammonium bicarbonate solution for 30 minutes. These digests were incubated overnight at 37°C.

For the MS analysis 1 μ l aliquot of each fraction was applied to the ground steel MALDI target plate. An equal volume of freshly prepared 5 mg/ml 4-hydroxy-a-cyano-

cinnamic acid (Sigma; in 50% aqueous (v:v), acetonitrile containing 0.1% trifluoroacetic acid (v:v)) was then added to the fractions. A positive-ion MALDI-MS spectra was obtained (Bruker Ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser) and the MS spectra were acquired over a m/z range of 800-4000. The final mass spectra were externally calibrated against an adjacent spot containing 6 peptides (des-Arg¹-bradykinin, 904.681; angiotensin I, 1296.685; Glu¹-fibrinopeptide B, 1750.677; ACTH (1-17 clip), 2093.086; ACTH (18-39 clip), 2465.198; ACTH (7-38 clip), 3657.929.). Monoisotopic masses were obtained using a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a S/N threshold of 2.

The ten strongest peaks of interest for each spot were then selected for MS/MS fragmentation (signal to noise ratio greater than 10). The fragmentation was performed without the introduction of a collision gas. A default calibration was used for the MS/MS spectra, which were first baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection used a SNAP averagine algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 3. Bruker FlexAnalysis software was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

The combined mass and tandem mass spectrum data were submitted to data searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.1), through the Bruker BioTools interface (version 3.1). Search criteria included: Enzyme, trypsin; variable modifications, oxidation (M); peptide tolerance,

200 ppm; MS/MS tolerance, 0.8 Da; instrument, MALDI-TOF-TOF. The search criteria also included carbamidomethyl (C) as a fixed modification for all alkylated samples.

For the *de novo* sequence interpretations from the tandem mass spectra PredictSequence routine (BioMultiview 1.4 software, MDS Sciex) was used. The complete and partial peptide sequences obtained were then searched with MS BLAST using PAM30 matrix and the nrdb95 database (NCBI). The search was performed with the WU-BLAST2 program (Gish W (1996-1999) <http://blast.wustl.edu>) on an EMBL server <http://dove.embl-heidelberg.de/Blast2/>.

The available expressed sequence tags (EST) libraries for hard ticks: *Amblyoma variegatum*, *Boophilus microplus*, *Ixodus scapularis* and *Rhiphicephalus appendiculatis*, were used to search for matches. The sequences obtained from the *de novo* sequencing were used and each was subjected to a database search. The searches were carried out with tBLASTn and the NCBI database was searched, Gen Index databases (<http://compbio.dfci.harvard.edu/tgi/>).

3.3. Results and Discussion:

3.3.1. 1D-analysis of hemolymph obtained from unchallenged ticks

Various factors can influence the number and resolution of bands or spots that are seen on 1DE or 2DE. The first is biological variation that is caused by differences that are present between ticks. The second is experimental variation, which is caused by variation in the sample preparation, small differences in each polymerized gel and the staining protocol used. Biological variation can be reduced by using ticks of the same life stage and of similar feeding and immune status. The only way to achieve this is to collect tissues from ticks that are reared in the laboratory. This, however, is problematic as the ticks in our studies were collected from the field, making their feeding status and degree of challenge unknown.

In order to assess the extent of biological variation, hemolymph was obtained from field collected ticks that were not challenged in the laboratory. Five different samples of hemolymph were collected, four samples containing hemolymph from 5 ticks each and a fifth sample containing hemolymph from 25 ticks. Equal amounts of protein (10 μ g) were used for analysis in each case. The 12.5% polyacrylamide gels were used to separate proteins with molecular weights ranging from 14-97 kDa. The gel was first stained with colloidal Coomassie, destained and then re-stained with silver stain. As can be seen from the gels that were stained with colloidal Coomassie (Figure 3.1A) and silver stain (Figure 3.1B), the higher molecular weight protein bands were the most prominent in all of the samples.

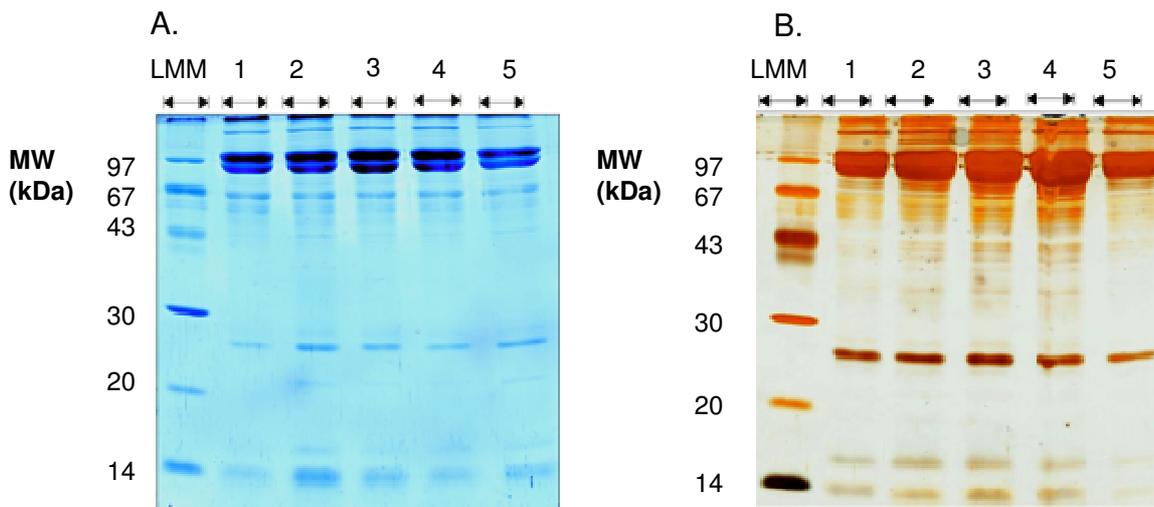


Figure 3.1: 1DE with hemolymph from unchallenged ticks. (A.) Gel stained with colloidal Coomassie, destained and restained with MS-compatible silver stain (B.). Low molecular mass markers (LMM) were loaded into the first lane. Lanes 1-4 were loaded with hemolymph obtained from 5 ticks each and lane 5 was loaded with hemolymph obtained from 25 ticks. Polyacrylamide gels (12.5%) were used and 10 μg of each sample was loaded.

These bands are possibly vitellin (VN), which has subunits of 45-200 kDa or vitellogenin (VG), which has large subunits of 150-200 kDa (James and Oliver, 1997). A band at 14 kDa was observed and it was present in all of the samples. This band could correspond to lysozyme, which has antibacterial activity (Fogaça *et al.*, 1999). A band was also seen at 16 kDa in the silver stained gel, but was not visible in colloidal Coomassie stained gel. The overall pattern of the bands was very similar, but variations could be seen in the 30-66 kDa range when stained with silver. More protein bands were visible in the silver stained gel and lanes 2-4 had a similar pattern. The duplicate gel that was stained with colloidal Coomassie was found to be more reproducible (results not shown), than the corresponding silver stained duplicate gel.

3.3.2. Comparative 1D analysis of hemolymph proteins

Ticks were induced with β -1,3-glucan and hemolymph obtained 2, 24 or 72 hours post-challenge was analyzed. In this experiment the gel was stained with a non-MS compatible silver stain. As can be seen from Figure 3.2, more bands are visible on this gel when compared to the gel stained with a MS-compatible silver stain (Figure 3.1 B).

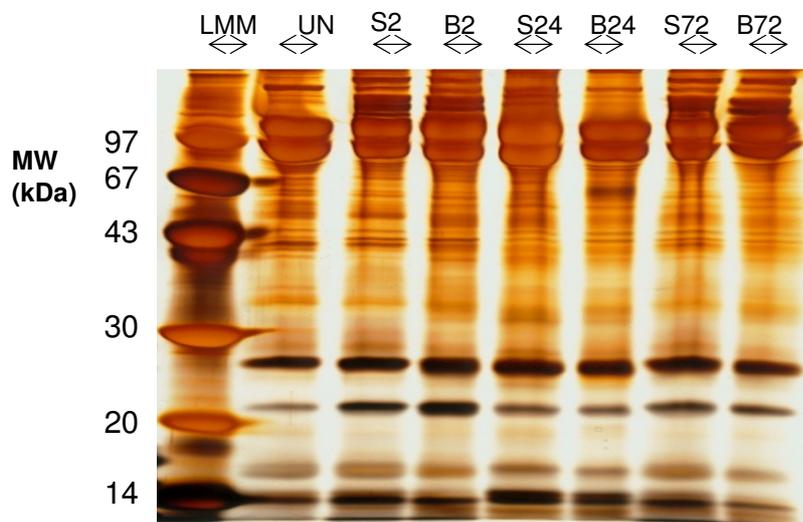


Figure 3.2: Comparison of hemolymph proteins obtained after challenge.

The lanes were loaded with LMM: Low molecular mass markers, UN: hemolymph obtained from unchallenged ticks. S2, S24, S72: hemolymph obtained from ticks injected with saline for 2, 24 or 72 hours and B2, B24, B72: hemolymph obtained from tick induced with β -1,3-glucan for 2, 24 or 72 hours. Hemolymph was collected from 5 ticks for each sample and 10 μ g of protein was loaded into each lane. A 12.5% polyacrylamide gel was used and the gel was stained with a non-MS compatible silver stain.

From this experiment it was seen that 1DE is not suitable for comparison between hemolymph samples from challenged and unchallenged ticks. These gels were done in duplicate and similar results were obtained for each experiment.

3.3.3. 2D analysis of hemolymph proteins from unchallenged ticks

Since, 1DE could not be used for differential analysis and better resolution can be obtained with 2DE, it was used in further experiments. Although 2DE is a powerful technique it also has some limitations: decreased spot resolution on small gels, gel-to-gel and run-to-run variations with the same protein set. It should also be kept in mind that this is a very time consuming process and the duration of one procedure is about five days. Equipment availability is also a large limiting factor as to how many gels can be run at a time (Carette *et al.*, 2006).

2DE was performed on two separate samples of hemolymph collected from 5 ticks each. The two gels were stained with a non-MS compatible silver stain. The differences that were observed could be due to biological variation between the two different samples that were used or it could be due to experimental error. Although the gels were stained with the same silver stain solution and for the same time, differences in the staining intensity of the two gels were observed. As can be seen from the Figure 3.3A the gel stained lighter than the gel in Figure 3.3B.

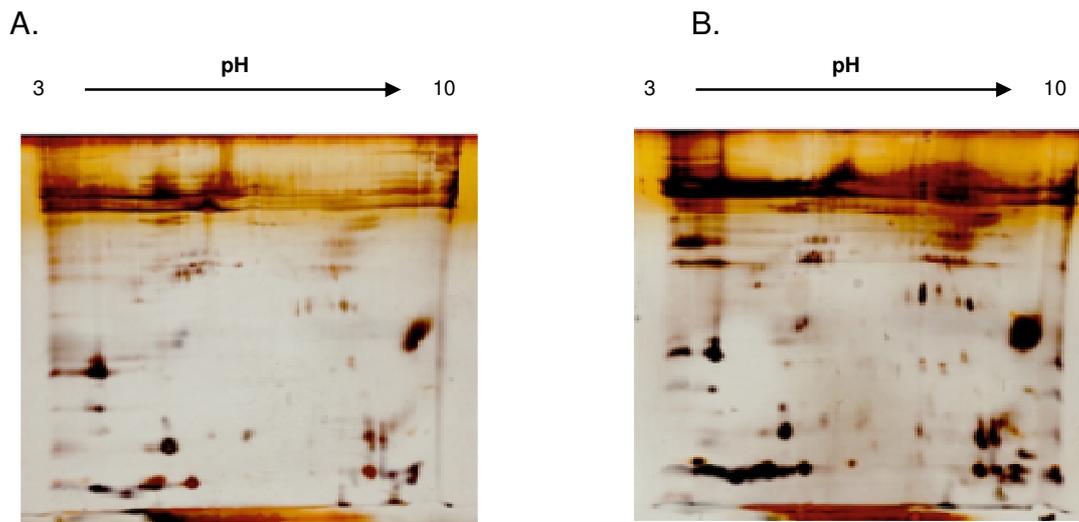


Figure 3.3: 2DE of hemolymph obtained from unchallenged ticks. *The gels in (A.) and (B.) were run from two different hemolymph samples collected from 5 ticks each. IEF was carried out with an Ettan IPGphor II using 7 cm IPG strips (pH 3-10, NL) and 60 μ g was loaded onto each IPG strip. The proteins were then separated on a 12.5% polyacrylamide gel and the gels were stained with non-MS compatible silver stain.*

The overall pattern of the spots was the same, but variation between the two gels could also be seen. This variation in spot intensity is largely due to the silver stain that was used. As the higher abundance proteins stained more intensely the lower abundance proteins are also overshadowed and cannot be visualized. Some streaking was also observed and this is largely caused by the presence of salt in the samples. This could have been prevented by desalting the samples before IEF was performed. This step was included in previous experiments, but it was found that the protein concentration of samples decreased significantly. A non-linear pH gradient was used as better separation can be obtained as the proteins separated more evenly over the gel.

3.3.4. Evaluation of a MS-compatible silver stain

To confirm that the variation that was seen between the gels in Figure 3.3 was largely due to the staining procedure used, the experiment was repeated by running 4 separate 2D-gels, using the same sample. The gels in this experiment were stained with a MS compatible silver stain. From Figure 3.4 it was seen that although the overall protein patterns of the different gels stained with silver are similar, some differences were still observed between the 4 gels.

These gels also stained lighter than the gels in Figure 3.3 as formaldehyde was excluded from the staining process and this decreased the sensitivity of the stain (Shevchenko *et al.*, 1996). The spots on the gels that were stained with non-MS compatible silver stain were more prominent than on the gels in this experiment. No comparison between proteins present can be made when the gels are stained with silver, because silver stain does not give consistent results and cannot be used for quantitative analysis. Streaking was again observed as the samples were not desalted before IEF. Thus, the variation that was seen between the gels could be due to some biological variation, but mostly as a result of the staining procedure used. Silver was the first choice of stain to be used with 2D-gels due to its high sensitivity and relatively low cost, when compared to other fluorescent stains. Although silver is a labour intensive staining method, spots can easily be visualized after a few hours. Another problem associated with silver is that with an increase in protein concentration used, the number of spots

detected decreases as the spots blend and they cannot be distinguished from each other (Smejkal *et al.*, 2004).

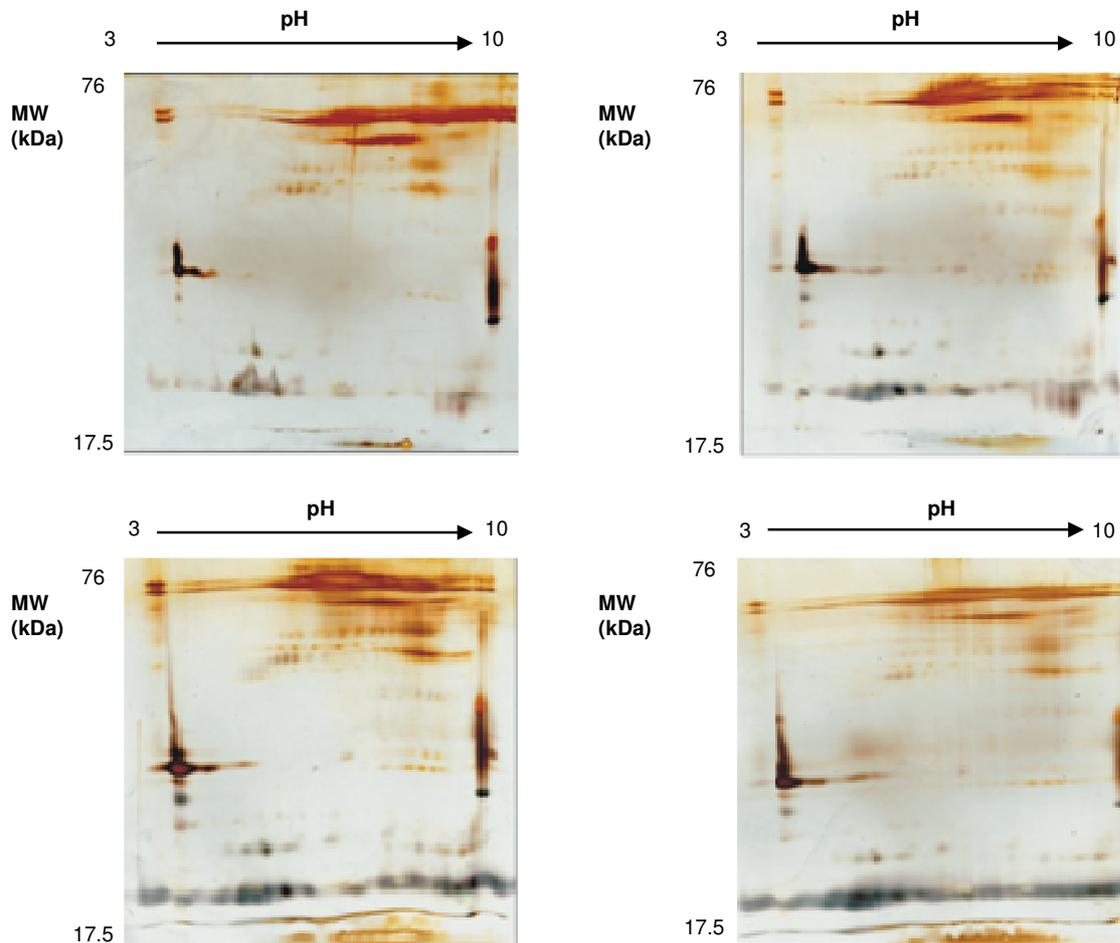


Figure 3.4: MS-compatible silver stained 2DE of hemolymph obtained from unchallenged ticks. For each gel 60 μ g of protein was loaded onto each IEF strip (IPG strips, 7 cm, NL, pH 3-10) and 12.5% gels were run. Four gels were run from one hemolymph sample collected from 25 ticks. The gels were stained with a MS-compatible silver stain.

3.3.5. 2DE of standard proteins

2DE was performed using a standard mixture of proteins. This was done as the gels that were run were too small to include a standard next to the IEF strip that

was run on the gel. These molecular weights and pH values were used to approximate the values on the 2D-gels (Figure 3.5).

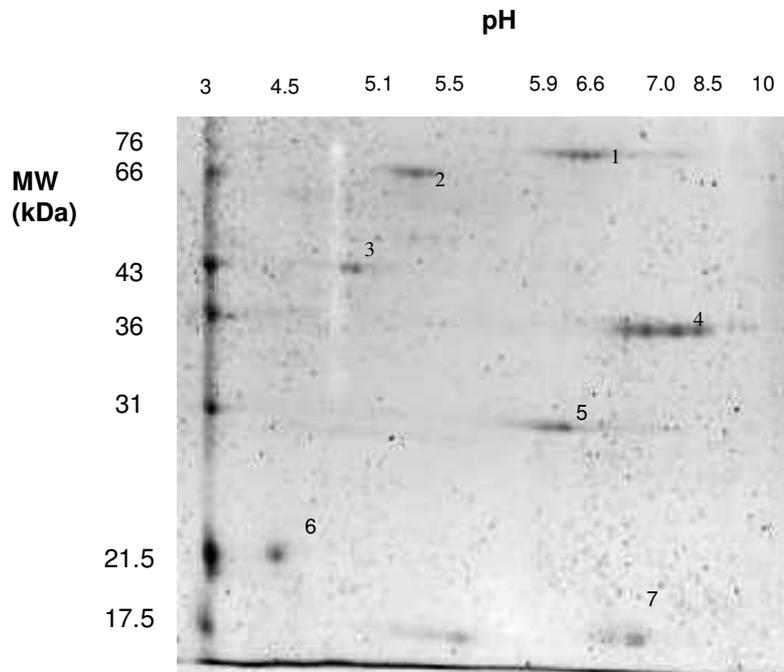


Figure 3.5: 2DE of standard proteins. The IEF strip (7 cm IPG strips, NL pH 3-10) was first rehydrated with 60 μ g of the protein standard and then separated on a 12.5% polyacrylamide gel. The MW and pI values of the following proteins correspond to the proteins spots on the 2D-gel: 1. conalbumin (MW= 76 kDa, pH 6.6), 2. albumin (MW= 66 kDa, pH 5.5), 3. actin (MW= 43 kDa, pH 5.1), 4. GAPDH (MW= 36 kDa, pH 8.5), 5. carbonic anhydrase (MW= 31 kDa, pH 5.9), 6. trypsin inhibitor (MW= 21.5 kDa, pH 4.5), 7. myoglobin (MW= 17.5 kDa, pH 7.0). The gel was stained with flamingo fluorescent stain.

3.3.6. Evaluation of Flamingo fluorescent stain

2DE was repeated and two separate gels were run using the same hemolymph sample (collected from 25 unchallenged ticks). As can be seen from Figure 3.6A and 3.6B the two gels stained similarly and the same spots could be seen on both gels.

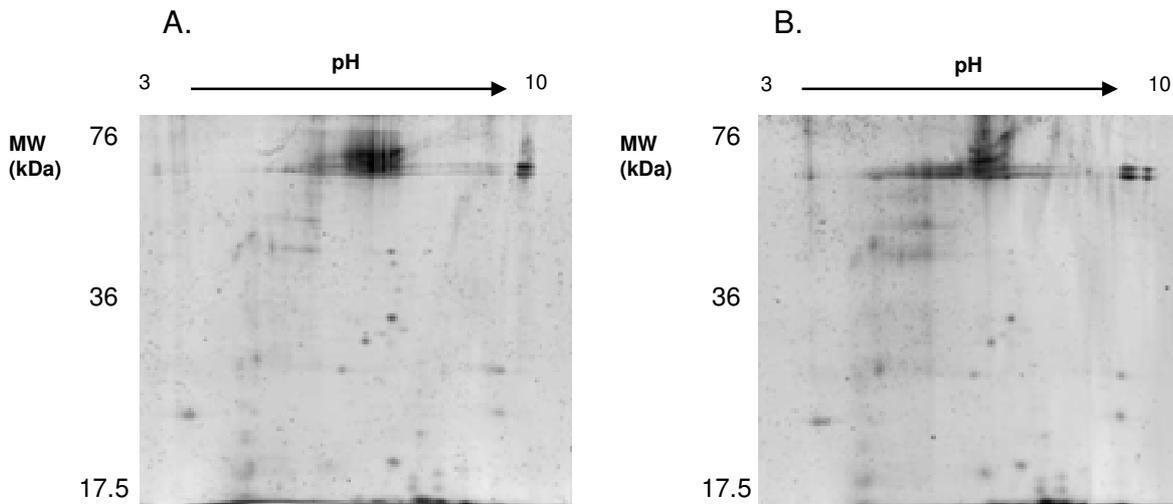


Figure 3.6: Flamingo stained 2D-gels of hemolymph collected from unchallenged ticks. The gels in (A.) and (B.) were run from one hemolymph sample collected from 25 ticks. For each gel 60 μg of protein was used, 7 cm IPG strips, NL pH 3-10 and the gels were stained with Flamingo fluorescent stain.

3.3.7. Comparative 2D-analysis of hemolymph proteins

After establishing the reproducibility of the Flamingo staining technique, the proteins that are differentially expressed in the tick hemolymph of challenged versus unchallenged was determined (Figure 3.7). As a control, hemolymph from unchallenged ticks was used to analyze the proteins that are present in the hemolymph before any challenge. Ticks were injected with sterile saline (injury induced response), β -1,3-glucan or heat killed *C. albicans* and the hemolymph was collected 2 hours after challenge. Each experimental condition was performed in quadruplicate (4 biological repeats) and 2DE were stained with Flamingo fluorescent stain and their images were analyzed with PDQuestTM software.

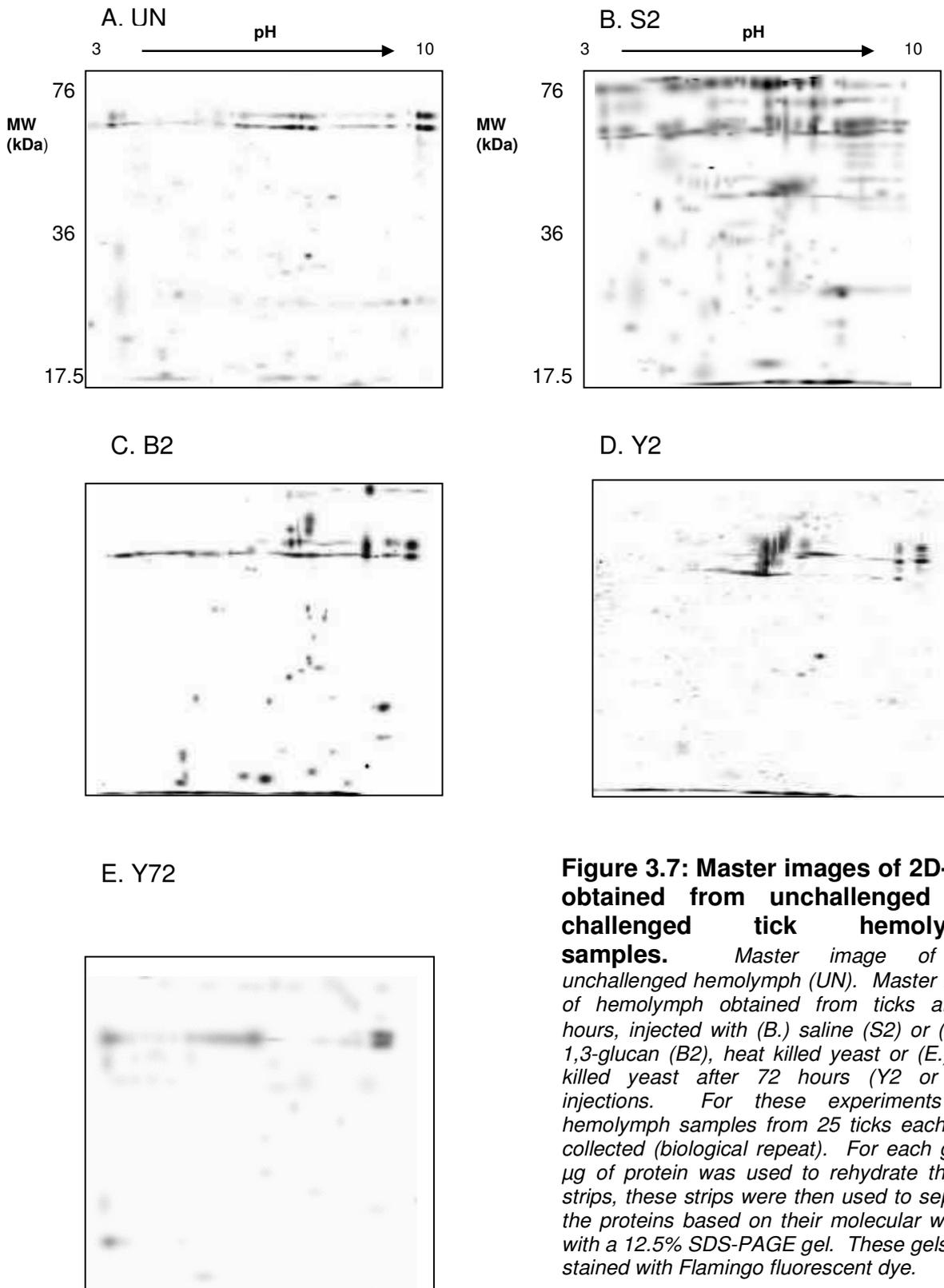


Figure 3.7: Master images of 2D-gels obtained from unchallenged and challenged tick hemolymph samples.

Master image of (A.) unchallenged hemolymph (UN). Master image of hemolymph obtained from ticks after 2 hours, injected with (B.) saline (S2) or (C.) β -1,3-glucan (B2), heat killed yeast or (E.) heat killed yeast after 72 hours (Y2 or Y72) injections. For these experiments four hemolymph samples from 25 ticks each were collected (biological repeat). For each gel 60 μ g of protein was used to rehydrate the IEF strips, these strips were then used to separate the proteins based on their molecular weights with a 12.5% SDS-PAGE gel. These gels were stained with Flamingo fluorescent dye.

All of the gels were analyzed with PDQuest™ software and the spots that were statistically significant ($p < 0.05$) were determined. These spots were indicated as dark spots on the master image (Figure 3.8) and the light spots indicate all of the spots present on the gel.

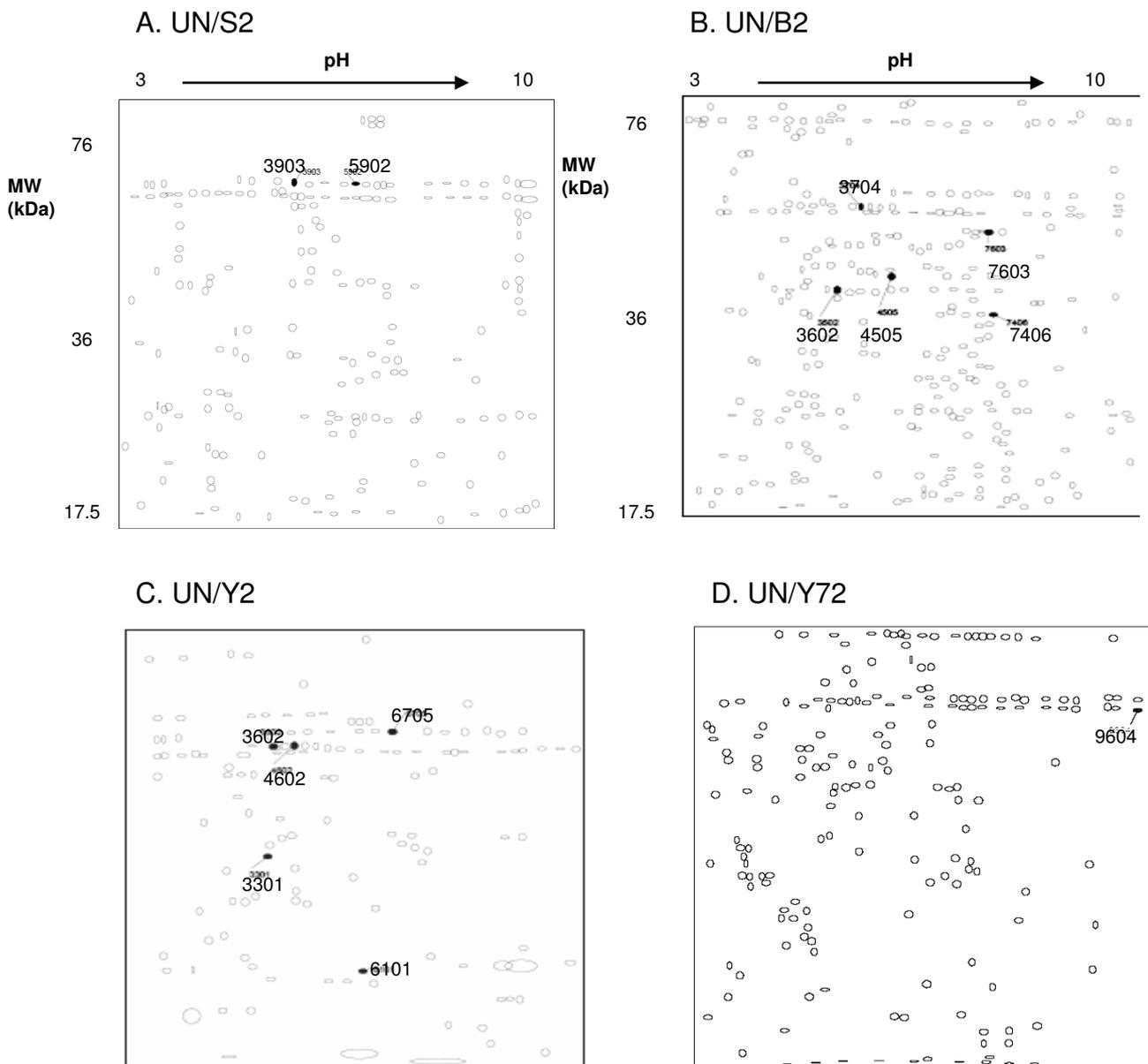


Figure 3.8: Master image showing differentially expressed proteins ($p < 0.05$).

(A.) Master image comparing gels from unchallenged with hemolymph from ticks injected with saline for 2 hours, (B.) b-1,3-glucan for 2 hours, (C.) heat killed yeast for 2 hours and (D.) heat killed yeast for 72 hours. The dark spots indicate the statistically significantly spots and the light circles indicate all the spots present when comparing unchallenged and challenged hemolymph.

When determining the statistically significant spots (Table 3.1), unchallenged hemolymph and hemolymph from saline injected ticks was compared and it was seen that no spots were up-regulated with injury alone, whereas two spots were down-regulated. When comparing unchallenged with β -1,3-glucan induced hemolymph, 5 spots were up-regulated in the β -1,3-glucan induced hemolymph. Challenge with yeast (Y2), resulted in the up-regulation of four spots and the down-regulation of one spot. Only one spot was found to be down-regulated in the hemolymph 72 hours post-challenge with yeast.

Table 3.1: Statistically significant spots when comparing unchallenged with challenged

Unchallenged vs. challenged	Spot number		Approximate Molecular Mass (kDa) values
	Up-regulated	Down-regulated	
Saline 2 hours		3903	54
		5902	54
β -1,3-glucan 2 hours	3502		38
	3704		52
	4505		40
	7406		35
	7603		48
Yeast 2 hours	3301		35
	3602		53
	4602		53
	6101		22
Yeast 72 hours		6705	54
		9604	53

The spots that differ significantly (Student t-test) in the gels was determined by comparing Y2 with UN. These spots were excised and subjected to MS/MS analysis at the University of York (UK). MASCOT software was used to identify and list the proteins in which peptides from the MS/MS spectra matched exactly to sequences in the database (a score higher than 52 indicates identity). The results

that were obtained from the database searches are listed in Table 3.2, showing only four of the matches with the highest ion scores obtained. As can be seen from Table 3.2 these spots could not be positively identified using the MASCOT data, as none of the scores were above 52.

Table 3.2: MASCOT results of protein spots

Spot	Hit	m/z ^a	Score ^b	Protein name ^c	Peptide sequence ^d
3301	1	1794.82	25	Conserved hypothetical protein (<i>Pseudomonas aeruginosa</i>)	R.NHAIADAVLLSGDEDV R.V
	2	1385.73	25	Phytanol-CoA diowygenase (<i>Solibacter usitatus</i> Ellin6076)	M.SNFIPSAEDLHR.V
	3	1147.56	18	Methylase, putative (<i>Chlorobium tepidum</i> TLS)	-MQIHAGRYR.G + Oxidation (M)
	4	1492.72	18	General secretion pathway protein K (<i>Desulfuromonas acetocidans</i> DSM 684)	M.SVFFAKKPLGNER.G
3602	1	1546.72	24	Similar to Cantactin-3 precursor (Brain-derived immunoglobulin superfamily protein 1, BIG-1) Plasmacytoma-associated neuronal glycoprotein) (<i>Monodelphis domestica</i>)	R.EPSNNIFPVGSEEK.K
	2	1115.62	25	Hypothetical protein FBALC1_16342 (<i>Flavobacteriales bacterium</i> ALC-1)	K.HLEPETSFR.S
	3	1234.69	18	Sensor histidine kinase, putative (<i>Oceanibulbus indolifex</i> HEL-45)	R.RDEATRPFSR.L
	4	1475.76	18	Hypothetical protein PFL_4711 (<i>Pseudomonas fluorescens</i> PF-5)	K.ALMLNRMLITGSR.E
4602	1	1122.63	38	To-component sensor histidine kinase (<i>Bacillus cereus</i> B4264)	R.HLEDRLVK.R
	2	1115.60	28	Unnamed protein product (<i>Tetraodon nigroviridis</i>)	R.HLEILGYDR.V
	3	1189.61	28	Polyphosphate kinase (<i>unidentified Eubacterium</i> SCB49)	K.TNYALIEIPR.S
	4	1491.73	28	Hypothetical protein, conserved in <i>Plasmodium</i> species (<i>Plasmodium knowlesi</i> strain H)	K.MYNYLMKLLDR.E + 2 Oxidation (M)
6101	1	1115.62	32	Predicted protein (<i>Aspergillus terreus</i> NIH2624)	R.LHELYPGMR.Q
	2	883.45	29	Thiamine monophosphate kinase (<i>Idiomarina loihiensis</i> L2TR)	R.LHYPTPR.V
	3	910.59	25	Hypothetical protein BPSS0216 (<i>Burkholderia pseudomallei</i> K96243)	R.LLVGAQPGR.A
	4	1011.53	25	Similar to Katanin p60 subunit A-like 1 (<i>Gallus gallus</i>)	R.KESPLQPR.G
6705	1	1575.85	37	Conserved hypothetical protein (<i>Comamonas testosteroni</i> KF-1)	R.DLLVQDVINMMER.H
	2	1147.53	36	Iron permease FTR1 (<i>Exiguobacterium sibiricum</i> 255-15)	R.QTGLAMNLAGR.S + Oxidation (M)
	3	1085.59	30	Similar to hypoxia associated factor (<i>Tribolium castaneum</i>)	R.YTGPIMEFK.E
	4	1492.69	30	Hypothetical protein LOC780085 (<i>Xenopus tropicalis</i>)	K.STMERNLVLEER.S + Oxidation (M)

a. m/z - mass to charge ratio, b. Score – Highest scores obtained with MASCOT (Scores above 51 indicates homology, $p < 0.05$), c. Protein to which the peptide sequence were matched with MASCOT and d. Sequence information obtained for matched proteins from tandem mass spectrometry as determined by searching with MASCOT.

As no significant matches were obtained with MASCOT searching the *de novo* sequences were then determined. PredictSequence software was used to interpret the MS/MS spectra that were obtained and matches were searched for with BLASTP (Table 3.3). The four highest scoring peptide sequences are listed in Table 3.3 for each spot that were analyzed, but no positive matches were obtained. This is due to the fact that only single fragments of our sequence could be matched to sequences that are in databases.

Table 3.3: MS-BLAST results for the five spots

Spot no	Hit	Score ^a	Peptide sequence ^b	Protein name ^c
3301	1	110	451 DTFRPHIWNVA 461	Membrane bound O-acyl transferase, MBOAT (<i>Bacillus cereus</i> subsp.)
	2	108	225 WDMFVQDVTM 234	ENSANGP00000012356 (<i>Anopheles gambiae</i> str. PEST)
	3	106	48 FTWTASPPR 56	putative phosphomethylpyrimidine kinase (<i>Myxococcus xanthus</i>)
	4	90	178 TTWRSSST 184	Microneme antigen (<i>Sarcocystis muris</i>)
3602	1	99	572 YHNYNDNYC 581	Hypothetical protein (<i>Danio rerio</i>)
	2	96	86 YYYDNNYDY 95	Hypothetical protein (<i>Danio rerio</i>)
	3	88	318 SDAEHYND 325	Hypothetical protein PF10_0350 (<i>Plasmodium falciparum</i> 3D7)
	4	88	105 SNWYYS 110	Cell wall binding repeat domain protein (<i>Clostridium perfringens</i>)
4602	1	127	155 EGHLDVVECL 164	Similar to ankyrin 2,3/unc44 (<i>Strongylocentrotus purpuratus</i>)
	2	95	214 AHDIEHAV 221	Hypothetical protein Erum0250 (<i>Ehrlichia ruminantium</i> str)
	3	93	96 EGHMDVEAML 105	Conserved hypothetical protein (<i>Metallosphaera sedula</i>)
	4	53	158 EGHLDVVECL 167	Similar to ankyrin 2,3/(<i>Strongylocentrotus purpuratus</i>)
6101	1	73	287 AGDEQCRRWAT 297	Hypothetical protein [<i>Oryza sativa</i> (japonica cultivar-group)]
	2	69	1 MNYRRTNPTGI 11	Hypothetical protein RB2501_01336 (<i>Robiginitalea biformata</i>)
	3	68	479 NRIGYKRLGSGV 491	ABC-type branched-chain amino acid transport systems periplasmic component-like (<i>Chlorobium phaeobacteroides</i>)
	4	49	216 HELPTSS 222	Mucin (<i>Homo sapiens</i>)
6705	1	112	121 SFVTVIMPYLVTM 134	Hypothetical protein (<i>Mus musculus</i>)
	2	75	45 YTTLMIDIMTTMT 57	Hypothetical protein PC103780.00.0 (<i>Plasmodium chabaudi chabaudi</i>)
	3	48	22 PPCRCSP 28	Similar to CG13731-PA (<i>Homo sapiens</i>)
	4	48	55 SLSYNI 61	Polyubiquitin (<i>Amoeba proteus</i>)

a. Score - HSP as determined by BLAST, using sequences obtained from *de novo* sequencing, matches higher than 64 were significant b. Peptide sequence of the *de novo* data (only sequence with highest value were given), the numbers gives the residues to which the query sequenced matched to the protein sequence in the database and c. Identity of the protein to which the *de novo* sequences were matched

No EST or genomic sequences are available for the soft tick, *O. savignyi*, making the chances to identify our proteins small. As new information becomes available and databases are updated, our proteins may someday be identified

Chapter 4

Yeast binding proteins in the hemolymph of the soft tick

4.1. Introduction

4.1.1. Lectins as pathogen recognition molecules

The most important function of the innate immune system is the recognition of microbial cell components of invading pathogens by PRRs (Medzhitov and Janeway, 2000). PRRs include the PGRPs, β GRP, Toll-like receptors in mammals and the lectins. Recognition leads to the activation of innate immune responses, such as encapsulation, melanisation, phagocytosis, coagulation and the production of AMPs, as described in Chapter 1 (Brennan and Anderson, 2004; Zhu *et al.*, 2005).

Lectins (agglutinins) play an important role as pathogen recognition receptors in mammals (Matsushita *et al.*, 1996). These molecules bind reversibly to specific carbohydrates that are present on microorganisms causing their aggregation (Peumans and van Damme, 1995). Lectins have been studied extensively in invertebrates, especially in the horseshoe crab and scorpions (Olafsen 1986, 1996; Vasta and Marchalonis, 1984). They were found to play a role in cell adhesion, opsonisation, phagocytosis and cytolysis (Vasta and Marchalonis, 1983).

Three major types of lectins have been distinguished by Peumans and van Damme (1995) based on their overall structure: the first class is the merolectins

with a carbohydrate-binding domain, the second class is the hololectins with two or more identical or highly homologous domains and the third class is the chimerlectins with a sugar-binding domain together with an unrelated domain having a different biological activity or binding specificity for another carbohydrate. Lectins have been identified as both soluble and/or membrane-bound proteins consisting of a number of identical subunits that have been modified post-translationally (Drickamer, 1988).

Tick lectins have not received the same level of attention as that of insects and much less are known about their role in the immune system (Grubhoffer *et al.*, 2004). Tick lectins have been found to play an important function in hemagglutination (HA) and opsonisation of microorganisms. The first lectin studies were conducted on hemolymph of the soft ticks *Ornithodoros tartakovskii*, *O. papillipes* and *Argas polonicus* (Grubhoffer *et al.*, 1991). It was observed that these lectins are sialic acid binding proteins and have extended binding affinity for N-acetylamino-D-hexosamines and D-galactose. HA activity has also been reported in the hemolymph, gut and salivary glands of *Rhipicephalus appendiculatus* (Kamwendo *et al.*, 1993).

Midgut HA activity was found in *Ixodes ricinus* and it was partially characterized (Uhlir *et al.*, 1996). This activity was found to be present only in fed ticks and it had an affinity for carbohydrates (N-acetyl-D-galactosamine, N-acetyl-D-glucosamine) and glycoconjugates (especially lipopolysaccharides). A lectin from the hemolymph of *I. ricinus*, with specificity for sialic acid has been characterized

by Kuhn *et al.* (1996). Immunolocalization studies indicated that this lectin is present in hemocytes, midgut and other tissues.

Two lectins were isolated from the gut of *I. ricinus*, by affinity chromatography, using bovine submaxillary mucine (BSM) or laminarin (β -1,3-glucan) as the immobilized ligands (Durnova, 1998). Analysis with SDS-PAGE revealed two proteins, a 65 kDa protein with affinity to BSM and a 37 kDa protein with affinity to laminarin. This 37 kDa protein functions as a β -1,3-glucan binding protein and may play a role in the defence against fungal infections.

Dorin M (a glycoprotein with sialic-acid binding ability) is a lectin that was characterized from the hemolymph plasma of the soft tick, *Ornithodoros moubata* (Kovar *et al.*, 2000). It was purified to homogeneity using affinity chromatography and anion exchange chromatography. In previous studies, it was shown that this lectin has high HA activity (Grubhoffer and Kovar, 1998). Purified Dorin M was found to form aggregates with molecular mass of 640 kDa. Rego *et al.* (2006) cloned and sequenced this plasma lectin and determined that it may play a role in non-self recognition. Immunofluorescent localization studies showed that Dorin M is present in the hemocytes and salivary glands.

Rego *et al.* (2005) cloned two fibrinogen-related proteins, Ixoderin A and B, from the hard tick, *Ixodes ricinus*. Ixoderin A was primarily expressed in the hemocytes and salivary glands, with expression also being present in the midgut. It was also suggested that Ixoderin A could play a role in microbial recognition. Ixoderin B showed tissue specific expression in the salivary glands. Both Ixoderin A and B

showed high homology to the lectin Dorin M of *O. moubata*. These lectin-like sequences from the hard tick contained a fibrinogen-domain which may have a possible role as recognition molecules in the innate immune system of the tick.

1.4.2. Identification of micro-organism binding proteins from hemolymph

In previous studies β GRPs have been isolated by affinity precipitation (Fabrick *et al.*, 2004). Tissue samples were incubated with a β -1,3-glucan-affinity resin (e.g., curdlan or laminarin). After binding, bound proteins were eluted from the resin with a buffer. The eluted proteins were then analyzed with SDS-PAGE.

Zhu *et al.* (2005) developed a novel method to define molecules present in the hemolymph plasma of the horseshoe crab that bind to microorganisms. The assay was first optimized with 'bacterial beads' and they found that urea affected higher extraction efficiency than the acidic or alkaline buffers that were used. Gram-positive bacteria (*Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli*) and a marine fungus (*Kluyveromyces marxianus*) were used to identify molecules that bind to microorganisms. Similar profiles of the plasma proteins were found when the different microbes were used. Various homologs (CL5a, 70 kDa; CL5b, 75 kDa and CrC3, 200 kDa) to vertebrate complement system were found, indicating the presence of a complement system in primitive protostomes. These proteins were found to be involved in the recognition of microorganisms and they play a role in defending against bacterial or fungal infection. Their findings indicated a platform to map pathogen recognition and the subsequent activation of defence mechanisms. The defence mechanisms included phagocytosis and coagulation followed by the clearance of the microbial invaders.

This study is based on the fact that recognition molecules are present in the innate immune system of invertebrates. These molecules are able to bind to specific molecules and initiate a reaction to eliminate foreign molecules. Thus when yeast is incubated with hemolymph, proteins will recognize and bind to the yeast. This can then initiate a reaction that will eliminate the yeast.

4.1.4. Hypothesis

Yeast binding proteins are present in the hemolymph of the tick.

4.1.5. Aims

- To determine the best conditions to elute bound proteins
- To determine whether hemolymph proteins bind to yeast
- To determine whether challenge of ticks leads to the release of additional binding proteins

4.2. Materials and Methods

4.2.1. Materials

All reagents used were of analytical grade and for all experiments double distilled deionised water (Millipore system Q, Millipore, USA) was used. Ticks were collected and handled as described in 2.2.2.

4.2.2. Eluent determination for hemolymph binding assays

The method that was followed was adapted from Zhu *et al.* (2005). *Candida albicans* was grown overnight (1 ml of yeast in 9.5 ml broth) at 30°C in a shaking incubator to obtain a saturated culture with $OD_{600nm} = 2.5$ (approximately 12.5×10^6 CFU). The yeast cells (100 μ l) were added to sterile Eppendorf tubes and washed once with 0.9% NaCl and twice with phosphate-buffered saline (PBS, 0.1 M NaH_2PO_4 , 0.1 M Na_2HPO_4 , 0.15 M NaCl, pH 7.2) by repeated centrifugation at 12 000 x g for 2 minutes each. The pellet was then re-suspended with 25 μ l of PBS (1/4 of original volume).

Unchallenged hemolymph from 100 ticks was collected into tick bleeding buffer [TBB (50 mM sodium hydrogen phosphate (Na_2HPO_4), 0.15 mM sodium chloride (NaCl), 2 mM EDTA, 0.02% sodium azide (NaN_3), 2 mM phenylthiourea (PTU) and 1 complete protease inhibitor cocktail tablet (ROCHE), pH 7.0); (1:1 ratio)]. The hemolymph was centrifuged for 15 minutes at 8200 x g to remove the hemocytes. Hemolymph (100 μ l) and yeast cell suspension (100 μ l) was then incubated with rotation for 10 minutes at 4°C. The yeast cells were pelleted with centrifugation for 2 minutes at 12 000 x g to remove all unbound proteins. As a control, yeast cells were incubated with 100 μ l of TBB. The yeast pellet was then washed twice with

PBS and centrifuged for 2 minutes at 12 000 x g. Different conditions were used to elute the bound proteins. The proteins were eluted with 25 µl of either: 0.1 M citric acid, pH 2; 4 M urea, pH 8; 0.15 M triethylamine, pH 11.5; water; 0.1 M NaCl; 0.5M NaCl or 1 M NaCl. The eluted proteins were centrifuged for 2 minutes at 12 000 x g and the respective supernatants were transferred into sterile eppendorf tubes. The samples were centrifuged for a second time for 2 minutes at 12 000 x g and the supernatants were again transferred into sterile eppendorf tubes. The proteins were precipitated with acetone (1 part protein sample: 4 parts acetone) and the samples were placed at -70°C overnight. The samples were centrifuged for 1 h at 14 000 x g and dried in a rotor-evaporator. The precipitated proteins were re-suspended in 10 µl of 1DE reducing buffer (as described in 3.2.6).

For 1DE-analysis, two additional controls representative of hemolymph plasma and yeast proteins respectively, were prepared. For the hemolymph protein control, 5 µl of hemolymph plasma was added to 5 µl reducing buffer. For the yeast protein control, heat killed yeast was first sonified with a Branson sonifier (Branson Sonic Power Company, USA), 30% duty cycle and output control 6. The disrupted yeast cells were then centrifuged at 8200 x g for 15 minutes and the supernatant was collected into sterile eppendorf tubes. The yeast controls were prepared as follows: the first yeast control was prepared by adding 0.5 µl yeast to 9.5 µl of reducing buffer (YC1) and the second yeast control was prepared by adding 5 µl of yeast to 5 µl of reducing buffer (YC2). All the samples were boiled for 5 minutes at 97°C and 1DE was carried out on a 12.5% gel as described in 3.2.6 and a LMM was added as prepared in 3.2.6. The gels were stained with

Colloidal Coomassie as described in 3.2.8.1 or with silver stain as described in 3.2.8.3.

4.2.3. Yeast binding proteins from hemolymph

4.2.3.1. Challenge of ticks and hemolymph collection

For each sample 50 ticks were each injected with 1 μ l of 0.0054 g/ml β -1,3-glucan or heat killed yeast (as described in 2.2.3). Hemolymph was collected from ticks 2 hours post-challenge into TBB. As a control, hemolymph from 50 ticks that were not challenged in the laboratory was collected into TBB. For each sample 100 μ l TBB was used for every 100 μ l of hemolymph that was collected. The hemolymph samples were centrifuged for 15 minutes at 8200 x g to remove hemocytes and the cell free plasma was transferred into new sterile eppendorf tubes. Samples were stored at -70°C until needed.

4.2.3.2. Analysis of yeast binding proteins

Hemolymph samples prepared from challenged ticks and unchallenged ticks were incubated with yeast cells as described in 4.2.2. In one experiment the bound proteins were eluted with 0.5 M NaCl and then repeated using 0.15 M triethylamine (pH 11.5).

4.2.4. MALDI-quadrupole time-of-flight mass spectrometry (MALDI-QToF MS)

Bands were cut from a one-dimensional gel for MS/MS analysis at the University of York (UK) as described in 3.2.10.

4.3. Results and Discussion

4.3.1. Analysis of yeast binding proteins in hemolymph obtained from unchallenged ticks

In an attempt to define the proteins in the hemolymph plasma of ticks that play a role in binding to yeast cells, live *Candida albicans*, was used as 'affinity beads' to adsorb the yeast-binding proteins from the plasma. Hemolymph plasma obtained from unchallenged ticks was used in this assay and the bound proteins were eluted by using different conditions (water, 0.1, 0.5 and 1 M NaCl). Eluted proteins were analyzed by SDS-PAGE. For each eluent a control (yeast incubated with buffer alone) was added to determine if the proteins that were eluted from the hemolymph plasma, were indeed plasma proteins and not proteins of yeast origin. The results in Figure 4.1 show that two predominant bands (97 and 88 kDa) were present in the proteins extracted with 0.1, 0.5 or 1 M NaCl. This suggests that the interaction between the yeast and proteins is ionic in nature. No corresponding bands were seen in the controls, confirming that these proteins are of hemolymph origin.

Various bands with lower molecular weights were seen in the proteins extracted with water: bands at 60 and 35 kDa were very prominent, but the two bands at 88 and 97 kDa were not present.

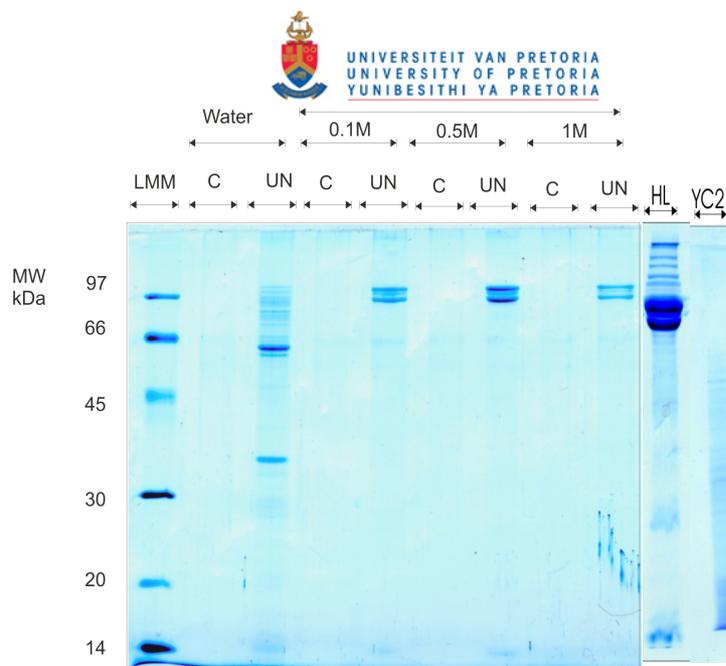


Figure 4.1: 1D-analysis of yeast binding proteins in unchallenged plasma. Yeast cells were incubated with buffer (C) or unchallenged hemolymph plasma (UN). Bound proteins were eluted with water, 0.1, 0.5 or 1 M NaCl. The proteins were precipitated with acetone, 10 μ l of reducing buffer was added to each sample and the proteins were separated on a 12.5% polyacrylamide gel. The gel was stained with colloidal Coomassie. Two separate controls were also included: one representing the hemolymph plasma protein (HL) and yeast proteins (YC2). Low molecular mass markers (LMM) were also loaded into the gel.

4.3.2. Analysis of yeast binding proteins from hemolymph obtained from challenged ticks

This assay was repeated to determine whether different yeast binding proteins will be released into the plasma after challenge when compared to the proteins present in unchallenged hemolymph. Hemolymph was collected 2 hours after ticks were injected with saline, β -1,3-glucan or heat killed yeast. Each hemolymph sample was incubated with live *C. albicans*. The bound proteins were eluted with 0.5 M NaCl. As can be seen in Figure 4.2, the two protein bands (97 kDa and 88 kDa), were present in all hemolymph samples, irrespective of challenge. These bands do not correspond to the bands present in the yeast controls, but they do correspond to the bands in the hemolymph control. These proteins are thus of hemolymph origin. The same results were observed when the experiment was repeated.

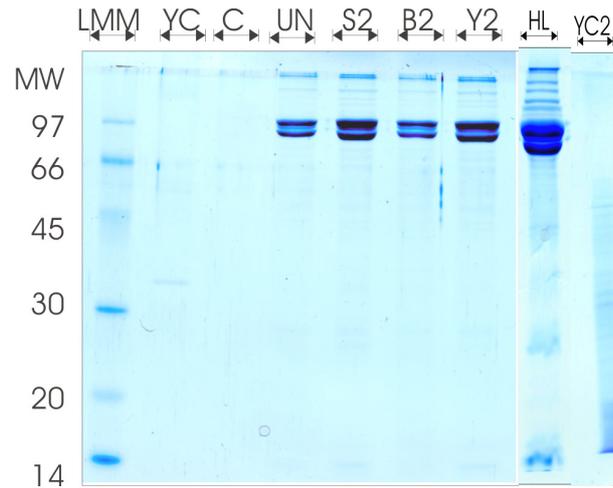


Figure 4.2: 1D-analysis of yeast binding proteins in unchallenged and challenged hemolymph plasma. Yeast cells were incubated with unchallenged (UN) or challenged hemolymph (hemolymph collected 2 hours after ticks were injected with saline (S2), β -1,3-glucan (B2) or heat killed yeast (Y2)). Bound proteins were eluted with 0.5 M NaCl. The released proteins were precipitated with acetone, 10 μ l of reducing buffer was added to each sample and the proteins were analyzed with a 12.5% polyacrylamide gel. Low molecular mass markers (LMM), yeast incubated with buffer (C), hemolymph plasma proteins (HL) and yeast controls (YC1 and YC2) were also run on the gel. The gel was stained with colloidal Coomassie.

4.3.3. Elution of bound proteins using other conditions

As only two bands were seen when 0.5 M NaCl was used to elute hemolymph proteins that bind to yeast, elution was attempted using other conditions. Different eluents with various pH values were employed to extract hemolymph proteins bound to yeast cells. In this experiment yeast cells were incubated with hemolymph obtained from unchallenged ticks. Different eluents were used to release the bound proteins: 0.1 M Citric acid, pH 2; 4 M urea in 10 mM Tris-HCl, pH 8 or 0.15 M triethylamine, pH 11.5. From Figure 4.3 it can be seen that only two bound proteins were eluted with citric acid, whereas more proteins were eluted with urea and triethylamine. These bands were also present in higher concentrations than those eluted with citric acid.

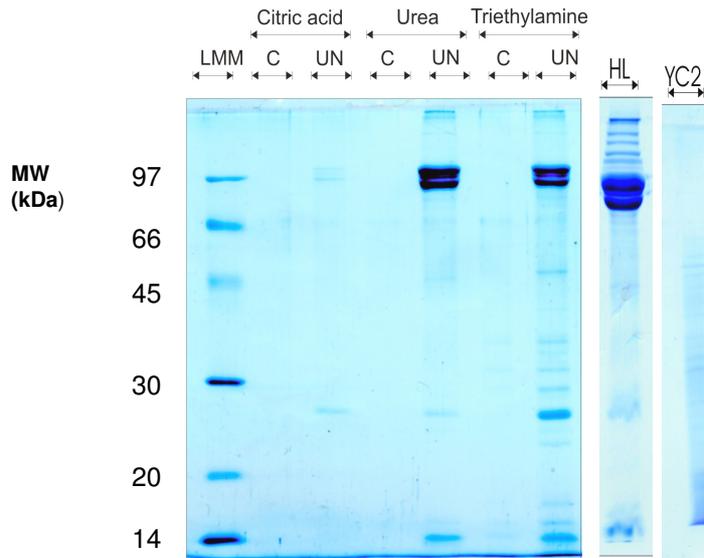


Figure 4.3: 1D-analysis of yeast binding proteins in unchallenged hemolymph under different conditions. Yeast cells were incubated with unchallenged hemolymph (UN) and the bound proteins were eluted with 0.1 M citric acid, pH 2; 4 M urea in 10 mM Tris-HCl, pH 8 or 0.15 M triethylamine, pH 11.5. The eluted proteins were precipitated with acetone and then separated with a 12.5% polyacrylamide gel. The gel was stained with colloidal Coomassie. Low molecular mass markers (LMM) were loaded. Different controls were included: one representing hemolymph plasma (HL) and the other yeast proteins (YC2).

4.3.4. Yeast binding proteins in the hemolymph plasma of challenged ticks eluted with triethylamine

The experiment to identify hemolymph proteins from challenged ticks was repeated using triethylamine as eluent. The gel was first stained with colloidal Coomassie (Figure 4.4A) and then with silver (Figure 4.4B). On the colloidal Coomassie stained gel, 2 bands with high molecular weights as well as faint bands between 20-30 kDa were present. The same gel was then destained and stained with silver stain. More bands were visible above 97 kDa. Two bands were seen at 97 kDa and bands between 20-30 kDa were more prominent.

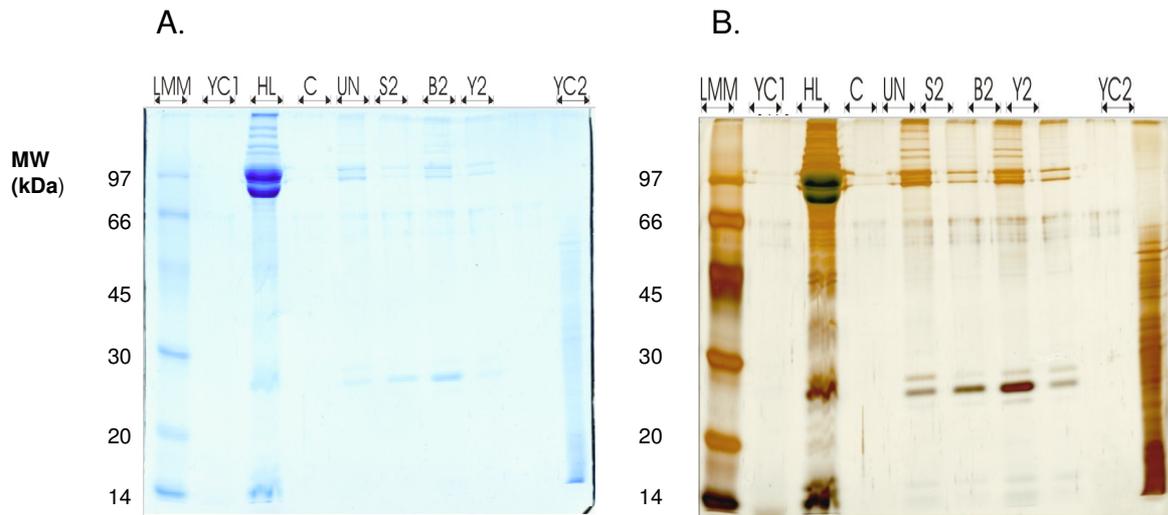


Figure 4.4: 1D-analysis of hemolymph proteins eluted with triethylamine. Yeast cells were incubated with hemolymph and the bound proteins were eluted with triethylamine pH 11.5. The released proteins were precipitated with acetone and then separated on a 12.5% polyacrylamide gel. The gel in (A.) was stained with colloidal Coomassie and the gel in (B.) with silver stained. The gel was loaded with low molecular mass markers (LMM), yeast protein controls (YC1/YC2), total hemolymph protein (HL), yeast incubated with buffer (C), yeast cells incubated with hemolymph collected from ticks that were injected with saline (S2), β -1,3-glucan (B2) or yeast (Y2).

As the prominent bands were seen in all of the hemolymph samples, regardless of challenge the assay was repeated with unchallenged hemolymph only (Figure 4.5). The three most prominent bands (97, 88 and 26 kDa) were excised and subjected to MS/MS analysis.

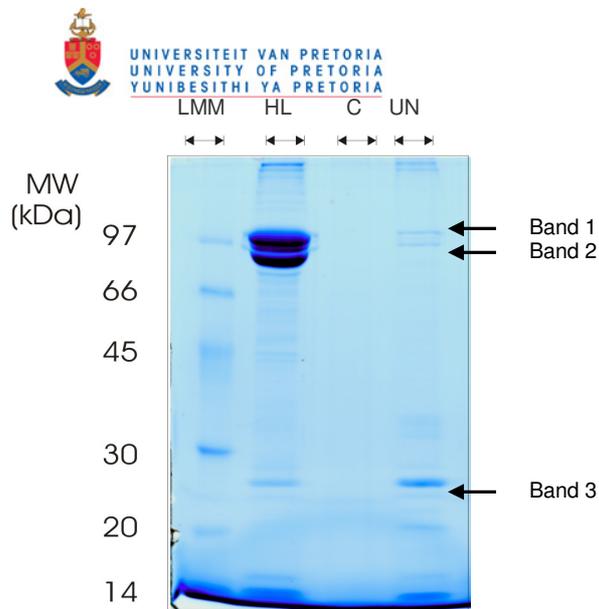


Figure 4.5: Protein bands subjected to MS/MS analysis. *Yeast cells were incubated with unchallenged hemolymph and the bound proteins were eluted with triethylamine, pH 11.5. The eluted proteins were precipitated with acetone and the proteins were separated on a 12.5% polyacrylamide gel. Low molecular mass markers were loaded, together with total hemolymph proteins (HL), yeast cells incubated with buffer (C) and yeast cells incubated with unchallenged hemolymph. The gel was stained with colloidal Coomassie.*

The MS/MS results were first analyzed with a MASCOT database search (Table 4.1). It was found that protein band 1 gave four different hits of which only two hits were significant (score > 51, $p < 0.05$). As the different fragments from protein band 1 did not match the same protein in the database this protein could not be identified. Protein bands 2 and 3 gave four different hits each, but in each case the scores were lower than 51.

Table 4.1: MASCOT results of protein bands

Protein band	Hit	m/z ^a	Score ^b	Protein name ^c	Peptide sequence ^d
1	1	1175.6067	56	Peptidase M48, Ste24p [<i>Burkholderia</i> sp. 383]	R.LPDLMPYAK.A
	2	1175.6067	54	Hypothetical protein Bxe_A4392 [<i>Burkholderia xenovorans</i> LB400]	R.LPDMLPLYAK.A
	3	1048.5978	51	Gamma-butyrobetaine,2-oxoglutarate dioxygenase, putative [uncultured bacterium 578]	R.LVSELVAYR.A
	4	1048.5978	51	Cytochrome P450 monooxygenase [<i>Nocardia farcinica</i> IFM 10152]	R.LVSELVMSR.S
2	1	1010.4865	40	Similar to Katanin p60 subunit A-like 1 [<i>Gallus gallus</i>]	R.KESPLQPR.G
	2	1010.4865	40	vacJ lipoprotein [<i>Methylococcus capsulatus</i> str. Bath]	K.QEGPEAGAPR.T
	3	1010.4865	40	hypothetical protein Bpse1_03001421 [<i>Burkholderia pseudomallei</i> 1655]	K.KADSLPKPR.Y
	4	1121.6078	39	NT02FT1147 [synthetic construct]	R.IHEILTAVVK.A
3	1	930.49	45	Hypothetical protein LOC735239	R.TPTISKER.T
	2	930.49	44	Hypothetical protein Bd2369	K.TPVDSKER.N
	3	930.49	43	Hypothetical protein MGG_13328	R.TPLTSQER.A
	4	902.57	41	Predicted protein	-.MAARLPTK.A

a. m/z - mass to charge ratio, b. Score – Highest scores obtained with MASCOT (Scores above 51 indicates homology, $p < 0.05$), c. Protein to which the peptide sequence were matched with MASCOT and d. Sequence information obtained for matched proteins from tandem mass spectrometry as determined by searching with MASCOT

De novo sequencing was then performed as no matches were obtained from the MASCOT search results. PredictSequence software was used to further interpret the MS/MS spectra that were obtained. These scores must be above a threshold value of 64 (value obtained from a scoring table). The matches that scored above this threshold value are listed in Table 4.2. It was again seen that no matches were found as single fragments of each protein band were matched to different protein sequences in the database.

Table 4.2: MS BLAST results for the protein bands

Band	Hit	Score ^a	Peptide sequence ^b	Protein name ^c
1	1	107 (70	69 DYFVRMTNVKVAFL 82	Unknown
		+37)	120 FLWVY 124	
	2	72	144 KPTFCWNSPFLHP 156	Beta(1,3)galactosyltransferase (<i>Lactobacillus delbrueckii</i>)
	3	70	26 AGTDIPNNIAANMIP 40	N-formylglutamate amidohydrolase (EC3.5.1.68) (<i>Burkholderia Mallei</i>)
	4	69	185 MEYLFWDER 193	Hypothetical protein.//:sptrembl Q7UJE7 Q7UJE7_RHOBA
	5	67	51 ELFWYVNYAR 60	Human mRNA for T-cell receptor alpha-chain HAVP36
	6	67	46 ELFWYVNYAR 55	TCRAV9S1"; product: "T-cell receptor alpha-chain V-region (V-J-C)
2	1	69	42 WRWFLHG 49	Hypothetical protein OSJNBa0004P12.8 (<i>Oryza sativa</i> (japonica cultivar-group))
	2	69	29 WPELFDEY 36	ORF.//:sptrembl O32454 O32454_9ACTO ORF (<i>Actinomadura hibisca</i>)
	3	68	4 YLVFKLLHVL 14	Unknown
	4	65	34 VHKDVEDFE 42	Conserved hypothetical protein (<i>Bacillus cereus</i>)
	5	65	21 VHKDVEDFE 29	Conserved hypothetical protein (<i>Bacillus cereus</i>)
	6	65	42 LVTGHIGFGEVEE 54	NrdI protein.//:sptrembl Q6GBA0 Q6GBA0 Hypothetical protein (<i>Staphylococcus aureus</i>)
	7	64	73 SEVIFDEYYR 82	T32E20.26.//:sptrembl Q9LP94 Q9LP94_ARATH T32E20.26. (<i>Arabidopsis thaliana</i>)
3	1	71	373 AGIEFNTIVGAHIKK 387	Unknown
	2	70	1026 YLAKLGTVEH 1035	Hypothetical protein CBG08584
	3	69	232 IEYTAFFPAV 240	Hypothetical protein CBG07969
	4	67	213 GRLPNTLVATLK 224	Unknown
	5	67	137 TAEADITHR 145	Putative transposase (<i>Mycobacterium fortuitum</i>)
	6	66	243 KELFNTLVGAHLK 255	Unknown

a. Score - HSP as determined by BLAST, using sequences obtained from de novo sequencing, matches higher than 64 were significant b. Peptide sequence of the de novo data, the numbers gives the residues to which the query sequenced matched to the protein sequence in the database and c. Identity of the protein to which the de novo sequences were matched

As no hits were found with the MASCOT or de novo sequencing, the peptide sequences were searched against expressed sequence tags (EST) libraries of hard tick species (Table 4.3): *Amblyoma variegatum*, *Boophilus microplus*, *Ixodus scapularis* and *Rhipicephalus appendiculatis*. These are the only genome

sequences of ticks that have been published up to date. The three protein bands could not be identified with the EST libraries, indicating that these proteins are novel as their sequences are not yet available in any known databases.

Table 4.3: Fragments that were matched to the EST libraries of tick species

Band	Peptide sequence ^a	Score ^b	Tick specie ^c	Protein ^d	
1	DYFVRMTNVKVAFL	64	<i>Ixodes scapularis</i>	TC10487	
		50	<i>Boophilus microplus</i>	TC4696 weakly similar to zinc-finger protein; African clawed frog	
	KPTFCWNSPFLHP	58	<i>Amblyoma variegatum</i>	BM290192 Graf protein; <i>Homo sapiens</i>	
		60	<i>Ixodes scapularis</i>	TC5809	
		62	<i>Boophilus microplus</i>	TC3982	
		50	<i>Rhipicephalus appendiculatus</i>	CD783570	
	AGTDIPNNIAANMIP	52	<i>Ixodes scapularis</i>	G894OP536RA6 weakly similar to UPIQ5T268_DROSE	
		52	<i>Rhipicephalus appendiculatus</i>	CD788566	
	MEYLFWDER	53	<i>Boophilus microplus</i>	CK191074	
	LFWYVNYA	60	<i>Ixodes scapularis</i>	G894P518RH23 weakly similar to PIQ9R167_mouse, zinc finger protein; Mouse	
2	WRWFGLHG	58	<i>Boophilus microplus</i>	TC5652	
		52	<i>Rhipicephalus appendiculatus</i>	TC692 similar to SP Q12931 Human heat shock protein	
	YLVFKLLHVLVLF	54	<i>Ixodes scapularis</i>	TC3942 weakly similar to RF NP_497589.2 32565196 nm_06188Y71H2B.5; <i>C. elegans</i>	
		55	<i>Boophilus microplus</i>	TC2335 similar to GP 1850098 dbj BAA11319.1 D78225 PAP-1; Mouse	
	LVTGHIGFGEVEE	53	<i>Amblyoma variegatum</i>	TC3184 weakly similar to RF NP_192967.1 15234514 NM_117300CYP706A4, heme binding; <i>Arabidopsis thaliana</i>	
		52	<i>Boophilus microplus</i>	TC5750 similar to SP P70354 MSX3_Mouse Homeobox protein MSX-3; Mouse	
		51	<i>Rhipicephalus appendiculatus</i>	TC2236 GP 14592910 gb AAK69655.1 AF343090 HSF-27 protein; Human	
	SEVIFDEYYR	60	<i>Ixodes scapularis</i>	G894P544RE6	
	3	AGIEFNTIVGAHIKK	54	<i>Ixodes scapularis</i>	DN971130 similar to UP Q6B8C&_9ACAR (Q6B8C7) KUN-4
			56	<i>Boophilus microplus</i>	TC4701 similar to GP 1943773 gb AAB52432.1 U97191 nuclear pore complex protein 11; <i>C. elegans</i>
IEYTAFFPAV		53	<i>Ixodes scapularis</i>	G89P58RB11	
		53	<i>Boophilus microplus</i>	CK185854	
GRLPNTLVATLK		55	<i>Ixodes scapularis</i>	TC6086	
KELFNTLVGAHLK		53	<i>Ixodes scapularis</i>	G894P530RK9 weakly similar to UP Q76KT1_NEIME gamma-glutamyl transpeptidase	
		54	<i>Rhipicephalus appendiculatus</i>	CD782012 similar to GP 10039541 gb AAG12172.1 AF074968_1 AF074968 p47ING3 protein; Human	

a. Peptide sequence as determined by de novo sequencing, b. Score - HSPs as determined by tBLASTn, matches higher than 51 were listed, c. Tick species to which the de novo sequences were matched, as published in the NCBI database and d. Genbank association number and protein name as identified by BLAST, searching the NCBI databases

As can be seen from the tables above these three protein bands could not be positively identified, as they do not match to any known proteins that are present in the databases. Due to the limited number of available genome sequences that are available of ticks, it is very difficult to identify these proteins.

Chapter 5

Concluding discussion

Compared to other invertebrates little is known about the recognition and signaling pathways that play a role in the innate immune system of the tick. Research to date has mostly focused on the antimicrobial peptides (AMPs) of ticks. These peptides were found to have mostly anti-Gram positive activity and only a few were found to also have anti-Gram negative or anti-fungal activity (Fogaça *et al.*, 1999, 2004; Takeuchi *et al.*, 2004; Yu *et al.*, 2005; Liu *et al.*, 2007). To date no information is available on the antifungal response of soft ticks.

As the resistance to antibiotics and antifungal agents is increasing, the search for novel antimicrobial agents is urgently under way (Marshall and Arenas, 2003). AMPs, expressed in many invertebrate, vertebrate and bacterial species, provide a new strategy to fight microbial infections. These molecules include cationic peptides, anionic peptides, aromatic dipeptides, etc. AMPs could be used in the development of antifungal drugs to treat fungal infections. Proteins that are involved in the innate immune system play an important role in tick survival after a fungal infection. These proteins can potentially be used as antigens in vaccine development to control ticks.

In our first set of investigations a micro-broth dilution assay (Barchiesi *et al.*, 1994) was used to determine whether antifungal activity was present in the hemolymph, midgut and salivary glands. This assay proved to be unsuccessful as inconsistent results were obtained.

A new assay (Masoko *et al.*, 2007) was then used in which the antifungal activity of various tick tissue extracts were determined. Iodonitrotetrazolium chloride (INT) was used as an indicator of yeast growth. It was found that the midguts inhibited yeast growth the most, followed by the salivary glands and then hemolymph. Antifungal activity was seen in the tissue extracts from ticks that were not challenged in the laboratory, as well as in the tissue extracts collected from ticks 2 hours after injection with yeast. The decrease in inhibition that was seen with the different tick tissue extracts, when inducing ticks with *C. albicans* could be due to the fact that older ticks were used for this assay. The previous assay was done with freshly collected ticks and these ticks could have been exposed to microorganisms, thereby increasing the inhibition that was seen when using unchallenged ticks. When these ticks were kept in the laboratory for a month challenge with these microorganisms was found to be decreased and by challenging them with *C. albicans* for 2 hours the inhibition was not increased above that of the unchallenged tick tissue extracts. Another factor that could have influenced the results obtained is that only crude extracts were used to test the antifungal activity of the different tick tissues. This means that the antifungal component that kills or recognized fungi in the different tissue extracts is not constant even though the same protein concentration was used in each assay.

Various defense mechanisms play a role in limiting the spread of microorganisms which is ingested together with a blood meal (Munderloh and Kurtti, 1995). The midgut and salivary glands are the first to come into contact with pathogenic microorganisms and for this reason high antimicrobial activity is expected in these tissues. As the midgut is the organ where the blood meal is stored it is vulnerable

to microbial infection (Nakajima *et al.*, 2002). For this reason high antimicrobial activity is expected in this tick tissue. Foreign microbes can also enter the tick through an accidental puncture of the integument. It has been found that upon blood feeding the hemocyte population is increased and fights off microbial infection. These tick tissues play an important role in the expression and secretion of molecules (e.g., antimicrobial peptides) that limit microbial infection (Gillespie *et al.*, 1997).

In future studies other time points should be investigated to determine whether the antifungal response will be affected. These molecules with antifungal activity can then be isolated and their activity can also be tested against Gram-positive and Gram-negative bacteria. The active compounds can then be used in the development of antifungal drugs. Preliminary investigations suggested that of the three tissue extracts, the midguts are the best starting material for future isolation of an antifungal agent from ticks. Our results also seem to indicate that no prior microbial challenge will be required to enhance the antifungal activity present in this tissue extract.

Proteins in the hemolymph of *Drosophila melanogaster* have been studied extensively, using proteomics. The information that has been obtained during various studies has led to the construction of a 2D-map of hemolymph proteins (de Morais Guedes *et al.*, 2003; Vierstraete *et al.*, 2003). This map serves as a reference database for researchers investigating changes that occur at protein level, during different developmental stages, physiological conditions or after infection (Levy *et al.*, 2003; Vierstraete *et al.*, 2004).

Hemolymph plasma collected 2 hours after ticks were injected with fungi was investigated, these proteins include those that have been released from tissues of the innate immune system e.g., fat body and hemocytes. These proteins play an important role in the immune system after a fungal infection. Proteomics is a very useful tool to use in the analysis of proteins that are differentially expressed upon challenge and may play a role in the innate immune response. As one-dimensional electrophoresis is a quick, easy and relatively inexpensive method to use in the analysis of a protein mixture, it was the first method that was employed in this study. This technique was used to determine whether reproducible results could be obtained when using the same hemolymph sample. The gels that were stained with colloidal Coomassie proved to be more reproducible, but the lower abundance proteins could not be visualized on the gel. Even though silver stain is a more sensitive stain it was difficult to obtain reproducible gels. 1DE was also used to separate proteins from unchallenged hemolymph, as well as hemolymph from ticks that were induced with saline, β -1,3-glucan and yeast induced ticks (2 hours). All of these samples were run on a single gel in order to compare the differences that are present after an injury response (S2) or a response to a fungal cell wall determinant (B2) and a response to yeast (Y2). When the gels from the 1DE were analyzed it was difficult to determine which bands were up- or down-regulated in any of the samples. The gels were first stained with colloidal Coomassie and it was seen that not all of the bands were clearly visible as some of the protein bands were only present in low concentrations when compared to the bands with higher molecular weights. The gels were then stained with silver stain, which cannot be used for quantitative analysis and it could not be

determined which bands were up- or down-regulated. This made it difficult to determine whether proteins are differentially expressed after challenge with fungi.

High resolution can be obtained when separating a complex mixture of proteins with 2DE. A limitation of 2DE is that the proteins that were separated on the 2D-gels ranged from 17.5-76 kDa and not all of the proteins involved in the immune response could be seen on these gels. This technique was used to determine which spots are up/down regulated after ticks were injected with either saline, β -1,3-glucan or yeast. Better results were obtained when a sample containing hemolymph from 25 ticks were used, as the biological variation was decreased.

When comparing the gels from UN and S2 and looking at spots that are statistically significant, it was seen that two spots were down-regulated in the S2 gels. Comparing the gels from UN and B2 it was seen that 5 spots were up regulated in B2 gels. In the gels from UN and Y2 it was seen that 4 spots were up-regulated in the Y2 and one spot was down-regulated. To determine whether a fungal challenge of 72 hours would enhance the immune response of the tick, these experiments were repeated using hemolymph from ticks that were challenged with inactivated yeast. The spots that differed significantly were also determined and it was found that 1 spot was down-regulated in Y72. In all of these experiments it was found that different proteins play a role in the response to injury or when the ticks were challenged with a fungal cell wall determinant or intact fungi. It seems that each of these different challenges induced a different set of proteins that may play a role in the immune response of the tick. Most of these proteins had high molecular weights (higher than 31 kDa) and were acidic

(pI ranging from 5.5-6.6). Due to budget constraints only the differentially expressed protein spots that were statistically significant in the Y2 gels were analyzed with MS/MS, but these spots could not be identified. As no EST database or genomic information is available for the soft tick, *O. savignyi* the chances to identify our proteins were small. This problem was also encountered by Ranchinsky *et al.* (2007) as they could only identify 19 of their 43 differentially expressed proteins. They investigated the proteins that were differentially expressed in ovarian tissues collected from *Babesia bovis*-infected and uninfected southern cattle ticks (*B. microplus*).

In future studies hemolymph samples from ticks that were challenged for 24 and 48 hours should also be analyzed with 2DE, to determine which induction time would give the optimal response when ticks are challenged with fungi. As this technique has a few limiting factors: it is labor intensive, expensive and the availability of ticks together with their cost, one time point was chosen to investigate the antifungal response of the tick against fungi. The proteins that were to be differentially expressed when the ticks were injured or challenged with fungi can play an important role in the immune response of ticks to fight of a fungal infection. Further investigation into these proteins are required as they can be used to study the immune response of the tick, as not enough studies have been done on this subject. These proteins can also be used as vaccine targets to limit tick populations and novel antifungal drugs could also be developed.

The most important function of the innate immune system is the recognition of invading microorganisms. In *Drosophila* pattern recognition molecules with the

ability to recognize fungi have been characterized. These molecules play a role in fighting a fungal infection by recognizing the fungi and also activating down-stream pathways that are able to eliminate the intruders. A few fungi recognizing molecules; known as lectins have been isolated from ticks. They have been found to play an active role in the cellular and humoral immune recognition mechanism. Dorin M is a fibrinogen-related lectin indicating its function as a non-self recognizing molecule and may also function in pathogen transmission by this vector (Kovar *et al.*, 2000; Rego *et al.*, 2006).

An assay that uses *C. albicans* as affinity beads was developed, to identify proteins from the hemolymph that might play a role in the recognition/elimination of fungi. The hemolymph binding assay was first done with 0.5 M NaCl as elution buffer, to compare the results obtained with experiments done with Gram negative bacteria. The bands that play a role in bacteria recognition was found to have molecular weights that are higher than 97 kDa (personal communication, Paul Cheng). The hemolymph binding assay was then repeated with triethylamine as elution buffer, as bands with lower molecular weights were also seen on these gels and not when 0.5 M NaCl was used as an elution buffer. The three most prominent bands were sent for MS/MS analysis, as they were only present in the binding assay experiments and not in the yeast controls. The peptide mass fingerprints that were produced were subjected to database searching with MASCOT. Because of the inconclusive results that were obtained with the MASCOT search de novo sequencing was also done and the sequences that were obtained were searched for matches in the databases with BLASTP software. Only single fragments of the proteins were matched to fragments of other known

proteins and our proteins could not be positively identified. Thus these proteins are novel and they might play a role in the recognition or elimination of yeast from the hemolymph of the tick, protecting ticks from a fungal infection. These fragments were also subjected to a search of the EST libraries of hard ticks and the scores that were obtained were again relatively low. This could be due to the differences that exist between the soft and hard ticks. Due to budget constraints the other protein bands could not be further analyzed, this is also the reason why N-terminal sequencing was not performed.

Another approach that could have been followed in isolating yeast binding proteins is affinity chromatography. This technique uses laminarin as a matrix and proteins with β -1,3-glucan affinity are retained by the matrix. The bound proteins are then eluted from the matrix and separated with SDS-PAGE. These proteins can then be visualized, isolated and analyzed further.

As databases are constantly updated and new information becomes available, matches to our unknown proteins may someday be found. Their conserved regions can then be used for primer design, by which the gene can be cloned and the sequence of the gene could be determined. The N-terminal sequence as well as the *de novo* sequence can be used in primer design. This information can then be used in determining the amino acid sequence, the isoelectric point and the molecular mass of the protein. Studies can also be done in determining whether this is a secreted or non-secreted protein and whether it is glycosylated. Bioinformatics can then be used to do structural modelling, by which the three dimensional structure of the protein could be determined. Further studies could

include expression of the proteins by which the mode of action could be determined. Antibodies could be produced and immunolocalization studies can be done. To evaluate the immuno-protective role of this protein, RNAi studies can also be employed.

Summary

Ticks are blood feeding ectoparasites that ingest large volumes of vertebrate blood. They are the most important arthropods that are capable of transmitting pathogens which cause disease in humans and domestic animals. Ticks are exposed to various microorganisms during feeding as well as in their habitat. They therefore must have a very good immune system to recognize and destroy these microorganisms.

In the present study a micro-broth dilution assay was used to determine whether antifungal activity was present in different tick tissue extracts with or without challenge. The midguts gave the highest inhibition of yeast growth, followed by the salivary glands and then the hemolymph. This was seen with unchallenged tick tissue extracts, as well as tissue extracts collected after yeast challenge (2 hours). Thus all of the tick tissue extracts that was analyzed in this study had antifungal activity.

Proteomics was used to determine whether proteins were differentially expressed in the hemolymph plasma, after a fungal challenge. 2DE was used since proteins are not only separated by molecular mass, but also by their charge. The proteins that were separated on the 2D-gels ranged between 17.5-76 kDa and not all proteins present on the 1D-gels (14-97 kDa) could be seen on the 2D-gels. Ticks were challenged for 2 hours to define the proteins that play a role in the short term innate immune response during a fungal infection. Various proteins were differentially expressed in the hemolymph samples that were collected 2 hours after ticks were injected with saline, β -1,3-glucan or yeast (or 72 hours). Injury and

fungal challenge play a role in producing proteins that might play a role in the fungal response of the tick. Five spots that were statistically significant in the hemolymph collected 2 hours after ticks were injected with yeast cells were analyzed with MS/MS. No matches were found with MASCOT database searching or with EST searching. This can be due to the limited information that is available on the soft ticks, as only hard tick ESTs have been published.

It was also attempted to identify hemolymph proteins that might play a role in the recognition of fungi. Hemolymph was incubated with live *Candida albicans* cells and eluted with buffer. Three protein bands (97, 88 and 26 kDa) were found to be present whether ticks were challenged or unchallenged. These proteins were subjected to MS/MS analysis and database searching was performed revealing no matches to other known proteins.

The antifungal response was found to be present in the soft tick *O. savignyi* and might play a vital function in the innate immune response during a fungal infection. These proteins may serve as lead molecules that could be used in the development of novel antifungal drugs, as well as in vaccine development.

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