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Biochemical and molecular characterization of putative immunoprotective molecules of the soft tick, *Ornithodoros savignyi* Audouin (1827)

By

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Abbreviations

A	
A5C	Actin 5C
AA	Arachidonic acid
ACN	Acetonitrile
AEBSF	4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride
AMP	Antimicrobial peptide
APS	Ammonium persulphate
ATCC	American type culture collection
α2M	α2-Macroglobulin
B	
BAB	Biogenic amine-binding
BLAST	Basic Local Alignment Search Tool
BLASTP	Protein-protein BLAST
C	
C	Control
CAPS	3´-cyclohexylamino-1-propanesulfonic acid
cB	Bacterial protein control
CDD	Conserved domain database
cHL	Total hemolymph plasma protein
CID-MS	Collision-induced dissociation mass spectrometry
D	
Da	Dalton
DAE	*Dermacentor andersoni* embryonic cell line
$dddH_2O$	Double distilled deionized water
DEPC	Diethylpyrocarbonate
DGNBP	*Drosophila* Gram-negative binding protein
DTE	Dithioerythritol
DTT	1,4 Dithiothreitol
E	
E-64	N-(trans-epoxysuccinyl)-L-leucine-4-guanidinobutylamide
EDTA	Ethylenediaminetetra-acetic acid
EST	Expressed sequence tag
F	
FB	Fat body
G	
GNBP	Gram-negative binding protein
GST	Glutathione S-transferase
H	
HBP	Histamine binding protein
HC	Hemocyte
HL	Hemolymph
HMM	High molecular mass protein
HPLC	High performance liquid chromatography
HSP	High score pairing

I	
IAA	**Iodoacetamide**
IDE 12	***Ixodes scapularis* embryonic cell line 12**
IMD	**Immunodeficiency pathway**
IPTG	**Isopropyl β-D-1-thiogalactopyranoside**
Ir-LBP	***Ixodes ricinus* lipocalin leukotriene B4 protein**
L	
L	**Transmembrane**
LB	**Luria-Bertani broth**
LPS	**Lipopolysaccharide**
LBP	**LPS binding protein**
LIR6	**Lipocalin of *I. ricinus***
LMM	**Low molecular mass marker**
LTB_4	**Leukotriene B4**
LTC_4	**Leukotriene C4**
M	
MALDI-MS	**Matrix assisted laser desorption ionization mass spectrometry**
MG	**Midgut**
MS	**Mass spectrometry**
MS/MS	**Mass spectrometry/ Mass spectrometry**
N	
NaCl	**Sodium chloride**
NAG	**N-acetylglucosamine**
NAID	**National institute of allergy and infectious diseases**
NAM	**N-acetylmuramic acid**
NaN_3	**Sodium azide**
N/D	**Not determined**
NEG	**Negative control**
NF-κB	**Nuclear factor kappa-light chain enhancer for B cells**
NIH	**National institute of health**
NJ	**Neighbour joining**
O	
OD	**Optical density**
OMCI	***Ornithodoros moubata* complement inhibitor**
P	
PAGE	**Polyacrylamide gel electrophoresis**
PAMPs	**Pathogen associated molecular patterns**
PBS	**Phosphate buffered saline**
PDB	**Protein databank**
PGBP	**Peptidoglycan recognition protein**
PGRP	**Peptidoglycan recognition receptor**
PGN	**Peptidoglycan**
PMM	**Peptide mass marker**
PO	**Phenol oxidase**
proPO	**Prophenoloxidase**
PRRs	**Pathogen recognition receptors**
PSI-BLAST	**Position-Specific Iterated BLAST**

PTU	**Phenyl thiocarbamide or Phenyl thiourea**
PVDF	**Polyvinylidene fluoride**
	R
RMSD	**Root mean square deviation**
ROS	**Reactive oxygen species**
RP-HPLC or RPHPLC	**Reversed phase- High performance liquid chromatography**
RT-PCR	**Reverse transcription polymerase chain reaction**
	S
S	**Secreted**
SDS-PAGE	**Sodium dodecyl sulfate polyacrylamide gel electrophoresis**
SERPIN	**Serine proteinase inhibitor**
SG	**Salivary gland**
SGE	**Salivary gland extract**
SHBP	**Serotonin and histamine binding protein**
	T
TAF	**Tick actin fragment**
TAM	**Tick alpha-macroglobulin**
TBB	**Tick bleeding buffer**
TBLASTN	**Search translated nucleotide database using a translated nucleotide query**
TEMED	**N,N,N',N'-Tetramethylethylenediamine**
TFA	**Trifluoroacetic acid**
TOF	**Time-of-flight mass spectrometer**
TPL	***Tachypleus* lectin**
Tris-HCl	**Tris (hydroxymethyl)aminomethane Hydrochloride**
TSGP	**Tick salivary gland protein**
TXA_2	**Thromboxane A2**
	U
UN	**Unchallenged ticks**
U/mg	**Unit per milligram**
UTR	**Untranslated region**
	X
X-GAL	**Bromo-chloro-indolyl-galactopyranoside**

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Summary

Most studies on innate immunity in ticks have focused on the antimicrobial peptides from hemolymph, such as defensins and lysozyme, while less is known about bacterial recognition molecules, or antimicrobial mechanisms in other tissues. The current study attempted to identify novel antimicrobial mechanisms, with a focus on bacterial recognition by hemolymph proteins and antimicrobial activity in salivary gland extracts.

Using bacteria as affinity beads, two high molecular mass molecules (Protein X and Protein Y) have been identified in tick hemolymph. These proteins are thought to interact with the bacterial surface via ionic interactions. Tandem mass spectrometry analysis followed by *de novo* sequencing indicated that these proteins are novel as no homologs could be identified from sequence databases.

In an attempt to clone Protein X, using a degenerate primer obtained from a *de novo* sequence, an unrelated hemocyte protein was identified. This protein, named savicalin, was shown to belong to the lipocalin family based on bioinformatical analysis. Transcriptional profiling indicated that savicalin is found in hemocytes, midgut and ovaries, but not in the salivary glands. To date, this is the first tick lipocalin not derived from salivary glands. Interestingly, up-regulation of its mRNA transcript in response to bacterial challenge suggests that this protein could be involved in antimicrobial activity. Up-regulation after feeding also suggests a role in the post-feeding development of the tick.

Two different approaches were used to purify the Gram-positive antibacterial activity from salivary gland extracts. The first attempt entailed a two-step separation approach. Tricine SDS-PAGE of the active fraction showed 3 components (~20, ~10 and ~7 kDa). BLAST searches using the N-terminal sequences of the latter proteins identified the ~20 kDa protein as savignin, while the other two proteins could not be matched. The second strategy included an

ultrafiltration step (10 kDa cut-off) and MS-analysis of the active fraction in this case indicated the presence of various components with molecular masses ranging from 0.99 – 7.182 kDa, with 12 predominant components ranging from 0.99 - 4.448 kDa. Further tandem mass spectrometry analysis of the active fraction revealed the presence of three tick actin-derived fragments. This is of interest as actin fragments have been implicated in innate immunity of other invertebrates. In this study, synthetic peptides corresponding to one of the detected tick actin fragments as well as actin5C (detected in *Drosophila* hemolymph) were found not to inhibit the growth of *Bacillus subtilis* when tested up to a concentration of 100 µg/ml.

It is envisaged that future studies of immunoprotective molecules of the tick, *O. savignyi*, may contribute to the development of novel anti-infective agents and potential targets for anti-tick vaccine design.

Chapter 1: Introduction

1.1 Problem statement

Ticks are obligate blood-feeding ecto-parasites that transmit pathogenic organisms such as viruses, bacteria and protozoa. They parasitize mammals, birds, amphibians and occasionally reptiles (Sonenshine, 1991). Tick-borne diseases greatly impact both human and animal health (Estrada-Peña & Jongejan 1999; Parola & Raoult, 2001).There are three distinct tick families, namely, the soft ticks (*Argasidae*), hard ticks (*Ixodidae*) and the *Nuttalliellidae*, containing only one species (Hill & Wikel, 2005; Sonenshine, 1991).

Much research has focused on control methods that include the use of acaricides and the development of anti-tick vaccines (de Castro & Newson, 1993; Sauer *et al.,* 1995; Vargas *et al.,* 2010; Willadsen, 2001). Both strategies are costly and suffer either from the emergence of acaricide resistance or ineffective cross-protection between different tick strains and species. Therefore, alternative methods to control ticks are needed (Sugumar *et al.,* 2010).

Recently, more investigations into tick immunity have been conducted and the regulation thereof could provide new insights into tick control that could be used for both medical and agricultural purposes (Nijhof *et al.,* 2010; Prudencio *et al.,* 2010; Sugumar *et al.,* 2010). These studies may also lead to the discovery of novel antibiotics (Steen *et al.,* 2006). Currently the widespread use of antibiotics has promoted the emergence of resistant bacteria. In the case of agricultural use this may lead to the spread of resistant bacterial strains to human populations (WHO 2002; Goossens *et al.,* 2005; Mathew *et al.,* 2007). The resistance problem demands that a renewed effort be made to seek antibacterial agents that are effective against resistant pathogenic bacteria. In this regard, ticks may provide a valuable source of antimicrobial agents that could be further developed for therapy. Furthermore, the relationship between tick immune responses and micro-organisms can be explored to limit disease transmission by ticks (Johns *et*

al., 2000, 2001a; Nakajima *et al.,* 2001, 2002, 2003a, b, c; Sonenshine & Hynes, 2008).

1.2 *Ornithodoros savignyi* as a model organism for this study

Ornithodoros savignyi is a soft tick that occurs throughout the North-western regions of Southern Africa. This tick is also known as "the eyed sand tampan". During feeding the uniform integument which folds in on itself allows the tick to obtain a large quantity of blood in a short period of time (Mans *et al.,* 2002). When these ticks feed they secrete toxic substances which can be lethal to young animals (Neitz *et al.,* 1969; Howell *et al.,* 1975; Mans *et al.,* 2001, 2002).

These ticks exhibit a very selective and low incidence of pathogen transmission. They have however, been proven to be responsible for the transmission of pathogens such as Alkhurma hemorrhagic fever virus and *Borrelia* (Shanbaky & Helmy, 2000; Charrel *et al.,* 2007). *Ornithodoros moubata,* a close relative to *O. savignyi,* is able to transmit *B. duttoni* which cause relapsing fever.

Little is known about the innate immune defense mechanisms of *O. savignyi*. In a previous study, Olivier (2002) purified a Gram-positive antimicrobial peptide (AMP) from the hemolymph of *O. savignyi*, and the N-terminal sequence was obtained. The project was continued by an Honours student who identified 2 defensin isoforms using degenerate primers based on the findings of Olivier (2002).

Recently, Stopforth *et al.* (2010) used a proteomics approach to identify proteins secreted into the hemolymph of these ticks 2 hours after immune-challenge with the yeast, *Candida albicans*. Profiling of the proteins present in hemolymph of unchallenged versus challenged ticks using heat-killed yeast revealed five differentially expressed proteins. The modulated protein spots were subjected to tandem mass spectrometry analysis (MS/MS), but could not be positively

identified. These proteins could be involved in the tick immune response as they were not induced following aseptic injury.

1.3 Invertebrate innate immunity

To accomplish the requirements necessary for survival in nature, arthropods must be aware of their surroundings and respond accordingly. Since they do not have an adaptive immune system like mammals, they utilize defense mechanisms similar to the innate immune system of vertebrates to protect themselves from pathogens (Iwanaga & Lee, 2005; Jiggins & Kim, 2005). The key questions of innate immunity are firstly how can pathogenic organisms be detected by arthropods and secondly what types of defense strategies against different types of infection are involved. Much work has been done to identify the sensing molecules and downstream components involved in the immunity of the fruit fly, *Drosophila melanogaster* (Royet, 2004; Kim & Kim, 2005).

Two general responses against the invasion of micro-organisms exist within arthropods. These include cellular and humoral responses. Cellular responses are generally mounted in the arthropod hemolymph by hemocytes. Recognition of micro-organisms by hemocytes generally occurs via specific arthropod-derived proteins that bind to the microbial surface (Lee *et al.,* 1996; Kim *et al.,* 2000; Janeway & Medzhitov, 2002). Humoral responses are generally mediated by peptides such as defensins and lysozyme.

In *Drosophila,* pathogens sensed by specific receptors initiate signals via the Toll and/or IMD (immune deficiency) signaling pathways. These events often lead to transcription of genes involved in the production of AMPs as well as trigger responses such as the production of reactive oxygen species (ROS), melanization, phagocytosis, nodulation, encapsulation and iron sequestration (Fig 1.1).

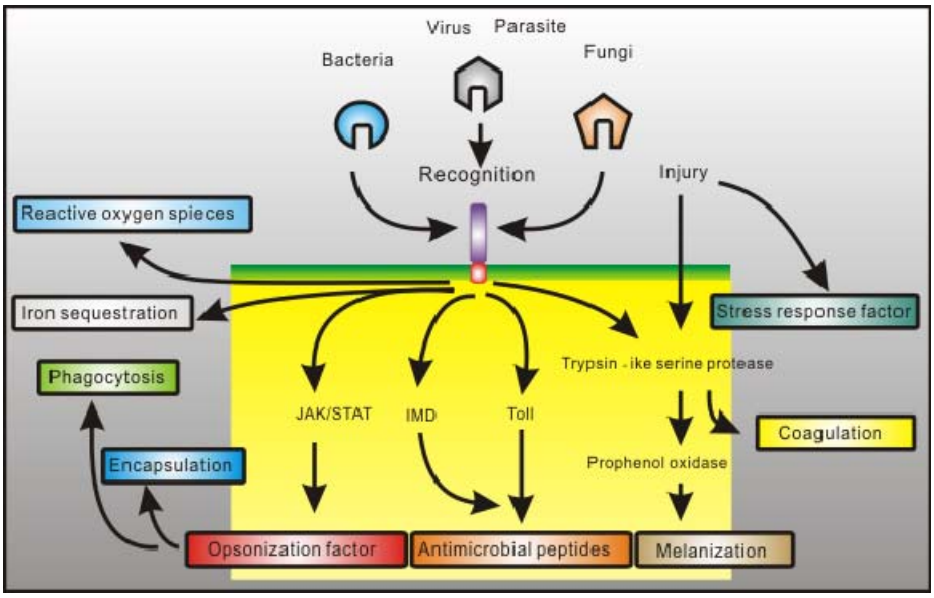

Figure 1.1 The intracellular and extracellular events taking place in arthropods after injury or pathogen recognition by cells.

1.3.1 Discrimination between self and non-self

The innate immune system in both vertebrates and non-vertebrates is able to identify micro-organisms as foreign by pattern recognition receptors (PRRs) of the host (Royet, 2004). The non-self molecules recognized by PRRs are generally present on the surface of microbes and are known as pathogen associated molecular patterns (PAMPs). Their recognition by PRRs activates the Toll-like and/or IMD pathways of host cells, especially in phagocytic hemocytes and the digestive cells lining the lumen of the midgut (Brennan & Anderson, 2004).

In arthropods such as *Drosophila*, PRRs which have been implicated in pathogen recognition include the peptidoglycan binding protein (PGBP) for Gram-positive bacteria (Kang *et al.,* 1998) and the Gram negative-binding protein (GNBP), which recognizes lipopolysaccharide (LPS) and β-1,3-glucan (Lee *et al.,* 1996; Kim *et al.,* 2000). PRRs differ in specificity due to the fact that different pathogenic organisms exhibit different PAMPs on their surface. In addition, a LPS binding protein (LBP) has been isolated from hemolymph of the American

cockroach *Periplaneta americana* (Jomori *et al.,* 1990). Lectins in turn recognize pathogen-specific carbohydrate moieties on the pathogen surface, thereby facilitating opsonization, phagocytosis and cytolysis (Janeway & Medzhitov, 2002).

The first peptidoglycan receptor protein (PGRP) was discovered in the silkworm, *Bombyx mori* (Yoshida *et al.,* 1996). Infection-induced proteins that bind to peptidoglycan (PGN), trigger the proteolytic melanization cascade. In *Drosophila* approximately 13 PGRPs have been identified which share a 160 amino acid PGN recognition domain. They occur as secreted (S) and transmembrane (L) forms (Kang *et al.,* 1998; Michel *et al.,* 2001; Werner *et al.,* 2000, 2003). PGRP-L consists of transmembrane and cytoplasmic domains and has different extracellular PGN recognition domains which trigger the IMD pathway (Choe *et al.,* 2002; Werner *et al.,* 2003). PGRP-L also plays an essential role in the recognition and distinguishing between Gram-negative and Gram-positive PGN (Werner *et al.,* 2003). PGRP-S consists of a single recognition domain and binds Gram-positive PGN with high affinity (Werner *et al.,* 2000; Michel *et al.,* 2001).

Lee *et al.* (1996) discovered a GNBP in the silkworm, *B. mori*. It is a 50 kDa hemolymph protein which has a strong affinity for the cell walls of Gram-negative bacteria, and is up-regulated during bacterial infection. The GNBP sequence contains a region displaying significant homology to the putative catalytic region of a group of bacterial β-1,3 and β-1,4 glucanases. Silkworm GNBP was also shown to have an amino acid sequence similar to the vertebrate LPS receptor CD14.

A cDNA sequence coding for GNBP has been characterized and identified as DGNBP-1 in *Drosophila*. It is either soluble or glycosylphosphatidylinositol-anchored and is expressed on *Drosophila* immunocompetent cells. It has a high affinity for LPS and β-1,3-glucan, but has no binding affinity for PGN, β-1,4-glucan or chitin (Kim *et al.,* 2000).

The LBP in the hemolymph of *P. americana* has a molecular mass of 450 kDa and is made up of 28 kDa subunits. This protein is highly specific for *Escherichia coli* K12 (Gram-negative bacteria), suggesting that this protein recognizes and binds to a specific carbohydrate structure of *E. coli* LPS (Jomori *et al.,* 1990).

Lectins are glycoproteins that play an important role in recognizing and binding to specific carbohydrate moieties commonly found on the cell walls of pathogens. They are also known as hemagluttinating agents. Lectins activate the complement pathway in both vertebrate and invertebrate animals, and also bind to microbes which enable hemocytes to recognize and engulf them in the opsonization process (Grubhoffer *et al.,* 2004). In addition, lectins are able to bind to one another. This was reported in the horseshoe crab, *Tachypleus tridentatus*, in which tachypleus lectin-1 (TPL-1) and tachypleus lectin-2 (TPL-2) form clusters with one another and bind to pathogens (Chen *et al.,* 2001). This clustering effect that immobilizes invading pathogens has been seen in insects and ticks (Gillespie *et al.,* 1997; Natori 2001; Rego *et al.,* 2005; 2006).

Tick lectins recognize a wide range of Gram-negative bacteria due to their specificity towards cell wall carbohydrate moieties such as LPS, sialic acid and N-acetyl-D-glucosamine (Grubhoffer *et al.,* 2004; Huang *et al.,* 2007). Several researchers have suggested their involvement in controlling pathogens and pathogen transmission. These molecules and other humoral immune factors in the hemolymph are produced and secreted into the hemolymph mainly by hemocytes and fat body cells (Grubhoffer *et al.,* 2004).

In hard ticks, *Ixodes ricinus* and *Rhipicephalus appendiculatus,* hemagglutination activity is found in the hemolymph, midguts and salivary glands (Kamwendo *et al.,* 1993, 1995; Kuhn *et al.,* 1996; Uhlír *et al.,* 1996). Lectins found in *I. ricinus* were later identified as Ixoderin A and Ixoderin B (Rego *et al.,* 2005).

In soft ticks, hemagglutination activity is found in the hemolymph of *O. tartakovskyi, O. tholozani (papillipes), O. moubata* and *Argas polonicus* (Grubhoffer *et al.,* 1991; Grubhoffer & Dusbábek, 1991; Kovář *et al.,* 2000). In *O. moubata*, Dorin M is expressed in hemocytes, salivary glands and malphigian tubules, but not in the midgut or ovary (Rego *et al.,* 2006). Within the same species, two other lectins OMFREP and OmGalec, were also discovered with similar tissue distribution to Dorin M (Huang *et al.,* 2007; Rego *et al.,* 2005).

1.3.2 Cellular immune responses

The exact mechanism of response and signaling in ticks is not known, but it is clear that hemocytes are recruited and specific responses are mounted upon infection or induction with an immunogen (Johns *et al.,* 1998; Matsuo *et al.,* 2003). The hemocytes have been shown to participate in a variety of responses, including opsonization by PAMPs and lectins, nodulation, encapsulation and phagocytosis (Fig 1.2).

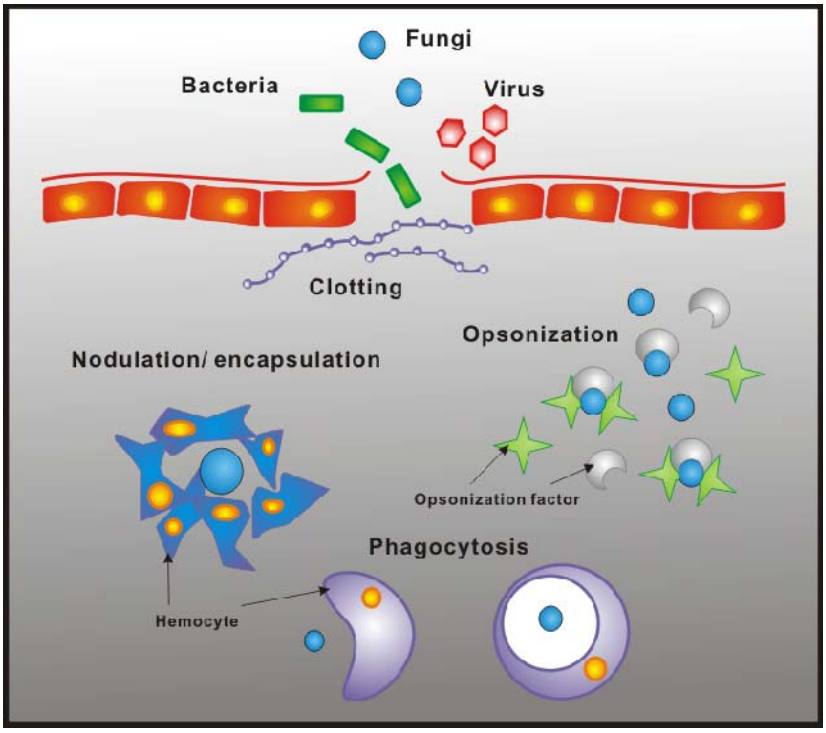

Figure 1.2: A schematic representation of cellular immune responses in arthropods. Once pathogens gain entry into the hemocoel, they activate a complex system of innate defense responses. Opsonization factor: PAMPs or lectins.

It has been reported that tick hemocytes function similarly to white blood cells. Four major cell types exist in tick hemolymph. These are the prohemocytes, non-granular plasmatocytes, granulocytes and spherulocytes (Kuhn 1996; Kuhn *et al.,* 1996; Borovicková & Hypsa, 2005). Hemocyte populations in the hard tick, *Dermacentor variabilis,* have been shown to increase 6.4 fold within 48 hours when challenged by injection with Gram-positive bacteria (*B. subtilis*) (Johns *et al.,* 1998). A similar finding was also observed in *D. variabilis* challenged with the spirochete, *Borrelia burgdorferi* (Johns *et al.,* 2000).

1.3.2.1 Phagocytosis

Phagocytosis is the cellular process of engulfing foreign molecules, which is similar to receptor-mediated endocytosis (Fig 1.2). The pathogens or foreign molecules are recognized and bind to the plasmatocyte or granulocyte cell surface receptors. This is followed by extrusion of pseudopods from the cells that

surround and engulf bound foreign molecules or pathogens (Lamprou *et al.,* 2007). The trapped molecules are internalized by endocytosis into a vesicle that fuses with lysosomes to form a phagolysosome. The foreign body is then destroyed via digestion with enzymes such as acid phosphatase and lysozyme. In ticks, both plasmatocytes and granulocytes are involved in phagocytosis of microbes (Johns *et al.,* 2001a). Three morphologically different cell types, granulocytes, plasmatocytes, and prohemocytes, were detected in hemolymph of *O. moubata*, and granulocytes and plasmatocytes showed phagocytic activity (Inoue *et al.,* 2001). In a study by Pereira *et al.* (2001) phagosomes from hemocytes of *Rhipicephalus* (*Boophilus*) *microplus* were able to use reactive oxygen species to eliminate the bacteria. More recently, Buresova *et al.* (2009), demonstrated that IrAM, an α2-macroglobulin (α2M) protease inhibitor, is involved in phagocytosis of *Chryseobacterium indologenes* by hemocytes in the hard tick *I. ricinus*.

1.3.2.2 Nodulation and encapsulation

In general nodulation is an event involving the clustering of hemocytes that attach to aggregated bacteria or microbes (Fig 1.2). Nodulation in insects is mediated by eicosanoids, prostaglandins and lipoxygenase-derived products (Miller *et al.,* 1994). Encapsulation occurs when the arthropod host is exposed to pathogens such as parasitoids and nematodes which are too large to be eliminated by phagocytosis or nodulation. During this process granulocytes degranulate and deposit a matrix-like material around the foreign molecules. Subsequently, plasmatocytes attach to the matrix, undergo apoptosis, become highly flattened and form a thick layer encapsulating the particle (Ceraul *et al.,* 2002). In insects, encapsulation is also associated with melanization.

Encapsulation in ticks has been demonstrated by Eggenberger *et al.* (1990). Plastic particles were injected into the hemocoel of *D. variabilis* and this resulted in a sequence of encapsulation events as seen previously in insects, but without melanization.

1.3.2.3 Melanization

Melanization is an encapsulation reaction mediated by phenol oxidase (PO) and plays a major role in the defense reactions of insects during wound healing and against pathogen transmission (Lackie, 1988; Sugumaran, 2002). After the recognition of PAMPs, a serine protease cascade activates a prophenoloxidase (proPO)-activating enzyme. This enzyme activates PO in a similar fashion causing the production of quinones in the presence of oxygen. The production of quinones leads to the formation of melanin (Fig 1.3). PO activity was found to be present in hemolymph plasma during ecdysis in 4th instar nymphs of *O. moubata* (Kadota *et al.,* 2002). The enzyme activity was not detected in the hemolymph or tissues of three ixodid ticks (Zhioua *et al.,* 1997).

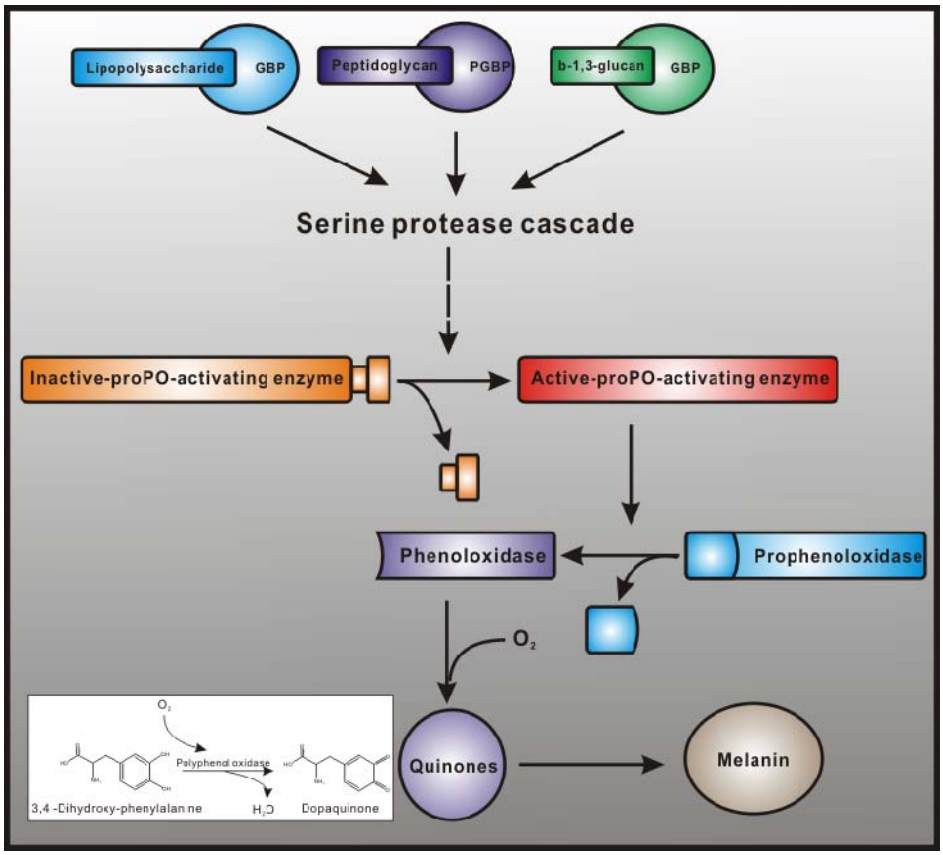

Figure 1.3 The prophenoloxidase activating system in arthropods (Adapted from Cerenius & Söderhäll 2004).

1.3.3 Humoral immune responses

Aside from the cellular responses of phagocytosis, encapsulation, nodulation, and melanization, the innate immune system of arthropods also involves humoral factors that not only identify foreign micro-organisms, but also play a role in the response to the micro-organism. These molecules can act directly on the microbe, or act as an adaptor to allow other molecules to act. They may also serve as opsonins, facilitating the effect of hemocytes on the microbe. Several classes of these molecules have been defined, including AMPs, serine proteases, protease inhibitors, lysozymes, lectins and iron sequestration molecules (Johns *et al.,* 2001a; Nappi & Christensen, 2005).

Peptide-mediated responses in *Drosophila* depend on the production of AMPs expressed in the fat body (the equivalent of the liver in mammals) and stored in the hemocytes (Kim & Kim, 2005; Lemaitre & Hoffmann, 2007). It remains unclear as to whether regulation of AMP levels is due to transcriptional up-regulation by the IMD or a similar pathway, or whether it is based on the release of AMPs from storage vessels in the hemocytes (Kim & Kim, 2005; Lemaitre & Hoffmann, 2007). In ticks, AMPs have been identified and characterized in hemolymph and midguts, but recently they have also been discovered in salivary glands (Sonenshine *et al.,* 2005; Fogaça *et al.,* 2006; Yu *et al.,* 2006).

1.3.3.1 AMPs

These have been found in various tissues and cell types in all species of life (Ganz, 1999; Hancock & Diamond, 2000; Lehrer & Ganz 2002; Zaiou & Gallo, 2002; Bulet *et al.,* 2004). AMPs are conserved components of the innate immune response (Brogden, 2005; Zasloff, 2002) and have been isolated from both hard and soft ticks (This aspect of the tick immune response will be extensively discussed in section 4.1).

AMPs are divided into subgroups on the basis of their amino acid composition and structure. They are generally small peptides consisting of 12 to 50 amino acids (Yeaman & Yount, 2003). These peptides include two or more positively charged residues provided by arginine, lysine and histidine in acidic environments, as well as containing a large proportion of hydrophobic residues (Sitaram & Nagaraj, 2002; Papagianni, 2003; Dürr *et al.,* 2006). Very often AMPs are unstructured in free solution and fold into their final conformation upon partitioning into biological membranes (Dhople *et al.,* 2006).

Bacterial cell membranes are rich in acidic phospholipid head groups (Chou *et al.,* 2008; Matsuzaki, 2008). Therefore, the outermost leaflets of the bacterial membrane bilayer are more prone to attack by the positively charged AMPs. For helical peptides, hydrophilic amino acid residues of AMPs are aligned along one side while the hydrophobic amino acid residues are aligned along the opposite side (Yeaman & Yount, 2003). This amphipathicity of the AMPs allows partitioning into the membrane lipid bilayer. The ability to associate with membranes is a definitive feature of AMPs although membrane permeabilization is not necessary. These peptides have a variety of antimicrobial activities ranging from membrane permeabilization to reacting with a range of cytoplasmic targets (Hancock & Rozek, 2002; Brogden, 2005).

The mechanism of action of AMPs is dependent on its amino acid composition (amphipathicity and cationic charge) and size (Oren & Shai, 1998; Huang *et al.,* 2004; Brogden, 2005). Most pore forming peptides cause depolarization of the normally energized bacterial membrane (Westerhoff *et al.,* 1989). Another proposed mechanism is the creation of physical holes that cause leakage of cellular contents (Shai, 1999). Other mechanisms include the activation of deadly processes such as induction of hydrolases that degrade the cell wall (Bierbaum & Sahl, 1985, 1987) and scrambling of the usual distribution of lipids between the leaflets of the bilayer, resulting in disturbance of membrane functions

(Matsuzaki,1999). Three models have been proposed to explain the interaction of AMPs with biological membranes, namely the “barrel-stave”, “carpet” and “toroidal-pore” models (Fig 1.4).

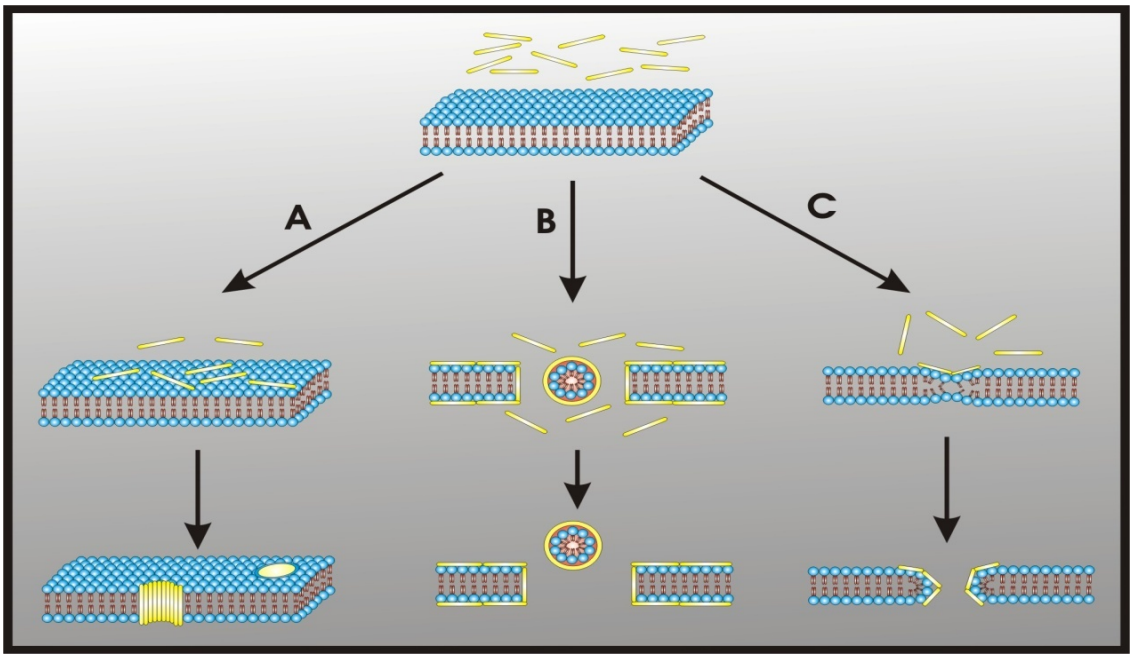

Figure 1.4: Extracellular mechanism of action of AMPs. A: barrel stave model. B: Carpet model. C: Toroidal-pore model (Adapted from Yang *et al.,* 2001; Brogden, 2005; Oren & Shai 1998; Shai, 1999).

In the barrel-stave model, peptide helices form a bundle in the membrane with a central lumen (Fig 1.4A). The hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore (Yang *et al.,* 2001).

The carpet model suggests that peptides are electrostatically attracted to the anionic phospholipid heads at numerous sites covering the surface of the membrane in a carpet-like manner (Fig 1.4B). At high peptide concentrations, the bilayer is disrupted in a detergent-like manner, eventually leading to the formation of micelles (Shai, 1999; Ladokhin & White, 2001).

A toroidal-pore is formed when AMP helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that the water pore is lined by both the inserted peptides and the lipid head groups as shown in Fig 1.4C (Matsuzaki *et al.,* 1996, Yamaguchi *et al.,* 2002).

There have been increasing reports from studies on various organisms illustrating that AMPs also have intracellular targets (Fig 1.5). Intracellular targets include the activation of autolysins, inhibition of cell wall synthesis, alteration of the cytoplasm membrane polarity, inhibition of DNA, RNA and protein synthesis and inhibition of enzymatic activity (Brogden, 2005).

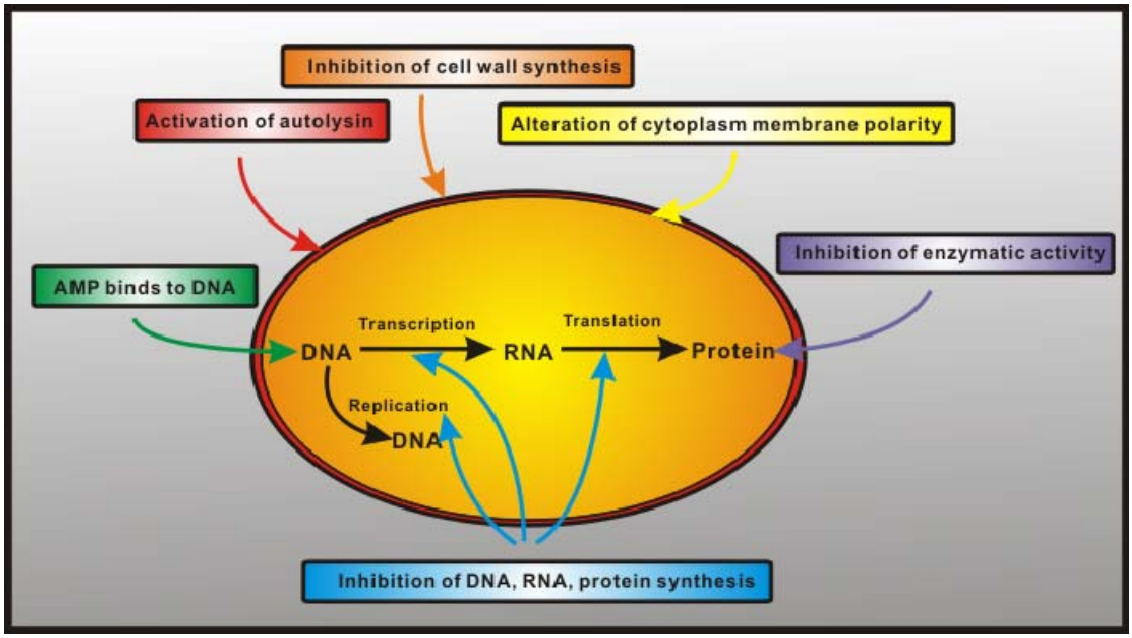

Figure 1.5 Intracellular mode of action of antimicrobial peptides. The intracellular targets and their effects are indicated in boxes (adapted from Brogden, 2005).

The defensins are a major family of AMPs. They are usually small in size and range from 67 – 92 amino acids (3 - 6 kDa) and include a region (37- 61 amino acids in length) which is cleaved to release the mature peptide. The arginine-rich and cationic peptides usually contain 6 – 8 cysteine residues which form the disulphide bridges. These bridges play a vital role in stabilization and

maintenance of the tertiary structure known as the “defensin fold” (Ganz, 2003). Ticks defensins will be discussed in section 4.1.

1.3.3.2 Lysozymes

These are small proteins with a molecular mass of ~ 14kDa, that catalyze the hydrolysis of β-1,4- glycosidic linkages between N-acetylmuramic acid (NAM) and N-acetyl glucosamine (NAG) in PGN, resulting in the disruption of the integrity of the cell wall of Gram-positive bacteria. Gram-negative bacteria are protected from lysozyme attack because of the presence of the outer cytoplasmic membrane (Salton & Kim, 1996).

Lysozymes are generally characterized as chicken (c), goose (g), or invertebrate (i) based on their possession of type specific amino acid sequence features and their species of origin (Nilsen & Myrnes, 2001). In arthropods, lysozyme is mostly found in hemocytes, midguts and salivary glands. While lysozymes function mainly as antimicrobials, other functions such as digestion, development, communication and reproduction have been described. In some insects such as *Drosophila* and house flies (*Musca domestica*), lysozymes are acidic proteins, which do not respond to bacterial infection (Kylsten *et al.,* 1992; Daffre *et al.,* 1994; Ito *et al.,* 1995).

In tick hemolymph, lysozyme may act synergistically with defensin to destroy Gram-negative bacteria, thereby broadening the range of bacteria inhibited by the activity of AMPs (Matsuzaki *et al.,* 1998; Johns *et al.,* 2001b). It is proposed that defensin affects the integrity of the outer membrane which allows lysozyme to reach and hydrolyze the inner PGN cell wall (Fig 1.6).

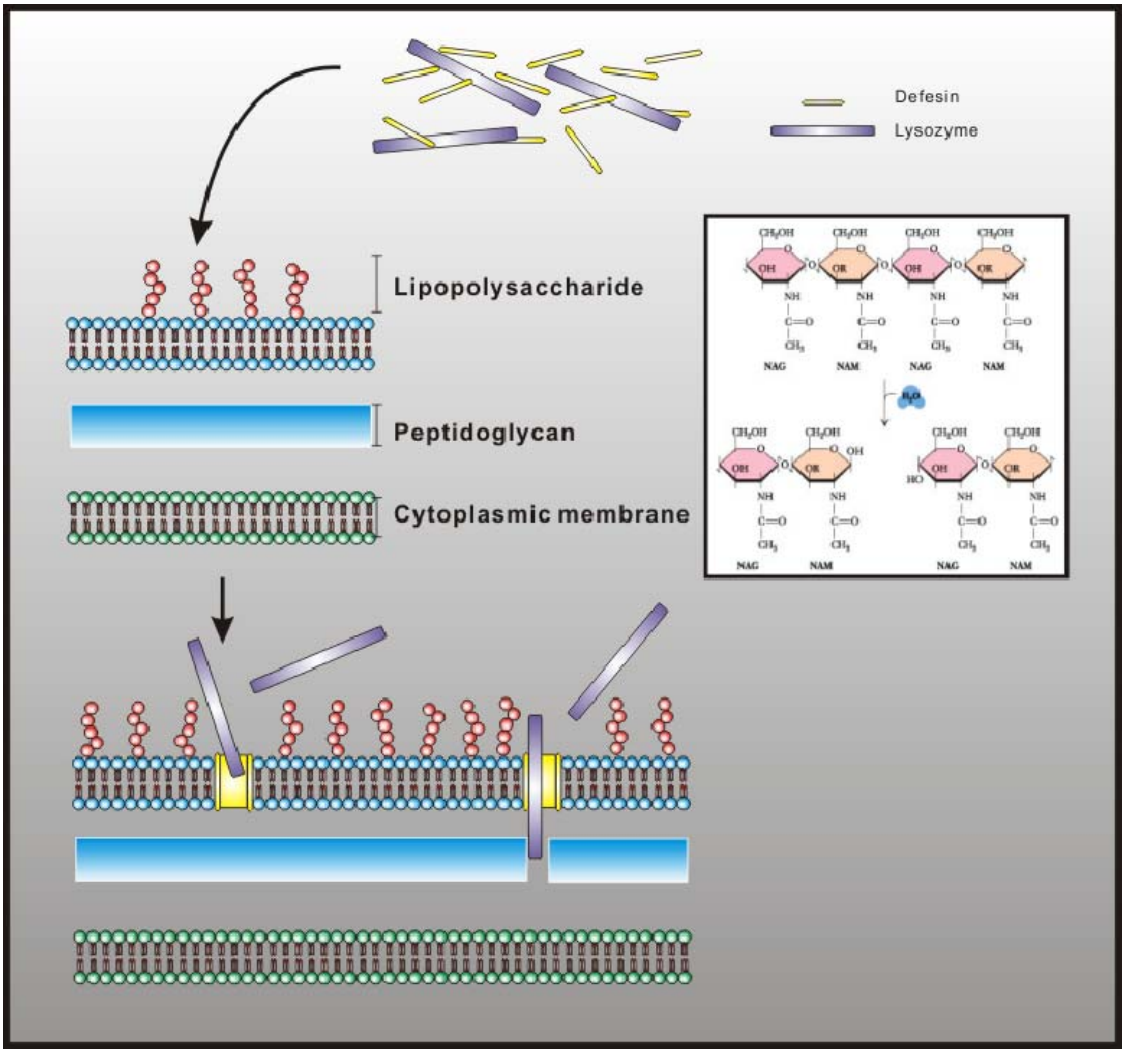

Figure 1.6 A schematic representation of the synergistic action of defensin and lysozyme against Gram-negative bacteria. Defensins permit lysozyme to infiltrate the outer LPS layer of Gram-negative bacteria and thus cleave the β-1,4-glycosidic bond between NAM and NAG in PGN.

In most ticks, lysozyme of the c-type is involved either in humoral innate immunity (induced in response to bacterial challenge) or has been evolutionary adapted to serve as digestive enzymes within the alimentary system (Table 1.1).

The mature lysozyme from digestive tissues in *O. moubata* contains 124 amino acids and belongs to the c-type. Transcript levels were elevated in response to feeding (Kopáček *et al.,* 1999; Grunclová *et al.,* 2003). This finding suggested

that c-type lysozyme possesses a combination of immune and digestive functions. However, its regulation in response to bacterial challenge has not been assessed.

Mattila *et al.* (2007) reported that lysozyme expression was not up-regulated in tick cell lines (IDE12 and DAE15) when challenged with *Rickettsia peacockii*. Expression was however up-regulated after stimulation of the cells with *E. coli* and *Micrococcus luteus*.

The mature c-type lysozyme of *D. variabilis* is 121 amino acids long. Transcript levels in hemolymph were increased 17 fold within 72 hours post-injection with *E. coli* (Simser *et al.,* 2004a). Lysozyme expression profiles showed levels of expression to be most abundant in hemolymph but present in very low levels in the midgut or other organs. Feeding of *D. variabilis* did not result in increased lysozyme expression (Simser *et al.,* 2004a; Sonenshine *et al.,* 2005). Lysozyme antimicrobial activity has been reported in the hemocytes of *I. ricinus*, *I. persulcatus* and cell lines derived from both *I. scapularis* and *D. andersoni* (Kuhn & Haug, 1994; Mattila *et al.,* 2007).

Table 1.1: Tick lysozyme

Species	Found	Antimicrobial	Gene bank accession code	Reference
D. andersoni	DAE 100 cell line, DAE 15 cell line	**+**	28883557	Simser *et al.,*2004a; Mattila, *et al.,* 2007
D. variabilis	Hemocyte	**+**	27819105	Simser *et al.,*2004b
I. persulcatus	Homogenate of tick	**+**		Podboronov, 1990; 1991
I. ricinus	Hemocyte	**+**		Kuhn & Haug, 1994
I. scapularis	IDE 12 cell line	**+**		Mattila *et al.,* 2007
O. moubata	Midgut	**-**	16326735	Kopáček *et al.,*1999; Grunclová *et al.,*2003

-: not determined **+ indicates antimicrobial activity**

1.3.3.3 Proteases and Protease inhibitors

Serine proteases play an important role in the innate immune responses of hemolymph coagulation, melanization and AMP synthesis. In the mosquito, *Anopheles dirus*, serine proteases were up-regulated in response to invasion of the hemolymph by *Plasmodium yoelii* (Xu *et al.,* 2006). Similar findings were found in tick hemolymph when an immune-responsive factor D-like serine protease was expressed in hemocytes in response to *E. coli* challenge (Simser *et al.,* 2004b). The serine proteases are comprised of a clip domain, a feature that is highly conserved in *D. andersoni* and *D. variabilis* (Simser *et al.,* 2004a, b).

The arthropod host produces protease inhibitors that selectively target pathogen proteases in various ways (Armstrong, 2001). Some inhibitors that have been identified in ticks include both the serine proteinase inhibitors (serpins) and α2M-type protease inhibitors. The α2M functions in both entrapment and inactivation of the proteases secreted from the pathogen (Armstrong & Quigley, 1999). The α2M identified from hemocytes of *O. moubata* is known as tick α2M (TAM: Kopáček *et al.,* 2000). TAM inhibits trypsin and thermolysin cleavage of the high-molecular-weight substrate azocoll in a manner similar to that of bovine α2M (Kopáček *et al.,* 2000). The mature protein is cleaved to form two subunits similar to the C3 and C4 complement components. Phylogeny analysis revealed that TAM is closely related to *Limulus* α2M and displays the highest similarity to the partial sequence from *I. scapularis* (Valenzuela *et al.,* 2002; Saravanan *et al.,* 2003).

1.3.4 Coagulation

Coagulation is the first response to injury. In the horseshoe crab, *Tachypleus tridentatus*, transglutaminase and proxin are expressed in hemocytes during injury. Proxin is cross linked with the plasma protein coagulin to seal wounds. Stabilin, another coagulation factor derived from hemocytes interacts with proxin forming a fibril complex. In addition, stabilin can bind to both Gram-positive and Gram-negative bacteria to immobilize them within the clots (Matsuda *et al.,* 2007). To date, no coagulation studies in ticks have been performed and the molecular mechanism of coagulation remains unknown.

1.3.5 Iron sequestration

Iron plays an indispensable role in various physiological processes such as gene regulation and DNA synthesis. Since iron plays an instrumental role in organisms, including the host and invading pathogens, it is vital to understand how host proteins regulate both intracellular and intercellular iron levels (Andrews, 2000).

During an infection, both host and the invasive pathogen compete for iron. Pathogens usurp host iron by secreting siderophores that have a high affinity for iron. They are then transported into the bacteria through specific receptors (Andrews, 2000; Ratledge & Dover, 2000). Transferrin has been shown to inhibit the growth of bacteria by depleting iron which may be used for bacterial metabolism (Yoshiga *et al.,* 1997; Caccavo *et al.,* 2002; Paily *et al.,* 2007).

To date, invertebrate transferrin has been isolated from the cockroach (*Blaberus discoidalis*), mosquito (*Aedes aegypti*), silkworm (*B. mori*), fruit fly (*D. melanogaster*) and tobacco hornworm (*Manduca sexta*) (Jamroz *et al.,* 1993, Huebers *et al.,* 1988; Yoshiga *et al.,* 1997, 1999; Yun *et al.,* 1999, 2009). Transferrin synthesis and secretion was shown to be increased upon exposure to microbes and to elicit an immune response in *D. melanogaster*, *A. aegypti* and the honey bee, *Apis mellifera* (Yoshiga *et al.,* 1997, 1999; Kucharski & Maleszka,

2003). A recent study has shown that the promoter region of transferrin found in *A. aegypti* contains a putative NF-κB-binding site, which is consistent with the postulated role of transferrin in insect innate immunity (Harizanova *et al.,* 2005). In ticks, *R.* (*B.*) *microplus,* levels of transferrin decreased significantly after feeding (Carvalho *et al.,* 2008).

1.3.6 Oxidative stress

In *D. variabilis*, the enzymes $DvGST_1$ and $DvGST_2$ which belong to the Glutathione S-transferase (GST) family, play a central role in removal of ROS and other harmful radicals. (Dreher-Lesnick *et al.,* 2006). Both isoforms were identified in tick midguts and were up-regulated during blood feeding. $DvGST_2$ was also found in the ovaries (Dreher-Lesnick *et al.,* 2006). A similar finding was also observed in *I. ricinus* when infected with *B. burgdorferi* (Rudenko *et al.,* 2005).

1.4 Aims of this thesis

The overall aim of this study is the biochemical and molecular characterization of putative immunoprotective molecules of the tick, *O. savignyi*. The investigations in Chapter 2 were aimed at identifying hemolymph plasma proteins with a putative Gram-negative recognition role. In Chapter 3 molecular characterization, bioinformatic analysis and expression profiling of a lipocalin-like hemolymph protein was undertaken. In Chapter 4 the biochemical isolation and characterization of a salivary gland Gram-positive antibacterial activity was attempted.

Part of this study was published in:
Paul H. Cheng, Ben J. Mans, Albert W. H. Neitz, Anabella R. M. Gaspar. Experimental and Applied Acarology (2010) 52:313-326. Savicalin, a lipocalin from hemocytes of the soft tick, *Ornithodoros savignyi.* DOI 10.1007/s10493-010-9368-6.

Various aspects were presented at scientific meetings:
Cheng P, Saunders M, Exley A, Neitz A & Gaspar A (2005). Proteomic and peptidomic analyses of the innate immune response of the soft tick, *Ornithodoros savignyi*. 5th International Conference on Ticks and Tick-borne Pathogens, Neuchatel, Switzerland. 29 Aug 2 September 2005 (P66).

CHENG PH, STOPFORTH E, NEITZ AWH & GASPAR ARM (2006). Antimicrobial activity in the soft tick, *Ornithodoros savignyi*. 20th SASBMB Conference, Pietermaritzburg, 2-5 July 2006 (P71).

SAUNDERS MG, CHENG PH, NEITZ AWH & GASPAR ARM (2006). Investigations into mechanisms in the hemolymph of the soft tick *Ornithodoros savignyi* to combat infection by micro-organisms. 20th SASBMB Conference, Pietermaritzburg, 2-5 July 2006 (P70).

Chapter 2: Identification of tick proteins recognizing Gram-negative bacteria

2.1 Introduction

Several PRRs, which are highly specific to pathogens, have been identified in arthropods. These include PGRP (PGRP-S, PGRP-L), GNBP, LBP and lectins, (Kang *et al.,* 1998; Kim *et al.,* 2000; Werner *et al.,* 2000, 2003; Michel *et al.,* 2001). They usually activate the Toll and/or IMD signaling pathways which leads to innate immune responses (Royet, 2004).

Grubhoffer *et al.* (2004), described how pathogen recognition in ticks is accomplished by lectins. Tick lectins have a high affinity for sialic acid, various derivatives of hexosamines and different glycoconjugates. Most of these tick lectins displaying hemagglutinin activities are blood meal enhanced, and allow discrimination between self and non-self, particulary against bacteria and fungi.

Tick lectins were first detected in the soft ticks, *A. polonicus*, *O. tartakovskyi* and *O. tholozani (papillipes)* (Grubhoffer *et al.,* 1991). In *O. tartakovskyi*, hemagglutinin activities found in hemolymph plasma and hemocytes, bind to sialic acid, NAG, N-acetyl-D-galactosamine, fetuin, asialofetuin and bovine submaxillary mucine. Lectins found in *O. tartakovskyi* have 2 subunits with molecular mass of 30 and 35 kDa, respectively (Grubhoffer *et al.,* 1991).

In *A. polonicus* and *O. tholozani* (*papillipes*), hemagglutinin activities were detected only in hemolymph plasma. The lectin isolated from the latter organism consists of 2 subunits with molecular masses of 37 and 40 kDa. Both lectins recognize sialic acid, NAG, D-galactose, fetuin and bovine submaxillary mucine (Grubhoffer *et al.,* 1991).

In the soft tick, *O. moubata*, Dorin M was identified in the hemolymph plasma and shown to have an affinity for sialic acid, N-acetyl-D-hexosamines and sialoglycoproteins. In the native state, Dorin M forms aggregates, each with a molecular mass of about 640 kDa. Under non-reducing conditions it consists of two non-covalently bound subunits, each with molecular mass of 37 kDa. More recently, Rego *et al.* (2006) found that Dorin M was also expressed in hemocytes, salivary glands and malphigian tubules, but not in the midgut or ovary. In another study, Rego *et al.* (2005) identified OMFREP within the same tick species which shows high sequence homology with Dorin M and is expressed primarily in the hemocytes and salivary glands.

Another lectin, OmGalec was identified in *O. moubata* and consists of 333 amino acids with a predicted molecular weight of 37.4 kDa (Huang *et al.,* 2007). Amino acid sequence analysis of OmGalec does not indicate a signal peptide or transmembrane domain. It is composed of repeated carbohydrate recognition domains, which are important for binding. Tissue expression profiling indicated that OmGalec is expressed both on the transcript and protein level in multiple organs at all stages of the tick life cycle, with most notable expression in hemocytes, midguts, and reproductive organs.

In the hard tick, *R. appendiculatus*, hemagglutinin activities were identified in hemolymph, midgut and salivary glands and the salivary gland lectin was found to be specific for mannose and turanose (Kamwendo *et al.,* 1993, 1995).

Hemagglutinin activities in *I. ricinus* were identified in the same tissues as in *R. appendiculatus*. The lectin found in hemolymph plasma and hemocytes is specific for sialic acid, NAG, fetuin, asialofetuin and bovine submaxillary mucine, while in the midgut it is specific towards N-acetyl-galactosamine, NAG, rhamnose, dulcet, fetuin, hyaluronic acid, laminarin, sialic acid, bovine submaxillary mucine and LPS (Kuhn *et al.,* 1996). The lectin consists of 4 subunits of 37, 60, 65 and

73 kDa. In the salivary glands of *I. ricinus* it is specific towards sialic acid, fructose, rhamnose, trehalose, fetuin, asialofetuin, bovine submaxillary mucine, laminarin, and heparin (Grubhoffer *et al.,* 2004).

Rego *et al.* (2005) showed that lectin-like cDNA sequences, Ixoderin A and Ixoderin B were present in *I. ricinus*. Tissue expression profiling showed that Ixoderin A was expressed in the midgut, whereas Ixoderin B was expressed in salivary glands. Ixoderin A showed high homology to the lectin Dorin M, from *O. moubata*.

IrAM, a protease inhibitor similar to α2M, is expressed in all stages of the tick life cycle of *I. ricinus* and in multiple organs except for the midgut (Buresova *et al.,* 2009). The authors demonstrated that silencing of IrAM reduced phagocytosis of *C. indologenes*, but not of the spirochete, *B. burgdorferi* or *Staphylococcus xylosus*. Furthermore, it was demonstrated that phagocytosis of *C. indologenes* required an active metalloprotease secreted by the bacteria.

Several strategies can be employed to isolate and characterize proteins involved in pathogen recognition. Zhu *et al.* (2005) described the identification of pathogen binding proteins from hemolymph of the horseshoe crab using live *S. aureus* as affinity beads. In this chapter, a similar approach was followed to identify hemolymph plasma proteins that bind to Gram-negative, *E. coli*.

2.2 Hypothesis

Tick hemolymph contains proteins that recognize *E. coli*.

2.3 Materials and methods

2.3.1 Ticks

Ornithodoros savignyi ticks were obtained from Upington (Northern Cape), South Africa by sifting of sand. Ticks were then transferred to sterile sand and kept at room temperature.

2.3.2 Reagents

All chemicals were of analytical grade and sterile double distilled deionized water was used in all experiments. Acetic acid, Acetonitrile (ACN), Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), trifluoroacetic acid (TFA), sodium chloride, citric acid, triethylamine, ethylene diamine tetraacetic acid (EDTA), urea, sodium azide, sodium phosphate monobasic, sodium phosphate dibasic, Coomassie blue G-250, ammonium sulphate, sodium dodecyl sulphate (SDS), methanol, ethanol, sodium acetate, diethylpyrocarbonate (DEPC), Bradford reagent, TRI Reagent, dithiothreitol (DTT), phenyl thiocarbamide (PTU), D-glucose, isopropanol, protease inhibitor tablet [2 mM of 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 1 mM of Ethylene diaminetetraacetic acid (EDTA), 130 uM of Bestatin, 14 µM of N-(trans-epoxysuccinyl)-L-leucine-4-guanidinobutylamide (E-64), 1 µM of Leupeptin, 0.3 µM of Aprotinin], chloroform, and N'N'N'N'-Tetramethylethylenediamine (TEMED) were obtained from Sigma Chemical Co, USA. Acrylamide, bisacrylamide, ammonium persulphate (APS), glycerol, bromophenol blue, β-mercaptoethanol, phosphoric acid, glutaraldehyde, silver nitrate, formaldehyde and sodium carbonate were obtained from Merck, Germany. Tryptone and yeast extract were purchased from Oxoid Ltd, England. The low molecular mass markers were phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactoalbumin (14.5 kDa). The peptide markers were intact myoglobin -17 kDA; myoglobin I and II – 14.6 kDa; myoglobin I – 8 kDa; myglobin II- 6kDa; myoglobin III- 2 kDa. Both markers were obtained from Amersham Bioscience, Uppsala, Sweden. A PageRuler

prestained recombinant protein Ladder obtained from Fermentas, Canada ranged from 11 kDa ~250 kDa.

2.3.3 *E. coli* binding proteins in hemolymph plasma from unchallenged ticks

2.3.3.1 Binding assay

For the preparation of the bacterial culture, lyophilized *E. coli* (American type culture collection, ATCC: 700928) was reconstituted with 10 ml of *Luria-Bertani* (LB) broth [(1 % (w/v) NaCl 1 % (w/v), tryptone, 0.5 % (w/v) yeast extract, pH 7.4] and grown overnight at 37 °C in a shaking incubator. Cells were pelleted by centrifugation (10 000 *g* for 10 min at 4 ^{o}C) and the supernatant discarded. The pellet was suspended in 10 ml LB broth containing 10 % (v/v) glycerol. Thereafter the cell suspension was divided into 100 µl aliquots and stored at - 70 ^{o}C.

A 1 µl aliquot of the glycerol stock was diluted with 100 ml of LB broth (1:1000 dilution) and the bacterial cells grown overnight. A 1:100 dilution of the overnight culture was prepared and cells were grown until an OD_{600} of 0.5 was reached. A 200 µl aliquot of the bacterial subculture was centrifuged at 10 000 *g* for 10 min. The pellet was re-suspended and washed 3 times with sterile saline (0.9 % w/v). The cell pellet was finally re-suspended with 20 µl of phosphate buffered saline (PBS; 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, 150 mM NaCl, pH 7.4).

For tick hemolymph extraction, 30 unchallenged ticks were dorsally immobilized on double-sided tape. Small nicks were made in the front legs, followed by the application of gentle pressure on the abdomen. Extruded hemolymph was then collected with glass capillaries into sterile eppendorf tubes containing pre-chilled tick bleeding buffer pH 7.0 (TBB; 50 mM Na_2HPO_4, 0.15 mM NaCl, 2 mM EDTA, 0.02 % NaN_3, 1 protease inhibitor tablet and 2 mM PTU) on ice and centrifuged at 10 000 *g* for 10 min at 4°C. The hemocyte free plasma was transferred to new sterile tubes.

For binding experiments, 100 µl of hemolymph plasma from unchallenged ticks was added to the 20 µl bacterial suspension. After incubation with rotation at 4 °C for ~ 10 min, the bacterial cells were pelleted by centrifugation (10 000 *g* for 10 min at 4°C) and washed three times with PBS. The bound proteins were eluted by adding 100 µl of either a) 4 M urea, in 10 mM Tris–HCl, pH 8.0 (Zhu *et al.,* 2005); (b) 0.1 M citric acid, pH 2.0 (Zhu *et al.,* 2005); (c) 0.15 M triethylamine, pH 11.5 (Zhu *et al.,* 2005); (d) sterile water or (e) 0.1 M, 0.5 M or 1 M NaCl and subjected to mild agitation for 10 – 15 min. As a control (C), bacterial cells were incubated with TBB instead of plasma and subjected to the same elution conditions. Following elution the samples were centrifuged (10 000 *g* for 30 min at 4 °C) to remove cells and the supernatants containing the eluted proteins were transferred to new eppendorf tubes. For the bacterial protein control (cB), sedimented cells obtained from 20 µl bacterial suspension were re-suspended in TBB buffer on ice and sonified with a Branson sonifier (Branson Sonic Power Co.), using 3 x 6 pulses at 30 % duty cycles on ice. The cell debris was sedimented by centrifugation at 10 000 *g* for 10 min on ice.

The eluted proteins were precipitated with acetone (1 part protein: 4 parts acetone) and incubated for 2 h at -20 °C. The air-dried protein pellet was resuspended in 10 µl of 2 × SDS–PAGE reducing buffer [0.06 M Tris-HCl, pH 6.8, 2 % SDS (w/v), 0.1 % glycerol (v/v), 0.05 % β-mercaptoethanol (v/v) and 0.025 % bromophenol blue (w/v)] for analysis.

2.3.3.2 SDS-PAGE analysis of bacteria binding proteins in plasma from unchallenged ticks

Electrophoresis was performed using the method of Laemmli (1970). A 12 % separating gel (0.375 M Tris-HCl, 0.1 % SDS, pH 8.8) and 4 % stacking gel (0.125 M Tris-HCl, 0.1 % SDS, pH 6.8) were prepared from a 30 % acrylamide/ 0.1 % bisacrylamide stock solution. The solutions were degassed for 30 min and polymerized by addition of 50 µl of 10 % APS and 5 µl of TEMED. Low molecular

mass and peptide markers (10 μg) and samples (10 μl) were loaded per well. A PageRuler marker (1 μg) was also loaded onto the gel.

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Electrophoresis was carried out in running buffer (0.02 M Tris-HCl, 0.1 M glycine and 0.06 % SDS, pH 8.3) using a Hoefer SE 300 miniVE system with an initial voltage of 60 V for 45 min and then a voltage of 100 V for a further 2 h. The gels were visualized with either silver stain or colloidal Coomassie stain.

For silver staining the procedure of Morrisey (1981) was implemented. The gel was fixed in 100 ml of fixing solution (40 % methanol, 10 % acetic acid) for 30 min. The fixing solution was poured off and the gel immersed in 50 % methanol and 10 % acetic acid in water and agitated slowly for 30 min. The solution was poured off and the gel was covered with 50 ml of a 10 % glutaraldehyde solution and agitated slowly for 10 min in a fume hood. After glutaraldehyde was poured off, the gel was washed thoroughly with several changes of water. The gel was then soaked in 100 ml of a 5 μg/ml DTT solution for 30 min.

The DTT was discarded and without rinsing, the gel was immersed in 100 ml of a 0.1 % silver nitrate solution and agitated for 30 min. The silver nitrate was decanted and the gel was thoroughly rinsed with water and then soaked in 100 ml of the carbonate developing solution (0.5 ml of a 37 % formaldehyde solution, 3 % sodium carbonate) and agitated slowly until the desired level of staining was achieved. The staining reaction was stopped by adding 5 ml of 2.3 M citric acid solution per 100 ml of carbonate developing solution. The gel was then rinsed in water for another 30 min.

Alternatively, visualization was achieved with colloidal Coomassie stain (0.1 % Coomassie blue G-250, 10 % of ammonium sulphate, 3 % phosphoric acid, 20 % methanol) as described Neuhoff *et al.* (1988). Bands of interest were excised for further analysis.

2.3.4 *E. coli* binding proteins in hemolymph plasma from challenged ticks

2.3.4.1 Challenge of ticks by hemocoelic injection

*Bacillus subtilis (*Gram-positive bacteria, ATCC: 13933) glycerol stocks were prepared as described in section 2.3.3. Hundred-fold dilutions of the overnight cultures for both *E. coli* and *B. subtilis* prepared and cells were grown until an OD_{600} of 0.5 was reached. For each strain, 1 ml of the subculture were centrifuged at 10 000 *g* for 10 min and the pellets was washed three times with sterile saline (0.9 % w/v). The cell pellets was then re-suspended in 1 ml of sterile saline and boiled for 10 min (heat killed bacteria).

For hemocoelic injection, 30 ticks were immobilized onto Petri dishes with double-sided tape with their ventral side facing up. Before inoculation ticks were surface sterilized by wiping their integuments with 70 % ethanol. The respective heat-killed bacterial suspensions (1 µl) or sterile saline (1 µl) were injected at the base of the trochanter of the fourth coxa using a 30 gauge needle. Hemolymph was collected from the ticks at 2, 12 and 24 h post-injection as described in section 2.3.3.

2.3.4.2 Challenge of ticks by artificial feeding

For artificial feeding, *B. subtilis* was cultured as described above. To calculate the amount of bacterial cells for tick feeding, 1 µl of the bacterial culture (OD_{600} of ~ 0.5) was diluted 1000 fold with LB broth and 10 µl plated on LB agar plates in quadruplicate. The plates were incubated overnight at 37^{o}C and the colonies counted on each plate. The CFU/ ml of the bacterial culture was calculated to be 3 x 10^8 CFU/ ml. Bacteria (2.5 x 10^6 cells) in 1 ml LB broth was centrifuged for 10 000 *g* for 10 min and the pellet was washed with sterile saline (0.9 % w/v) three times. The cell pellet was then re-suspended within 1ml of native bovine blood to obtain a final concentration of 2.5 x 10^6 cells/ml blood. Native blood (no bacteria added) was used for the control group.

The artificial feeding of ticks was performed as described by Schwan *et al.* (1991) and is illustrated in Fig 2.1. The feeding apparatus consisted of a conical 500 g yogurt container (diameter at the top: 85 mm, diameter at the bottom: 70 mm, height: 110 mm). A piece of parafilm (10 cm x 15 cm indicated in yellow) was stretched over the opened bottom end of the container and fixed with elastic bands, before placing up to 30 unfed ticks into the container. The container was fixed to the stand with tape (white). Subsequently the container was placed in a petri-dish (diameter: 87 mm, height 12 mm) containing either native or infected-blood samples (red). The petri-dish was placed in a water bath (blue) at a temperature of 36 ^{o}C. At 24 h and 10 days post feeding (time intervals were chosen based on Nakajima *et al.,* 2002, 2003a, b), hemolymph was then extracted as described in section 2.3.3.

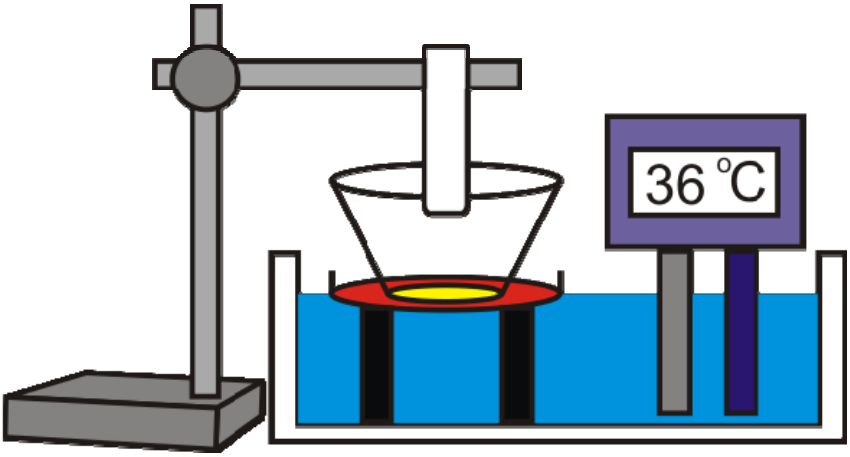


Figure 2.1 An illustration of the feeding apparatus.

2.3.4.3 SDS-PAGE analysis of bacteria binding proteins in plasma from unchallenged and challenged ticks

Hemolymph samples prepared from unchallenged and challenged ticks were incubated with *E. coli* as described in section 2.3.3. In each case the proteins were eluted with 0.5 M NaCl and analyzed using 7.5 % separating gels.

2.3.5 Protein identification by MS/MS analysis and *de novo* sequencing

For the identification of *E. coli* binding proteins, the experiment was repeated as described in section 2.3.4 using hemolymph plasma collected from 300 ticks at 24 h following aseptic hemocoelic challenge with sterile 0.9 % (w/v) saline. The bands were excised from the SDS-PAGE gel and analyzed at the Technology facility, Department of Biology, University of York, UK.

In-gel tryptic digestion was performed after reduction with dithioerythritol (DTE) and S-carbamidomethylation with iodoacetamide. Gel pieces were washed twice with 50 % (v : v) aqueous ACN containing 25 mM ammonium bicarbonate, then once with ACN 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 μg/μl. Gel pieces were rehydrated by adding 10 μl of trypsin solution, and after 30 min enough 25 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37 ^{o}C.

A 0.5 μL aliquot of each digest was applied directly to the matrix-assisted laser desorption/ionization (MALDI) target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/ml solution of 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50 % aqueous (v : v) ACN containing 0.1 % TFA (v : v). Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in reflectron mode. MS spectra were acquired over a mass range of m/z 800-4000. Final mass spectra were internally calibrated using the tryptic autoproteolysis products at m/z 842.509 and 2211.104. Monoisotopic masses were obtained from centroids of raw, unsmoothed data. The twenty strongest peaks with a signal to

noise greater than 40 were selected for collision-induced dissociation-MS/MS (CID-MS/MS) analysis.

For collision-induced dissociation mass spectrometry/ mass spectrometry (CID-MS/MS), source 1 collision energy of 1 kV was used, with air as the collision gas. The precursor mass window was set to a relative resolution of 50, and the metastable suppressor was enabled. The default calibration was used for MS/MS spectra, which were baseline-subtracted (peak width 50) and smoothed (Savitsky-Golay with three points across a peak and polynomial order 4); peak detection used a minimum signal-to-noise ratio (S/N) of 5, local noise window of 50 m/z, and minimum peak width of 2.9 bins. Filters of S/N 20 and 30 were used for generating peak lists from MS and MS/MS spectra, respectively.

Mass spectral data obtained in batch mode were submitted to database searching using a locally-running copy of the Mascot proGram (Matrix Science Ltd., version 2.1). Batch-acquired MS and MS/MS spectral data were submitted to a combined peptide mass fingerprint and MS/MS ion search through the Applied Biosystems GPS Explorer software interface (version 3.6) to Mascot. Search criteria included: Maximum missed cleavages, 1; fixed modifications, carbamidomethyl; variable modifications, oxidation; peptide tolerance, 100 ppm; MS/MS tolerance, 0.1 Da. A recent version of the National Center for Biotechnology Information (NCBI) non-redundant protein database was used. (The version and size of the database can be obtained from a Mascot result pages). MS/MS spectral data was also submitted to *de novo* sequencing using GPS Explorer TM software – DeNovo Explorer Version 3.6. The following parameters were used: enzyme: trypsin, Mass Tolerance; 0.2 Da, fixed modifications; carbamidomethyl, variable modification; oxidation. The top ten sequence matches were submitted to an MS Blast search using the current version at http://dove.embl-heidelberg.de/Blast2/msblast.html.

2.3.6 *E. coli* binding proteins in hemocyte extracts

Ticks (30) were injected with sterile saline and hemolymph collected after 2 h as described in section 2.3.4. After centrifugation at 10 000 *g* for 1min, the plasma was removed and the hemocyte pellet resuspended in TBB buffer on ice and sonified with a Branson sonifier (Branson Sonic Power Co.) using 3 x 6 pulses at 30 % duty cycles on ice. The cell debris was removed by centrifugation (10 000 *g* for 10 min) and the protein content of both the hemocyte extract and the plasma samples was determined by the Bradford method according to the manufacturer's manual (Sigma-Aldrich).

2.4 Results and discussion

2.4.1 Analysis of *E. coli* binding proteins in hemolymph from unchallenged ticks

In an attempt to identify proteins in hemolymph plasma that play a role in binding to Gram-negative bacteria, *E. coli*, was used as affinity beads to adsorb bacterial-binding proteins from plasma. Plasma obtained from unchallenged ticks was incubated with the bacteria and bound proteins were eluted using different conditions. Eluted proteins were analyzed by SDS-PAGE (Fig 2.2). For each eluent a control (bacteria incubated with buffer alone) was included to determine whether the eluted proteins were indeed plasma proteins and not of bacterial origin.

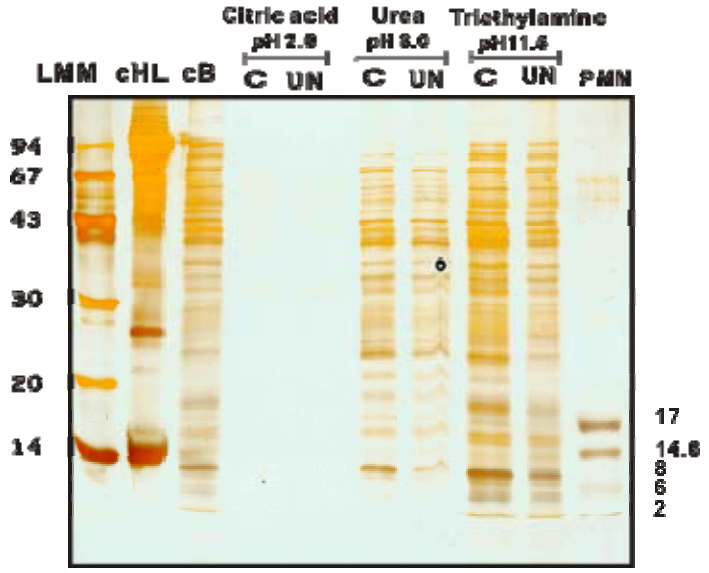


Figure 2.2 SDS-PAGE gel analysis of bacteria binding proteins in plasma of unchallenged ticks. Bacteria cells were incubated with buffer (C) or plasma from unchallenged ticks (UN). Bound proteins were eluted with citric acid (pH 2.0), urea in Tris-HCl (pH 8.0) or triethylamine (pH 11.5) in Tris-HCl. The eluted proteins were precipitated with acetone and analyzed on a 12.5 % gel. Proteins were visualized by silver staining. Two separate controls were included: total hemolymph plasma protein control (cHL) and total bacteria protein control (cB). Low molecular mass markers (LMM) and peptide mass markers (PMM) were also included.

The results in Fig. 2.2 showed that elution with citric acid (pH 2.0) did not have a detrimental effect on the bacterial cells as no proteins were observed in the control lane. These conditions failed to elute any bound plasma proteins

suggesting that the bound plasma proteins are positively charged at pH 2.0 and are strongly associated with the negative charges on the surface of *E. coli*. However, as can be observed in both control lanes and lanes in which cells were incubated with the plasma, elution with urea in Tris- HCl (pH 8.0) and triethylamine, (pH 11.5) released many bacterial proteins, making identification of any bound protein of plasma origin impossible. These results demonstrated that the elution conditions used in this study were unsuitable for further studies.

The experiment was repeated, but instead the bound proteins were eluted with water alone or various concentrations of NaCl (Fig 2.3).

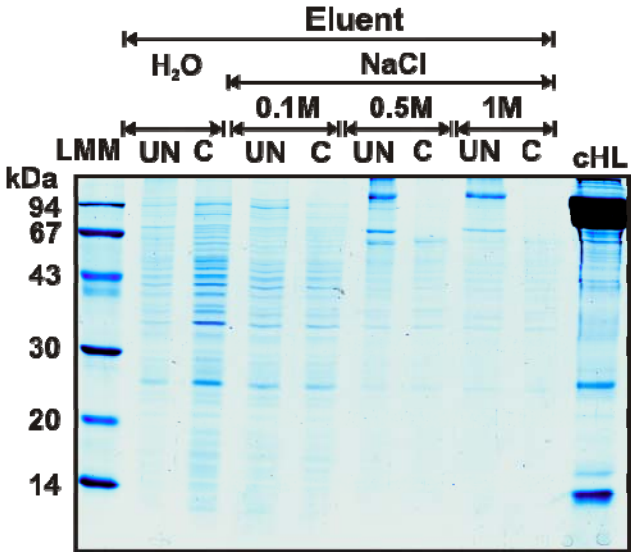


Figure 2.3 SDS-PAGE gel analysis of bacterial binding proteins in plasma of unchallenged ticks. Bacteria cells were incubated with buffer (C) or plasma from unchallenged ticks (UN). Bound proteins were eluted with dddH_2O and NaCl (0.1 M; 0.5 M; 1 M). The eluted proteins were precipitated with acetone and analyzed on a 12.5 % gel. Proteins were visualized by colloidal Coomassie staining. Total hemolymph plasma protein control (cHL) and low molecular mass markers (LMM) were included.

Using these elution conditions bacterial proteins were still visible in all the controls, however elution with both 0.5 and 1 M NaCl resulted in the release of significantly less bacterial proteins. These conditions also led to the release of two plasma proteins (> 94 and ~ 67 kDa) that were not detected in the controls. For subsequent experiments 0.5 M NaCl was used for elution of proteins.

2.4.2 Analysis of *E. coli* binding proteins in hemolymph from challenged ticks

2.4.2.1 Hemocoelic challenge

In order to determine whether additional bacteria binding proteins appear in the plasma following a challenge, the experiment was repeated using hemolymph plasma obtained from ticks that were injected with either saline (aseptic injury) or heat – killed *E. coli* or *B. subtilis* (septic injury). In each case hemolymph was collected from ticks at 2, 12 and 24 h post-challenge (Fig 2.4). The *E. coli* bound proteins were eluted with 0.5 M NaCl and the protein profiles compared to those obtained for unchallenged plasma.

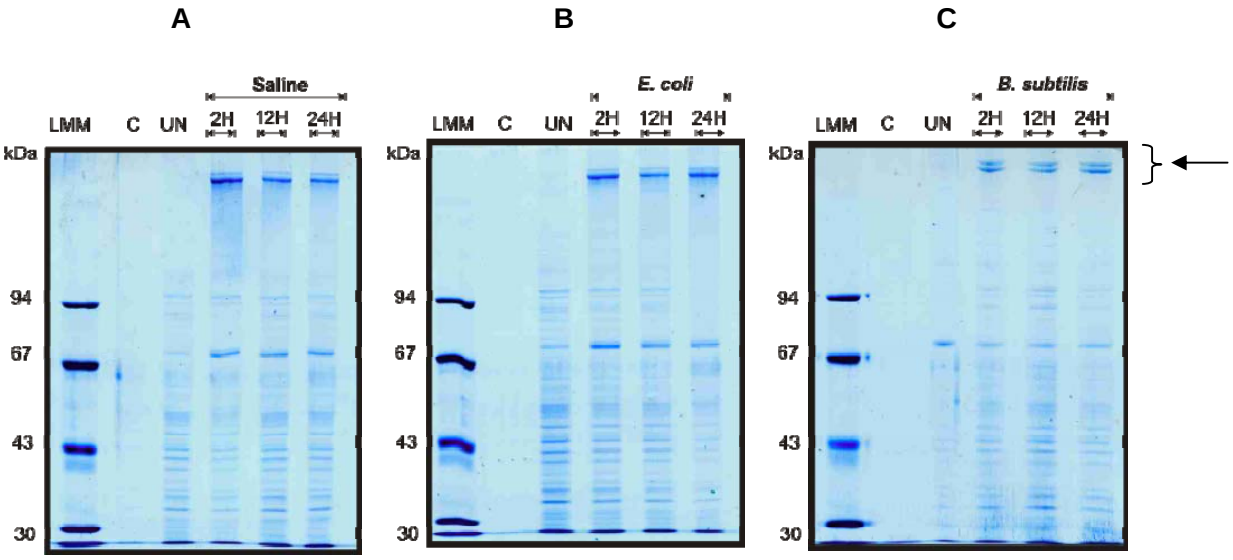


Figure 2.4 SDS-PAGE analysis of bacterial binding proteins, following hemocoelic challenge of ticks. Bacteria cells were incubated with hemolymph plasma obtained from unchallenged ticks (UN) or from ticks injected with either saline (A), heat-killed *E. coli* (B) or *B. subtilis* (C). Hemolymph was obtained from ticks at 2, 12 and 24 h post-challenge. Bound proteins were eluted with 0.5 M NaCl, precipitated with acetone, analyzed on 7.5 % gels and visualized by colloidal Coomassie staining. In the controls, C, cells were incubated with buffer alone. Low molecular mass markers (LMM) were loaded on each gel. Arrow indicates differentially binding high molecular mass proteins.

The results in Fig 2.4 show that both aseptic and septic challenge of ticks leads to the appearance of high molecular mass proteins (HMM; >> 94 kDa). In this

experiment these bands were not observed in the unchallenged plasma. The same trend was observed when the experiment was repeated. The two bacteria binding proteins with sizes of > 94 kDa and ~ 67 kDa observed previously in plasma of unchallenged ticks were present once again in both unchallenged and challenged plasma samples.

2.4.2.2 Challenge by artificial feeding

To investigate whether feeding has an effect on proteins that bind to *E. coli*, ticks were allowed to feed on native blood and blood infected with *B. subtilis* using an artificial feeding system. Hemolymph was collected from these ticks at 24 h and 10 days post-feeding (Fig 2.5).

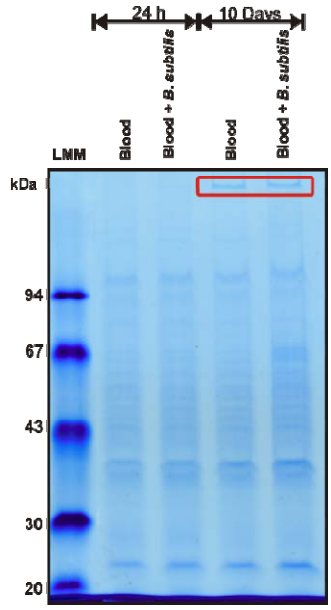


Figure 2.5 SDS-PAGE analysis of bacteria binding proteins following challenge of ticks by artificial feeding. Bacteria cells were incubated with hemolymph plasma obtained from ticks at 24 h and 10 days post-feeding with native blood or blood infected with *B. subtilis*. Bound proteins were eluted with 0.5 M NaCl, precipitated with acetone and analysed on a 7.5 % gel. Bands were visualized by colloidal Coomassie staining. Low molecular mass markers (LMM). Red box: high molecular mass proteins.

SDS-PAGE analysis of the eluted proteins showed that feeding on native blood alone led to the appearance of HMM proteins in plasma 10 days following feeding (Fig 2.5; indicated by red box). No HMM proteins were visible in plasma

24 h after feeding. It appears that tick feeding triggers the release of these two *E. coli* binding proteins and requires a longer period of time for their synthesis. The reason for this could be that the nutrients or pathogen ingested require time to diffuse from the midgut to the body cavity where most of the innate molecules are produced. This finding implies that feeding is also a type of stress and induces a response.

2.4.3 Protein identification

To gain a better understanding of the functions of the bacteria binding proteins and the role these proteins play in innate immunity of ticks it is essential to characterize them. For the identification of the differentially expressed HMM proteins, 30 ticks were injected with sterile saline and the hemolymph was collected from these ticks after 24 h. The binding experiment was repeated and eluted proteins analyzed by SDS-PAGE (Fig 2.6). For their identification, the 2 HMM bands (X and Y; > 250 kDa) were excised from the gels and subjected to MS/MS analysis.

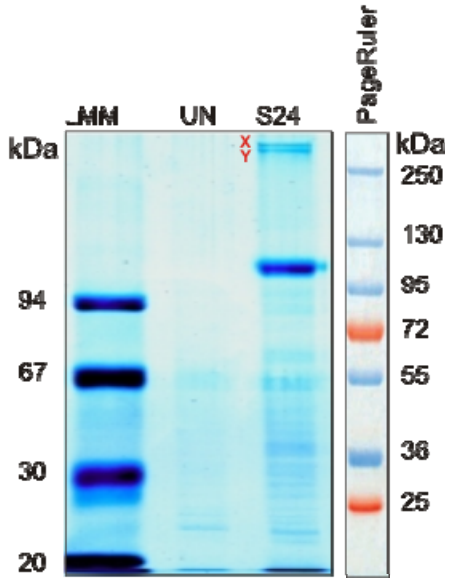


Figure 2.6 SDS-PAGE analysis of bacteria binding proteins following aseptic injury. Bacterial cells were incubated with hemolymph plasma obtained 24 h from ticks injected with sterile saline (S24). The experiment was also performed with plasma obtained from unchallenged ticks (UN). Bound proteins were eluted with 0.5 M NaCl, precipitated with acetone and analyzed on a 7.5 % gel. Bands were visualized by colloidal Coomassie staining and bands X and Y were excised for MS/MS analysis. Low molecular mass markers (LMM) are included.

Data obtained from MS/MS *and de novo* analysis of Protein X and Y are shown in Tables 2.1, 2.2, 2.3 and 2.4. For the *de novo* sequence interpretations from the tandem mass spectra Predict sequence software was used. The complete and partial peptide sequences obtained were then used to search the NCBI database with MS BLAST. The search was performed with the BLASTP2 program. BLASTP2 identification is based on all high-scoring pair (HSP) regions of high local sequence similarity between individual peptides in the query and a protein sequence from the database entry.

The scores obtained from MASCOT for both Protein X and Protein Y were very low (Tables 2.1 and 2.2). When proteins X and Y were run through BLASTP2, Protein X obtained a highest score of 76, while a score of 65 was obtained for Protein Y (Tables 2.3 and 2.4). No matches were found when these sequences were searched with BLAST against the NCBI and a tick database. The *de novo* sequence of Protein X (hit 1) was used to design a degenerate primer in order to identify its gene (Chapter 3).

Table 2.1: MS/MS ion search result for *E. coli* binding Protein X

Hit	Protein[a]	Accession number	Peptide sequence[b]	Observed Mass (M^+ H^+)/ Da	Theoretical Mass (M)/ Da	Mowse score[c]	Expect score
1	novel protein (zgc:92104) [*Danio rerio*]	123232855	R.GTINNDITERDQELQR.L	1901.9557	1900.9239	30	6.2
2	hypothetical protein ACTODO_00361 [*Actinomyces odontolyticus* ATCC 17982]	154507878	R.TMHRTESSVPSEASNPR.R	1900.9484	1900.8697	30	6.4
3	glutamyl(E)/glutaminyl(Q) tRNA Synthetase family member (ers-1) [Caenorhabditis elegans]	17543332	K.NGKFDEGEATLR.L	1336.6735	1335.6419	28	12
4	N-acetylgalactosamine 6-sulfatase (GALNS) [*Rhodopirellula baltica* SH 1]	32476005	R.TTNMNETRTIR.L	1336.6735	1335.6565	28	12
5	PREDICTED: similar to F-box only protein 43 (Endogenous meiotic inhibitor 2) [*Gallus gallus*]	50731779	K.DPDAEYKETLR.A	1336.6735	1335.6306	28	12
6	probable soluble lytic transglycosylase [*Psychrobacter arcticus* 273-4]	71065829	K.TTLTEKLPDYR.S	1336.6735	1335.7034	28	12
7	hypothetical protein Tc00.1047053510155.100 [*Trypanosoma cruzi* strain CL Brener]	71666309	R.GRNLLHSAVTLR.Q	1336.6735	1335.7735	28	12
8	50S ribosomal protein L21 [*Psychromonas sp.* CNPT3]	90408283	K.QHRVAVEQTLR.L	1336.6735	1335.7371	28	12
9	ribosomal protein S6 kinase, 90kDa, polypeptide 4, isoform CRA_d [*Homo sapiens*]	119594669	K.SAQSWMWATLR.R	1336.6735	1335.6394	28	12
10	hypothetical protein LinJ20.0600 [*Leishmania infantum* JPCM5]	146085147	R.ASRESCGTVTLR.S	1336.6735	1335.6565	28	12

[a] Protein to which the peptide sequence was matched with MASCOT

[b] Sequence information obtained for matched peptide from tandem mass spectrometry as determined by searching with MASCOT

[c] Mowse scores greater than 67 are considered significant

Table 2.2: *De novo* sequence results for Protein X

Hit	Score[a]	Accession number	Peptide sequence[b]	Protein name[c]
1	76	3378541	157 VDWTDYYDRM 166	Succinate dehydrogenase, subunit B (EC 1.3.99.1)
2	70	119704	140 DVWTDYYDRM 149	Cephalosporin biosynthesis expandase/hydroxylase [Includes: Deacetoxycephalosporin C synthetase (EC 1.14.20.1)
3	67	26987147	138 NGDVWTDY 145	Nucleotidyltransferase family protein
4	67	55833085	138 NGDVWTDY 145	"orf1"; product: "Orf1"; Pseudomonas fluorescens OstA (ostA) gene, partial cds; PtsP (orfT), Orf1 (orf1), DjlA (djlA), and hypothetical protein (orf2) genes, complete cds; and Kup (kup) gene, partial cds
5	67	28867784	138 NGDVWTDY 145	"nucleotidyltransferase family protein"; *Pseudomonas syringae* pv. tomato str. DC3000 complete genome.
6	63		157 VDWTDYYDRMN 167	"unknown"; Environmental sequence IBEA_CTG_UAAED28TF, whole genome shotgun sequence
7	62	32811191	106 DVWTDYYDTW 115	NTIR7"; *Helianthus annuus* NTIR7 (NTIR7) gene, partial cds.
8	61		158 DWTDYYDRMN 167	"unknown"; Environmental sequence IBEA_CTG_2050970, whole genome shotgun sequence
9	61		158 DWTDYYDRMN 167	"unknown"; Environmental sequence IBEA_CTG_1998552, whole genome shotgun sequence
10	61		158 DWTDYYDRMN 167	"unknown"; Environmental sequence IBEA_CTG_SVAEA17TF, whole genome shotgun sequence
11	61		158 DWTDYYDRMN 167	"unknown"; Environmental sequence IBEA_CTG_SVABU53TF, whole genome shotgun sequence
12	61		158 DWTDYYDRMN 167	"unknown"; Environmental sequence IBEA_CTG_UBAKV86TR, whole genome shotgun sequence
13	61		158 DWTDYYDRMN 167	"unknown"; Environmental sequence IBEA_CTG_SZAST41TR, whole genome shotgun sequence.
14	61		158 DWTDYYDRMN 167	"unknown"; Environmental sequence IBEA_CTG_2061864, whole genome shotgun sequence.
15	58	48734744	773 CRPCHLSR 780	HUMAN DHX37 protein
16	58	55295976	217 VPAWLPPR 224	Hypothetical protein P0038D11.14 (Hypothetical protein P0707D10.41

[a] Score (high scoring pair, HSP) as determined by BLAST, using sequences obtained from *de novo* sequencing. Matches higher than 64 are considered significant

[b] Peptide sequence of the *de novo* data. The numbers give the residues to which the query sequence matched to the protein sequence in the database

[c] Identity of the proteins to which the *de novo* sequences were matched

Table 2.3: MS/MS ion search result for *E. coli* binding Protein Y

Hit	Protein[a]	Accession number	Peptide sequence[b]	Observed Mass (M^+ H^+)/ Da	Theoretical Mass (M)/ Da	Mowse score[c]	Expect score
1	nef protein [Human immunodeficiency virus 1]	90192281	K.EKGGLDGLTHSQQR.Q	1525.6853	1524.7645	34	2.4
2	hypothetical protein SS1G_12076 [*Sclerotinia sclerotiorum* 1980]	156039880	K.ASSTPEPIGHVNGSTTKK.A	1810.8436	1809.9221	29	7.5
3	multidrug resistance protein [*Xanthomonas axonopodis pv.* citri str. 306]	21243571	R.LDAPISGRIGR.S	1154.5946	1153.6567	25	22
4	PREDICTED: similar to novel hemicentin protein [*Strongylocentrotus purpuratus*]	115893492	R.LDVQVSPTNLR.L	1241.6893	1240.6776	25	21
5	hypothetical protein pRL100313A [*Rhizobium leguminosarum bv. viciae* 3841]	116254753	R.RCCSPNSALR.I	1220.5745	1219.5550	25	22
6	membrane fusion protein TbtA [*Pseudomonas stutzeri*]	4321265	K.VLSPISGRIGR.S	1154.5946	1153.6931	25	23
7	GTP-binding protein-like protein [*Leishmania major*]	68127102	K.SVIIPYHVAR.V	1154.5946	1153.6608	25	23
8	hypothetical protein AN6115.2 [*Aspergillus nidulans* FGSC A4]	67539890	K.IEGGGVLFR.V	947.5540	946.5236	25	32
9	hypothetical protein STIAU_2391 [*Stigmatella aurantiaca* DW4/3-1]	115380533	K.CARTPAGVCR.G	1147.5493	1146.5386	24	31
10	GA21010-PA [*Drosophila pseudoobscura*]	125985689	K.GFTTPCGVCR.Q	1154.5946	1153.5009	24	29

[a] Protein to which the peptide sequence was matched with MASCOT

[b] Sequence information obtained for matched peptide from tandem mass spectrometry as determined by searching with MASCOT

[c] Mowse scores greater than 67 are considered significant

Table 2.4: *De novo* sequence results for Protein Y

Hit	Score[a]	Accession number	Peptide sequence[b]	Protein name[c]
1	65	38638613	482 WWPDWC 487	"Bcep1-06"; product: "gp06"; *Burkholderia cenocepacia* phage Bcep1, complete genome
2	63	52425080	408 LVFYFKTHF 416	"unknown"; *Mannheimia succiniciproducens* MBEL55E, complete genome
3	62	50415801	57 ADIGSEKDTH 66	"Unknown (protein for MGC:87732)"; Homo sapiens cDNA clone MGC:87732 IMAGE:5768316, complete cds
4	59	32472387	741 LTFVTNPR 748	"hypothetical protein"; *Pirellula sp.* strain 1 complete genome

[a] Score (high scoring pair, HSP) as determined by BLAST, using sequences obtained from de novo sequencing. Matches higher than 64 are considered significant

[b] Peptide sequence of the *de novo* data. The numbers give the residues to which the query sequence matched to the protein sequence in the database

[c] Identity of the proteins to which the de novo sequences were matched

2.4.4 The source of Protein X and Y

Most hemolymph proteins are derived from hemocytes and the fat body (Hoffmann, 1995; Hoffmann & Reichhart, 2002; Bulet *et al.,* 2004). The aim of this experiment was to investigate whether Proteins X and Y originate from hemocytes. Crude hemocyte protein extracts and total plasma protein samples prepared from hemolymph obtained from ticks at various time intervals following aseptic challenge were analyzed with SDS-PAGE and the results are shown in Fig 2.7.

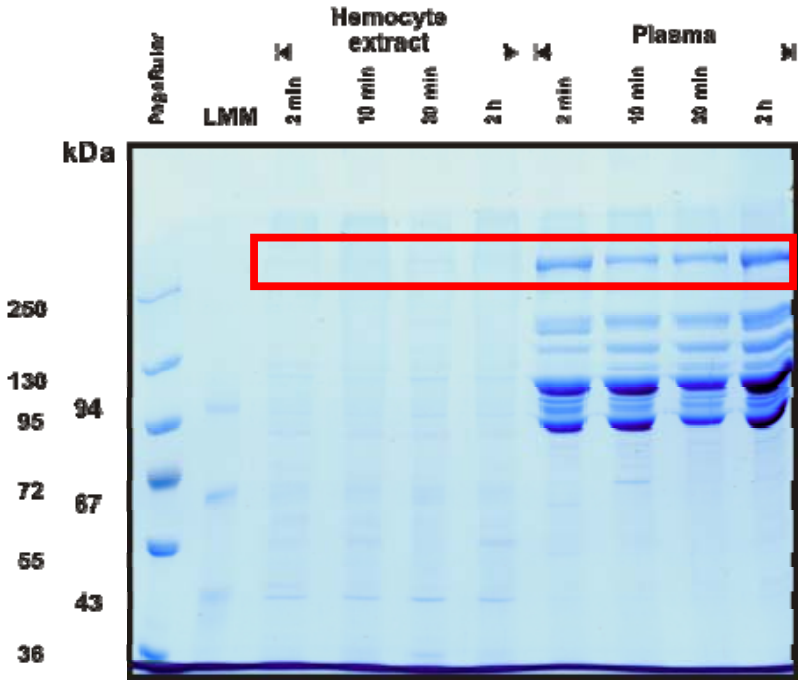


Figure 2.7 SDS-PAGE analysis of hemolymph plasma and hemocyte proteins obtained from ticks at various time intervals (2 min, 10 min, 30 min and 2 h) following aseptic challenge. Samples (10 μg protein per lane) were loaded onto a 7.5 % gel. Bands were visualized by colloidal Coomassie staining. Low molecular mass markers (LMM) are shown. Red box: HMM hemolymph bands (> 250 kDa).

The results indicated that the HMM proteins (> 250 kDa) are absent in the hemocyte extracts, but present in the hemolymph plasma suggesting that these proteins are not of hemocyte origin and are most likely synthesized in other tissues such as the fat body and subsequently released into the hemolymph. Whether this is indeed the case still needs to be confirmed by means of immunolocalization studies. One can also speculate that the HMM proteins are polymers composed of smaller proteins stored within the hemocyte which are released into the hemolymph. An interesting observation was the appearance of the > 250 kDa bands in plasma 2 min following aseptic injury and their subsequent up-regulation 2 h post-challenge (Fig 2.7).

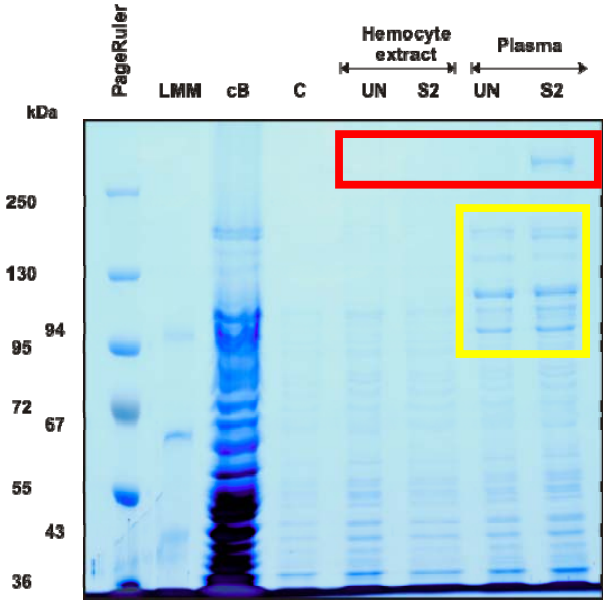


Figure 2.8 SDS-PAGE analysis of hemolymph plasma and hemocyte proteins obtained after 2 h following aseptic challenge. Bacterial cells were incubated either with hemolymph plasma or hemocyte extracts obtained 2 h from ticks injected with sterile saline (S2). The experiment was also performed with plasma obtained from unchallenged ticks (UN). Bound proteins were eluted with 0.5 M NaCl, precipitated with acetone and analyzed on a 7.5 % gel. Bands were visualized by colloidal Coomassie staining. Low molecular mass markers (LMM). Cells were incubated with buffer alone (C); Total bacteria protein control (cB). Red box: Differentially expressed plasma HMM proteins (> 250 kDa). Yellow box: Plasma proteins involved in the immediate defense

The bacterial binding experiment was repeated using both hemocyte extracts and plasma samples prepared from hemolymph obtained from both unchallenged ticks and ticks after 2 h post-aseptic challenge. SDS-PAGE analysis of the eluted proteins (Fig 2.8) showed that the HMM *E. coli* binding proteins were absent in the hemocyte extracts and were present only in the plasma obtained from challenged ticks. Also visible in the gel were *E. coli* binding proteins present in both unchallenged and challenged plasma samples in the size range of > 94 kDa and < 250 kDa. These proteins are most probably involved in the immediate defense response of these ticks.

Chapter 3: Protein X and its relation to savicalin, a lipocalin in hemocytes

3.1 Introduction

The limited number of tick protein sequences available in databases is a drawback to the identification of tick proteins (Madden *et al.,* 2002; Oleaga *et al.,* 2007). The number of tick sequences is however growing rapidly and MS/MS ion spectra data as well as *de novo* sequences may be used in the future to search databases (Blackburn & Goshe, 2009; Shevchenko *et al.,* 2009). However, until then *de novo* sequences may be employed for the design of degenerate primers for gene cloning. This PCR-based approach will allow for further functional characterization (Lingner *et al.,* 1997, Shevchenko *et al.,* 2001).

In this chapter, this approach was used in an attempt to further characterize Protein X described in Chapter 2. This strategy failed to identify the original tick hemolymph *E. coli* binding protein, but led instead to the discovery of a novel lipocalin-like protein in hemocytes.

Lipocalins play an important role in immune response, transport of hydrophobic molecules (such as pheromones, steroids, bilins, retinoids and lipids), cancer cell interactions and allergies (Pervaiz & Brew, 1987; Cowan *et al.,* 1990; Flower *et al.,* 1991; Peitsch *et al.,* 1991; Nagata *et al.,* 1992). These molecules have been detected across all living organisms and exhibit three characteristic features, namely, an unusually low amino acid sequence similarity (typically 15-25 % between paralogs), a highly conserved protein tertiary structure, and a similar arrangement of exons and introns in the coding sequence of their genes (Mans & Neitz, 2004a, b; Mans *et al.,* 2008b). Lipocalins are single modular proteins of around 150-200 amino acids that fold tightly to form a β-barrel that winds around a central axis. Small hydrophobic molecules are bound in a central pocket (Flower, 2000; Flower *et al.,* 2000; Skerra, 2000). These proteins usually have two helices, one at the N- and one at C-terminal end. The N-terminal helix closes

off one side of the barrel and the C-terminal α-helix packs against the outer surface of the barrel. Most lipocalins are classified based on variations observed in the length of the N- and C- terminal segments. These changes are not expected to alter the β-barrel core significantly, and could provide variation for functional diversification and specialization (Montfort *et al.,* 2000).

To date there are more than 300 lipocalin-like molecules in ticks (Tables 3.1, 3.2 and Appendix I, II). Tick lipocalins identified from saliva or salivary glands are distinct from other arthropod lipocalins and could only be assigned to the lipocalin family based on structural similarity (Paesen *et al.,* 2000; Mans *et al.,* 2003). The crystal structure of histamine-binding protein (HBP) from the hard tick, *R. appendiculatus*, established the first functional relationship of tick lipocalins and their ligands and indicated that tick lipocalins could function in an immunomodulatory capacity by scavenging histamine (Paesen *et al.,* 1999; 2000).

Since then, both hard and soft tick lipocalins have been implicated in the binding of a variety of bio-active ligands such as histamine, serotonin, leukotriene B4 (LTB_4), leukotriene C4 (LTC_4), arachidonic acid (AA), and thromboxane A2 (TXA_2) involved in immuno-modulation and platelet aggregation (Mans *et al.,* 2008a, b; Mans & Ribeiro, 2008a, b; Sangamnatdej *et al.,* 2002). Soft tick lipocalins have also been shown to target complement C5 as well as being associated with toxic effects (Mans *et al.,* 2002, 2003; Mans 2005; Mans *et al.,* 2008b; Mans &Ribeiro, 2008a, b; Nunn *et al.,* 2005).

Table 3.1 Lipocalin functions from hard ticks

Gene accession code	Species	name	Expression	Function	Ligand	Reference
18032205	*D. reticulatus*	SHBP	Salivary gland	Suppression of inflammation during feeding	Serotonin and histamine (Two hydrophobic binding pockets)	Sangamnatdej *et al.,* 2002
PDB: 1QFV 3452093	*R. appendiculatus*	HBP1-3	Salivary gland (Stage and gender specific)	Suppression of inflammation during feeding	Histamine (Two hydrophobic binding pockets)	Paesen *et al.,*1999
219935276	*I. ricinus*	LIR6 or Ir-LBP	Salivary gland	Inhibition of neutrophil chemotaxis and host inflammation, delayed LTB4 induced apoptosis, decreased activation of neutrophils	LTB_4	Beaufays *et al.,* 2008a, 2008b
67083329	*I. scapularis*	IXOSC	Salivary gland	Suppression of inflammation	Serotonin	Ribeiro *et al.,* 2006; Mans *et al.,* 2008b
67083266	*I. scapularis*	IXOSC	Salivary gland	Suppression of inflammation	Serotonin	Ribeiro *et al.,* 2006; Mans *et al.,* 2008b

Table 3.2 Lipocalin functions from soft ticks

Gene accession code	Species	name	Expression	Function	Ligand	Reference
114153055	*A. monolakensis*	AM-182	Salivary gland	Suppression of inflammation	Serotonin and histamine	Mans *et al.* ,2008a
114152973	*A. monolakensis*	AM-33	Salivary gland	Suppression of inflammation	LTC_4	Mans *et al.,* 2008a, b
114152975	*A. monolakensis*	Monotonin (AM-38)	Salivary gland	Anti-platelet aggregation	Serotonin	Mans *et al.,* 2008a, b
114152935	*A. monolakensis*	Monomine (AM-10)	Salivary gland	Suppression of inflammation	Histamine	Mans *et al.,* 2008a, b
159944	*O. moubata*	Moubatin	Salivary gland	Inhibition of collagen induced platelet aggregation	AA, TXA_2	Keller *et al.,* 1993, Waxman & Connolly 1993, Mans & Ribeiro 2008b
49409516	*O. moubata*	Lipocalin (OMCI)	Salivary gland	Inhibition of complement system	Complement C5, LTB_4	Nunn *et al.,* 2005
	O. moubata	Lipocalin (OMCI, Chain A)	Recombinant expression (*Pichia methanolica*)	Inhibition of complement system	Fatty acid	Roversi *et al.* ,2007
149287030	*O. parkeri*	Moubatin homolog 3 (OP-3)	Salivary gland	Suppression of inflammation	LTC_4	Francischetti *et al* , 2008a; Mans & Ribeiro 2008b
25991386	*O. savignyi*	TSGP1	Salivary gland	Salivary gland biogenesis	Histamine and serotonin	Mans *et al.,* 2001, 2003, 2004a; Mans & Ribeiro 2008b
25991388	*O. savignyi*	TSGP2	Salivary gland	Suppression of inflammation, Toxic to host cardiovascular system	LTB_4, AA, TXA_2 and Complement C5	Mans *et al.,* 2001; 2003; 2004a; Mans & Ribeiro 2008b
25991390	*O. savignyi*	TSGP3	Salivary gland	Anti-platelet aggregation	LTB_4, Complement C5	Mans *et al.* ,2001; 2003; 2004a; Mans & Ribeiro 2008b
25991437	*O. savignyi*	TSGP4	Salivary gland	Suppression of inflammation, Toxic to host cardiovascular system	LTC_4, LTD_4, LTE_4,	Mans *et al.,* 2001; 2003; 2004a

3.1.1 Lipocalins found in hard ticks

In *R. appendiculatus*, female specific HBPs, have been isolated from the salivary gland and were found to sequester histamine released by the host in response to tissue damage. HBPs fulfill one of the roles described for nitrophorin in hematophagous insects by reducing the immune and inflammatory host responses (Paesen *et al.,* 1999; Montfort *et al.,* 2000).

HBPs consist of two separate internal and binding sites for histamine. The high affinity and low affinity sites are lined with acidic residues, useful for binding a basic ligand. The hydrophobicity of these pockets represents another striking difference with the binding pockets of most lipocalins suited for binding hydrophobic ligands (Paesen *et al.,* 1999; 2000). The high affinity site occupies the position expected for other lipocalins, but the entrance of histamine to this site is anomalous when compared with the open side of other lipocalin pockets. The low affinity site occupies the closed end of the barrel (Paesen *et al.,* 2000).

The expression of HBPs is stage and gender specific in that HBP1 and 2 are secreted by adult females while HBP3 is secreted by larvae, nymphs and adult males. They also differ in their glycosylation and macromolecular complexes. HBP1 and HBP2 are non-glycosylated monomers, while HBP3 forms disulfide-linked dimers. The functional significance of their temporal and gender-dependent regulation and other molecular attributes are not fully understood (Paesen *et al.,* 1999, 2000).

In *D. reticulatus*, another type of lipocalin molecule known as a serotonin and histamine binding protein (SHBP) contains two internal binding sites. Binding of histamine to the high affinity site has been studied. In contrast a ligand for the low affinity site has not yet been identified. Analysis of its structure, however, suggests serotonin to be the most likely candidate ligand (Sangamnatdej *et al.,* 2002).

Lipocalins found in the hard tick, *I. ricinus,* are segregated into phylogenetic groups suggesting potential distinct functions. This was demonstrated by the lipocalin of *I. ricinus* (LIR6) later designated as *I. ricinus* lipocalin leukotriene B4 protein (Ir-LBP). As the name suggests it scavenges leukotriene B4. Other LIRs did not bind any of the other ligands tested. These included 5-hydroxytryptamine, ADP, norepinephrine, platelet activating factor, prostaglandins D2, E2 , LTB_4 and LTC_4 (Beaufays *et al.,* 2008a, b).

Ten putative lipocalin sequences were obtained from *I. pacificus* by Francischetti *et al.* (2005). The analysis of their primary sequences suggested that they are secreted proteins. So far there is no information on the structural or biochemical properties to understand their role in blood-feeding.

3.1.2 Lipocalins found in soft ticks

In *A. monolakensis,* 33 lipocalin-like sequences have been identified from the cDNA library of adult female salivary glands (Mans *et al.,* 2008a, b). Only 3 out of the 33 sequences have been identified as having lipocalin functions. The crystal structure of monotonin (AM-38) and monomine (AM-10) have only one single binding site rather than the two sites described for HBPs in the hard tick *R. appendiculatus*. The binding site of monotonin and monomine displays a similar low affinity binding site like that of HBP. The binding sites of monomine and monotonin are similar to the low affinity site of the female specific HBP. The interaction of the protein with the aliphatic amine group of the ligand is very similar for all of the proteins, whereas specificity is determined by interactions with the aromatic portion of the ligand. Protein interaction with the imidazole ring of histamine differs significantly between the low affinity binding site of HBP and monomine, suggesting that histamine binding has evolved independently in the two lineages (Mans *et al.,* 2008a, b). AM-33, another lipocalin-like molecule from *A. monolakensis*, is related to tick salivary gland protein 4 (TSGP4) of the soft

tick *O. savignyi*, which binds cysteinyl leukotrienes with high affinity (Mans & Ribeiro 2008a).

Four lipocalins were identified in *O. savignyi* and designated as tick salivary gland proteins (TSGPs). TSGPs have been proposed to have a role in salivary gland granule biogenesis and are stored in the secretory granules (Mans *et al.,* 2001, 2003; Mans & Neitz 2004a). They do not bind histamine nor any of the other mediators involved in the control of host response to tick bites. TSGPs do not affect the blood coagulation cascade or ADP- and collagen-induced platelet aggregation. TSGP2 and TSGP4 were identified as toxins that affect the cardiovascular system of the host and are therefore involved in the pathogenesis of toxicosis caused by the *O. savignyi* bite (Mans *et al.,* 2002, 2003). The toxicity of these lipocalins might be considered as detrimental for the feeding parasite especially for ticks that have to spend longer periods of time on the host to complete a meal. TSGPs were modeled using the known structure of HBP2 with a reasonable fit.

A lipocalin identified in *O. moubata* saliva, is moubatin. This molecule shows a similarity with HBPs and displays platelet aggregation inhibitory activity (Keller *et al.,* 1993; Waxman & Connolly, 1993). A recent study by Mans & Riberio (2008b) showed that moubatin and TSGP3 inhibit platelet aggregation by scavenging TXA_2 and thus act as potent inhibitors of TXA_2 mediated vasoconstriction. TSGP2, on the other hand, is unable to inhibit platelet aggregation due to an amino acid substitution in the lipocalin-binding cavity in position 85 (Mans & Ribeiro, 2008b). Moubatin, *O. moubata* complement inhibitor (OMCI), TSGP2 and TSGP3 scavenge LTB_4 which implicates them in the modulation of neutrophil function. As far as the C5 complement ligand is concerned, only TSGP2 and TSGP3 can bind, but not moubatin, in a similar manner as the OMCI. TSGP3 and moubatin have also shown high affinity toward arachidonic acid (Mans & Ribeiro, 2008a).

Recently, other lipocalin sequences have been identified from salivary gland cDNA of *O. coriaceus* and *O. parkeri* (Francishetti *et al.,* 2008a, b). Almost none of their structural and biochemical functions have been determined, except for moubatin homolog 3 (OP-3) which binds to serotonin and histamine (Mans & Ribeiro 2008b).

In general, tick lipocalins are very eccentric members of the lipocalin family, which highlights the versatility of the lipocalin fold to carry out many functions (Paesen *et al.,* 2000). Despite the structural and biochemical differences of tick salivary gland lipocalins, the resemblance to the β-barrel of standard lipocalins and the data of their gene structure show more similarities and therefore they are assigned as lipocalins.

All tick lipocalins described thus far are salivary gland derived and presumed to be involved in tick feeding (Mans *et al.,* 2008b; Valenzuela *et al.,* 2002; Francischetti *et al.,* 2008a, b; Ribeiro *et al.,* 2006). However, lipocalins have been described in other arthropods that are not involved in blood-feeding, but in processes such as development, coloration, defense mechanisms and transport of ligands (Sánchez *et al.,* 1995, 2000, 2006; Weichsel *et al.,* 1998; Andersen *et al.,* 2005; Kayser *et al.,* 2005; Mauchamp *et al.,* 2006). The possibility thus exists that lipocalins might play a much larger role in tick biology that is not limited to the feeding process alone. The current study describes such a lipocalin from the hemocytes of *O. savignyi*.

3.2 Hypothesis

Degenerate primers designed from a *de novo* sequence obtained from Protein X will enable its characterization as an immunoprotective agent.

3.3 Materials and methods

3.3.1 Hemolymph collection and RNA extraction

Ticks were dorsally immobilized with double-sided tape. A 30 gauge needle was used to puncture the first pair of coxae at the base of the trochanter followed by gentle pressure on the abdomen (Johns *et al.,* 1998). The exuding hemolymph was collected (~ 200 µl) from 20 ticks using a glass capillary and was immediately added to 800 µl of TRI-Reagent (Sigma-Aldrich). RNA was isolated according to the manufacturer's instructions.

3.3.2 Single stranded cDNA synthesis

Single stranded cDNA was prepared from total RNA (500 ng) using 7 µl of a 12 µM 5´ Smart IIA anchor primer AAG CAG TGG TAT CAA CGC AGA GTA CGC GGG, and a poly-T anchor primer GCT ATC ATT ACC ACA AAC CAC TCT TTT TT. DEPC-H_2O was then added to obtain a final volume of 64 µl. The reaction mixtures were then spun briefly in a microcentrifuge and incubated at 65 ^oC for 2 min in a thermal cycler, which allowed for the denaturation of RNA secondary structure. The samples were immediately placed on ice for 5 min in order to prevent the reformation of RNA secondary structure. To each reaction tube, 10 µl 50 X dNTP's (10 mM) (Roche Diagnostics, Indianapolis, USA), 20 µl 5 X first strand buffer, 2 µl of 100 mM DTT, 100 U RNase inhibitor (Promega, Madison, WI, USA), 500 U Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and 5 µl DEPC-H_2O were added. The above mentioned components were available with the Superscript III reverse transcriptase kit. The reaction mixtures were mixed by gentle pipeting and then spun down using a microcentrifuge. This was followed by incubation at 42 ^oC for 90 min in a Perkin Elmer Gene Amp PCR system 2400. At the end of the incubation period, 2 µl (0.5 M) EDTA was added to stop the reaction. Samples were stored at -20 ^oC until purification.

Purification of the first strand DNA was performed using the Nucleospin Extract II PCR clean up and Gel extraction kit (Macherey-Nagel, Duren, Germany). Two volumes of buffer NT was added to 1 volume of sample. This starting buffer contains chaotropic ions which provide the ideal environment for the first strand cDNA to bind to the silica. The

samples were then loaded onto the columns and centrifuged for 1 min at 11 000 *g*. Buffer NT3 (600µl) was added to wash the column. Contaminants were removed by centrifugation at 11 000 *g* for 1 min. Buffer NT3 contains ethanol and for this reason, the columns were centrifuged for an additional 2 min at 11 000 *g* to remove excess ethanol and to dry the silica column. Tubes were then incubated at 70 ^{o}C for 5 min in order to ensure complete removal of ethanol which would otherwise inhibit downstream reactions. Thereafter, 50 µl of elution buffer (Buffer NE: 5 mM Tris-HCl, pH 8.5) was added and columns were incubated at 37 ^{o}C for 5 min and subsequently centrifuged at 11 000 *g* for 1 min. The concentration of the DNA using a 10 times dilution, was determined using the Gene Quant and the remaining sample was stored in a low adhesion siliconized tube at -20 ^{o}C.

3.3.3 Degenerate primer design and 3´ RACE

To obtain the coding gene and 3´-untranslated region for Protein X, a degenerate primer TGG ACG GA(T/C) TA(T/C) TA(T/C) GA(T/C) (A/C)G (Integrated DNA Technologies, Coralville, USA) was designed from a *de novo* sequence WTDYYDRM, which had been previously obtained for Protein X (Table 2.2). Single-stranded cDNA, degenerate primer (50 µM) and poly-T anchor primer (10 µM) were used to optimize 3' RACE using Ex Taq polymerase (Takara, Japan). Optimized conditions consisted of an initial cDNA denaturation (94 ^{o}C, 3 min), hot start addition of enzyme (80 ^{o}C), followed by 35 cycles of DNA denaturation (94 ^{o}C, 30 s), annealing (52 ^{o}C, 25 s) and extension (72 ^{o}C, 2 min) followed by a final extension (72 ^{o}C, 7 min).

3.3.4 Cloning and sequencing of amplified cDNA

One-tenth of amplified products were analyzed by agarose gel electrophoresis and the rest of the amplified products were precipitated by adding 1/5 volume of sodium acetate (pH 5.0) and 3 volumes of 100 % ethanol. The solution was centrifuged for 45 min at 13 000 *g* at 4 ^{o}C. This was followed by washing the DNA pellet using 300 µl of 70 % ethanol and centrifuged for 45 min at 13 000 *g* at 4 ^{o}C. The supernatant was discarded and the pellet was washed with a final volume of 300 µl of 70 % ethanol and centrifuged

for a further 10 min at 13 000 *g* at 4 °C. Once the supernatant was removed the samples were dried in a vacuum concentrator (Bachoffer, Germany). The purified PCR products were reconstituted with 20 µl of dddH_2O (double sterilized, double distilled deionized water) and the concentration was determined using a Nanodrop spectrophotometer (Amersham Bioscience, USA).

In order to facilitate the downstream sequencing reaction the PCR product purified above was ligated into the pGEM T Easy vector system (Promega, Madison, WI, USA), using 5 µl of 2 x T4 DNA ligase buffer, 1 µl of 50 ng/µl pGEM-T easy vector. The amount of insert to be used was calculated using the formula:

$$\frac{\text{Size of insert (bp)} \times \text{Amount of vector used (ng)}}{\text{Size of vector (bp)}} \times \frac{3}{1}$$

PCR water was added to make up a final volume of 10 µl. The ligation reactions were precipitated by the addition of 1µl tRNA, 2 µl sodium acetate (pH 5.0) and 30 µl 100 % ethanol, to the 10 µl ligation reactions. The solutions were centrifuged for 45 min at 13 000 *g* at 4 °C and washed with 70 % ethanol and centrifuged for a further 10 min at 13 000 *g* at 4 °C. These steps were repeated twice to remove salts completely. Once the supernatant was removed the samples were dried in the vacuum concentrator and reconstituted in 20 µl PCR grade water.

Fresh colonies of *E. coli* (BL21) cells were used to inoculate 50 ml of SOB medium [2 % (w/v) tryptone; 5 % (w/v) yeast extract 10 mM NaCl; and 2.5 mM KCl, pH 7.0] in a 500 ml flask. Cells were proliferated with vigorous aeration overnight at 37 °C. Cells (2.5 ml) were diluted into 250 ml of SOB in a 1 L flask. They were grown for 2~3 h with vigorous aeration at 37 °C until the cells reached an OD_{600} ~ 0.5. Cells were harvested by centrifugation at 2 600 *g* for 10 min. The cell pellet was washed by re-suspension in 250 ml of sterile ice-cold wash buffer [10 % glycerol, 90 % distilled water, (v/v)]. The cell suspension was centrifuged at 2 600 *g* for 15 min. Cells were washed again in 250 ml of

sterile ice-cold double distilled water and the same wash process was repeated. The cell suspension was centrifuged at 2 600 *g* for 15 min and the supernatant was poured off. The cell pellet was resuspended in wash buffer to a final volume of 1 ml. The cell suspension was divided into 0.1 ml aliquots and stored at - 70 ^{o}C.

Electroporation cuvettes obtained from BioRad were kept at -20 ^{o}C, 1 h prior to use. *Escherichia coli* (BL21) cells were allowed to thaw on ice. The ligation reaction (20 µl) was added to cells and mixed by gentle swirling. This reaction mixture was then pipetted into the cold cuvette and inserted into a slot in the chamber rack and pulsed at 2000 mV for 5 ms [Electroporator 2510 (Eppendorf, Germany)]. Thereafter, 100 µl of LB-glucose [0.01 % (w/v) tryptone; 0.01 % (w/v) NaCl; 0.005 % (w/v) yeast extract and 2 M D-glucose] was added to the electroporated cells in order to allow for recovery of the cells. The cells were then transferred to the remaining 800 µl LB-glucose. These cell solutions were incubated at 37 ^{o}C for 45 min with shaking at 250 rpm of which 100 µl were plated onto LB-ampicillin-Xgal-IPTG plates (1 % (w/v) agar in LB-broth, 50 µg/ml ampicillin, 20 µl of 200 mg/ml IPTG and 4 µl of 50 mg/ml X-Gal). Plates were incubated at 37 ^{o}C for 16 h.

Colony PCR was performed to identify plasmids with the correct inserts. Random colonies were picked with a pipette tip and dipped into a tube that contained 1 µl of 50 µM degenerate primer and 1 µl of 10 µM SP6 primer and 12.5 µl of KapaTaq Ready mix (Taq polymerase, dNTP, buffer, 25 mM $MgCl_2$) from Kapa Bioscience and 10.5 µl PCR water for a total volume of 25 µl. The tip was then dropped into a 5 ml LB-ampicillin (LB-broth containing 5 µl of 50 mg/ml ampicillin) tube and the cells were allowed to proliferate at 37 ^{o}C in a shaking incubator at 250 rpm. Colonies containing the correct insert were grown overnight (16 h) for subsequent plasmid isolation.

Plasmid DNA was recovered using the NucleoSpin® Ready-to-use system for fast purification of nucleic acids (Macherey-Nagel,, Germany) as described in the manual. Overnight cultures (5 ml) that reached an optical density at 600 nm (OD_{600}) of approximately 3 were centrifuged for 10 min at 1 000 *g* in sterile test tubes. The cell

pellet was resuspended in 500 µl of resuspension buffer A1 containing RNases and vortexed. Lysis buffer A2 (500 µl) was then added and mixed by gentle inversion of the tubes 6 times. This solution was incubated at room temperature for 5 min. Neutralization buffer A3 (600 µl) was added and the solutions mixed by gentle inversion of the tubes. Since Buffer A3 is acidic it leads to the neutralization of the solutions. The solutions were then centrifuged at 11 000 *g* for 10 min at room temperature to remove cell lysates. The supernatant was loaded onto columns and centrifuged at 11 000 *g* for 1 min after which 600 µl of wash buffer A4 containing ethanol was added followed by centrifugation at 11 000 *g* for 1 min. An additional centrifugation step of 11 000 *g* for 2 min allowed the removal of excess ethanol and drying of the silica membrane. Purified plasmid was eluted with 50 µl PCR water followed by centrifugation for 1 min at 11 000 *g* after a 1 min incubation at 37 ^{o}C. The concentration was determined by using the Nanodrop spectrophotometer.

Plasmids (550 ng in 1 µl) with correct inserts were sequenced in both directions either using 1 µl of T7 forward primer with sequence 5´-TAA TAC GAC TCA CTA TAG GG-3´ of and SP6 reverse primer with sequence 5´-TAT TTA GGT GAC ACT ATA G-3´ at concentrations of 5 µM in 3 µl of sequencing buffer, reconstituted to a final volume of 18 µl with PCR water. The reaction mixtures were spun down briefly in a microcentrifuge and incubated at 94 ^{o}C for 2 min to allow denaturation of the plasmid DNA. The ABI Big Dye solution (2 µl) was added in a hot start addition at 80 ^{o}C after 1 min. The reactions were then cycled: 96 ^{o}C for 20 s, 50 ^{o}C for 30 s and 60 ^{o}C for 3 min for a total of 26 cycles. The sequencing reactions were precipitated by adding 4 µl of sodium acetate (pH 5.0) and 60 µl of 100 % ethanol was added to each 20 µl sequencing reaction. This solution was then centrifuged at 13 200 *g* at 4 ^{o}C for 45 min after which the supernatant was gently aspirated. The pellet was washed using 50 µl 70 % ethanol and centrifugation at 13 200 *g* at 4 ^{o}C for 10 min. The wash step was repeated twice. The nucleotide sequences were analyzed using an automated ABI 3130 DNA sequencer.

DNA sequences were analyzed for similarities with known sequences using the BLAST (Basic Local Alignment Search Tool) algorithm (www.ncbi.nlm.nih.gov). The BLAST algorithm (Altschul *et al.,* 1990; 1997) searches for local (as opposed to global) alignments and reports the significance of the search results as an expect value. The expect value is a parameter that describes the number of hits one can expect to see just by chance when searching a database. It essentially describes the random background noise that exists for matches between sequences. The lower the e-value of a similarity, the higher the probability that the hit is significant. Generally, an e-value of <0.0001 (1e-04) is considered highly significant. The amino acid sequence of the protein was deduced using Bioedit, while Expasy was used to predict amino acid composition, hydrophobicity profile and pI.

3.3.5 Sequence retrieval for multiple sequence alignments and phylogenetic analysis

Lipocalin sequences from both hard and soft ticks as listed below were retrieved from the NCBI Genebank database, by BLASTP, TBLASTN and PSI-BLAST analysis using the obtained amino acid sequence for Protein X (Altschul *et al.,* 1990, 1997). All sequences were NCBI database entries. NCBI entries are composed by a common description and Gene bank accession codes. Multiple sequence alignments of the tick lipocalins were performed using ClustalX with default parameters (Jeanmougin *et al.,* 1998; Larkin *et al.,* 2007). Sequences were manually checked and adjusted accordingly. Neighbor joining (NJ) analysis was conducted using MEGA version 4.0 (Saitou & Nei, 1987; Tamura *et al.,* 2007). Gapped positions were completely deleted so that 55 informative sites were used for analysis. Reliability of the inferred tree was evaluated by bootstrap analysis (100 000 replicates).

The sequences referred to are the following:

Lipocalin [*Amblyomma americanum*] (196476629), lipocalin [*Argas monolakensis*] (114153056), lipocalin [*Argas monolakensis*] (114153300), lipocalin [*Argas monolakensis*] (114153282), lipocalin [*Argas monolakensis*] (114153166), lipocalin [*Argas monolakensis*] (114153124), lipocalin [*Argas monolakensis*] (114153090), lipocalin [*Argas monolakensis*] (114153072), lipocalin [*Argas monolakensis*] (114153054), lipocalin [*Argas monolakensis*] (114153036), lipocalin [*Argas monolakensis*] (114152998), lipocalin [*Argas monolakensis*] (114152996), lipocalin [*Argas monolakensis*] (114152994), lipocalin [*Argas monolakensis*]

(114152990), lipocalin [*Argas monolakensis*] (114152982), lipocalin [*Argas monolakensis*] (114152974), lipocalin [*Argas monolakensis*] (114152960), lipocalin [*Argas monolakensis*] (114152958), Chain A, Crystal Structure Of Am182 Serotonin Complex [*Argas monolakensis*] (171849040), Chain A, Crystal Structure Of Monomine [*Argas monolakensis*] (171849042), Monomine [*Argas monolakensis*] (114152936), Monotonin [*Argas monolakensis*] (114152976), Chain A, Crystal Structure Of Monomine-Histamine Complex [*Argas monolakensis*] (171849043), lipocalin-like protein [*Rhipicephalus (Boophilus) microplus*] (45360102), serotonin and histamine binding protein [*Dermacentor reticulatus*] (18032205), putative secreted histamine binding protein of 25.9 kDa [*Ixodes pacificus*] (51011604), putative secreted histamine binding protein of 22.5 kDa [*Ixodes pacificus*] (51011586), IR1 [*Ixodes ricinus*] (219935266), LIR2 [*Ixodes ricinus*] (219935268), LIR3 [*Ixodes ricinus*] (219935270), LIR4 [*Ixodes ricinus*] (219935272), LIR5 [*Ixodes ricinus*] (219935274), LIR6 or Ir-LBP [*Ixodes ricinus*] (219935276), LIR7 [*Ixodes ricinus*] (219935278), LIR8 [*Ixodes ricinus*] (219935280), LIR9 [*Ixodes ricinus*] (219935284), LIR10 [*Ixodes ricinus*] (219935288), LIR11 [*Ixodes ricinus*] (219935290), LIR12 [*Ixodes ricinus*] (219935292), LIR13 [*Ixodes ricinus*] (219935294), LIR14 [*Ixodes ricinus*] (219935296), 25 kDa salivary gland protein A [*Ixodes scapularis*] (15428310), putative secreted salivary protein [*Ixodes scapularis*] (67083547), 25 kDa salivary gland protein B [*Ixodes scapularis*] (15428302), putative protein [*Ixodes scapularis*] (22164276), putative secreted protein with HBP domain [*Ixodes scapularis*] (67083737), putative salivary secreted protein [*Ixodes scapularis*] (67083439), 25 kDa salivary gland protein family member [*Ixodes scapularis*] (67083725), histamine binding protein [*Ixodes scapularis*] (67083717), histamine binding protein [*Ixodes scapularis*] (15428292), histamine binding protein [*Ixodes scapularis*] (67083721), salivary histamine binding protein [*Ixodes scapularis*] (67083719), 25 kDa salivary gland protein C [*Ixodes scapularis*] (67083485), 25 kDa salivary gland protein C, putative [*Ixodes scapularis*] (215509983), putative secreted protein [*Ixodes scapularis*] (67083637), putative salivary HBP family member [*Ixodes scapularis*] (67083407), secreted protein, putative [*Ixodes scapularis*] (215498016), secreted protein, putative [*Ixodes scapularis*] (215502003), putative 22.5 kDa secreted protein [*Ixodes scapularis*] (22164318), putative secreted salivary protein [*Ixodes scapularis*] (67083669), putative secreted protein [*Ixodes scapularis*] (67083623), secreted protein, putative [*Ixodes scapularis*] (215491831), putative salivary secreted protein [*Ixodes scapularis*] (67083682), nymphal histamine binding protein [*Ixodes scapularis*] (67083741), salivary lipocalin [*Ornithodoros coriaceus*] (172051218), salivary secreted lipocalin [*Ornithodoros coriaceus*] (172051166), salivary lipocalin [*Ornithodoros coriaceus*] (172051154), salivary lipocalin [*Ornithodoros coriaceus*] (172051146), salivary lipocalin [*Ornithodoros coriaceus*] (172051116), salivary lipocalin [*Ornithodoros coriaceus*] (172051090), moubatin-like lipocalin [*Ornithodoros coriaceus*] (172051084), salivary lipocalin [*Ornithodoros coriaceus*] (172051236), complement inhibitor precursor [*Ornithodoros moubata*] (49409517), Chain A, The Complement Inhibitor Omci In Complex With Ricinoleic Acid [*Ornithodoros moubata*] (146386434), Chain A, The Complement Inhibitor Omci In Complex With Ricinoleic Acid [*Ornithodoros moubata*] (146386433), salivary secreted lipocalin [*Ornithodoros parkeri*] (149287112), salivary lipocalin [*Ornithodoros parkeri*] (149287038), salivary lipocalin [*Ornithodoros parkeri*] (149287008), truncated salivary lipocalin [*Ornithodoros parkeri*] (149286990), salivary lipocalin [*Ornithodoros parkeri*] (149286978), salivary lipocalin [*Ornithodoros parkeri*] (149286916), salivary lipocalin [*Ornithodoros coriaceus*] (172051234), salivary lipocalin [*Ornithodoros coriaceus*] (172051222), salivary lipocalin [*Ornithodoros coriaceus*] (172051210), moubatin-like lipocalin [*Ornithodoros coriaceus*] (172051206), salivary lipocalin [*Ornithodoros coriaceus*] (172051204), salivary lipocalin [*Ornithodoros coriaceus*] (172051168), salivary lipocalin [*Ornithodoros coriaceus*] (172051114), salivary lipocalin [*Ornithodoros coriaceus*] (172051112), salivary lipocalin [*Ornithodoros coriaceus*] (172051078), salivary lipocalin [*Ornithodoros coriaceus*] (172051062), salivary lipocalin [*Ornithodoros parkeri*] (149287102), salivary lipocalin [*Ornithodoros parkeri*] (149287092), salivary secreted lipocalin [*Ornithodoros parkeri*] (149287088), salivary lipocalin [*Ornithodoros parkeri*] (149287084), salivary lipocalin [*Ornithodoros parkeri*] (149287076), salivary lipocalin [*Ornithodoros parkeri*] (149286974), salivary lipocalin [*Ornithodoros parkeri*] (149286972), moubatin-like 7 [*Ornithodoros parkeri*] (149287170), moubatin-like 4 [*Ornithodoros parkeri*] (149287126), moubatin-like 5 [*Ornithodoros parkeri*] (149287116), moubatin-like 5 variant [*Ornithodoros parkeri*] (149287118), moubatin-like 3 [*Ornithodoros parkeri*] (149287030), moubatin 1-like 2 [*Ornithodoros parkeri*] (149287000), cDNA sequence from whole ticks [*Ornithodoros porcinus*] (17510378), TSGP4 [*Ornithodoros savignyi*] (25991438), TSGP3 [*Ornithodoros savignyi*] (25991391), TSGP2 [*Ornithodoros savignyi*] (25991389), TSGP1/ lipocalin [*Ornithodoros savignyi*] (25991387), Chain A, Histamine Binding Protein From Female Brown Ear *Rhipicephalus appendiculatus* [*Rhipicephalus appendiculatus*] (7767032).

3.3.6 Homology modeling and quality assessment

For homology modeling of savicalin, female specific histamine binding protein (PDB ID: 1QFT, 1QFV; Paesen *et al.,* 1999) was used as a template using SWISS-PdbViewer (Guex & Peitsch, 1997). The initial model was submitted to the SWISS-MODEL automated comparative protein modeling server (Guex *et al.,* 1999). Savicalin's sequence was also submitted to the Phyre fold recognition server (Kelley & Sternberg, 2009) and analyzed using the conserved domain database (CDD) (Marchler-Bauer *et al.,* 2009).

3.3.7 Transcriptional profiling

Reverse transcription polymerase chain reaction (RT-PCR) was carried out to analyze gene expression of savicalin. *Bacillus subtilis* (ATCC: 13933) cells were resuspended in physiological saline to a final concentration of the 2.5 x 10^6 cells/ml and 1 μl of the suspension was heat-inactivated and injected into 20 unfed adult female ticks. Saline was injected into the same number of ticks as a control. Total RNA was isolated as described in section 3.3.1 from the midguts, ovaries, salivary glands and hemolymph obtained from both groups of ticks 24 h post injection.

In the second part of the experiment, 20 ticks were fed artificially on heparinized cattle blood (obtained from Experimental Farm, University of Pretoria, SA) infected with *B. subtilis* (2.5 x 10^6 cells/ml blood) as described section 2.3.4.2. Native blood (no bacteria added) was used for the control group. At 1 day and 10 days post feeding, total RNA was isolated from the same tissues as described for unfed, hemocoelic injected ticks.

First strand synthesis was performed as described in section 3.3.2. For expression analysis, first strand cDNA for each tissue (500 ng), 10 μM of both the gene specific primer TGG ACG GAT TAC TAC GAC CG and a poly-T anchor primer were used. The forward primer CAG ATC ATG TTT GAG ACC TTC AAC and reverse primer G(C/G)C CAT CTC (T/C)TG CTC GAA (A/G)TC at a concentration of 10 μM were used for the amplification of the housekeeping gene, actin. PCR reactions were performed using an

initial denaturation step (94 ^{o}C, 3 min), hot start addition of exTaq enzyme (80 ^{o}C) followed by 35 cycles of DNA denaturation (94 ^{o}C, 30s), annealing (54 ^{o}C, 25s) and extension (72 ^{o}C, 2 min) followed by a final extension (72 ^{o}C, 7 min).

3.4 Results and discussion

3.4.1 Sequence analysis

As described in the previous chapter, none of *O. savignyi* hemolymph proteins that recognize and bind to *E. coli* bacteria could be identified by searching the current databases with both the MS/MS ion spectra as well as the derived *de novo* sequences obtained for these proteins. In this study, a degenerate primer derived from a *de novo* sequence for one of these hemolymph proteins, was used for 3´-RACE and resulted in the amplification of a single 900 bp fragment from cDNA prepared from total hemocyte RNA. Sequencing did not reveal the sequence of Protein X but instead the full gene sequence of a non-related hemocyte protein (Fig 3.1). The sequence contains a 5'UTR, open reading frame, stop codon, poly-adenylation site and 3'UTR. The translated protein sequence has a signal peptide indicating that the hemocyte protein is targeted to the secretory pathway. The mature processed protein has a calculated pI of 4.37 and molecular mass of 21481.9 Da, that includes 10 cysteine residues predicted to be involved in disulphide bonds.

Failure to amplify Protein X from hemocyte RNA could be as a result of increased stability of the corresponding secondary mRNA structure or possibly that a primer in the degenerate mix may have a higher affinity for the lipocalin mRNA. However, in Chapter 2 the Gram-negative bacteria binding Proteins X and Y were only detected in plasma obtained from challenged ticks and not in the corresponding hemocyte extracts. Therefore, the results obtained here plus the latter results suggest that Protein X is not of hemocytic origin but is synthesized in other tissues and possibly in the fat body. Another possibility is that the HMM bacteria binding proteins observed in the plasma are polymers composed of monomeric proteins of hemocytic origin. For this reason and the fact that the fat body of argasid ticks is distributed throughout the connective tissue of the body and therefore difficult to dissect (Sonenshine, 1991), hemocytes were used in this study.

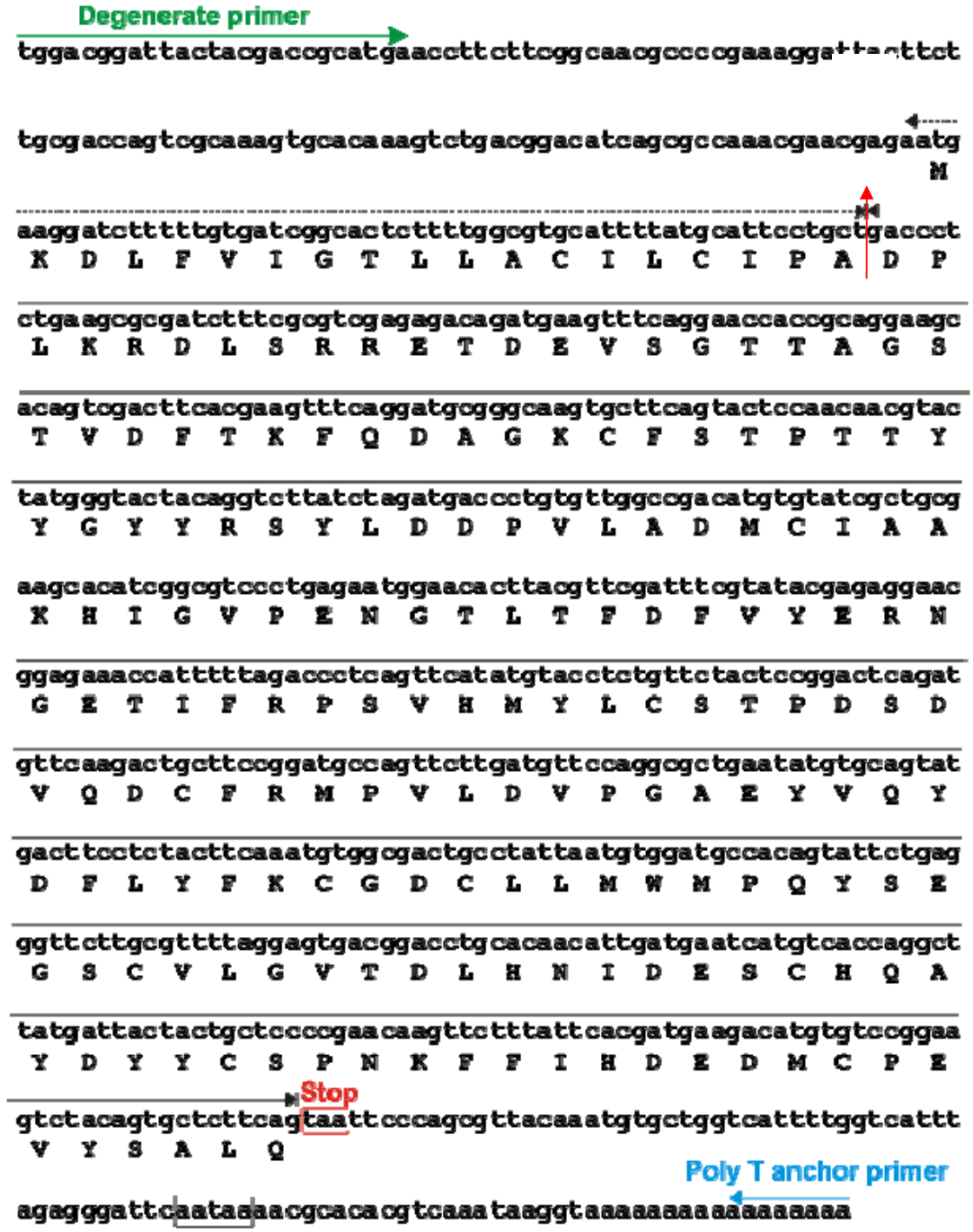


Figure 3.1: Nucleotide and deduced amino acid sequence of the amplified cDNA. The mature peptide is indicated by the solid line. The red arrow indicates the putative cleavage site of the signal peptide (broken line). The stop codon is marked with red box. The polyadenylation signal is indicated by the black box. Degenerate primer is indicated by the green arrow. Poly T anchor primer indicated by the blue arrow. Nucleotide sequence data has been submitted to the database (accession number: 298200310).

BLASTP analysis indicated similarity of the sequence to tick lipocalins and the name savicalin was coined for this protein. The three best hits included lipocalins from *R.* (*Boophilus*) *microplus* (E-0.004), *I. scapularis* (E-0.004) and *A. monolakensis* (E-0.003). In addition, a TBLASTN query of the non-redundant EST database retrieved EST sequences (5E-14) from a whole body cDNA library of the closely related tick, *O. porcinus*. The translated EST sequences showed 22 % sequence identity to savicalin. In addition, savicalin was submitted to the CDD and Phyre servers in order to confirm that it belongs to the lipocalin fold. In the case of the CDD analysis savicalin was assigned to the His-binding superfamily, which essentially describes all tick lipocalins. The top 4 hits obtained with the Phyre server were all tick lipocalins for which structures were previously solved and in all cases the estimated precision was greater than 95%, while all other hits corresponded to lipocalins from other organisms. As such, savicalin was assigned to the lipocalin family using three different algorithms that preferentially selected the lipocalin fold from a variety of known sequences and folds. This increased the confidence that savicalin belongs to the lipocalin fold, even if it is divergent.

3.4.2 Multiple sequence alignments of tick lipocalins

Multiple sequence alignments with these proteins as well as tick lipocalins that have been functionally characterized indicated that savicalin shows overall less than 20 % sequence identity to these tick lipocalins (Fig 3.2).

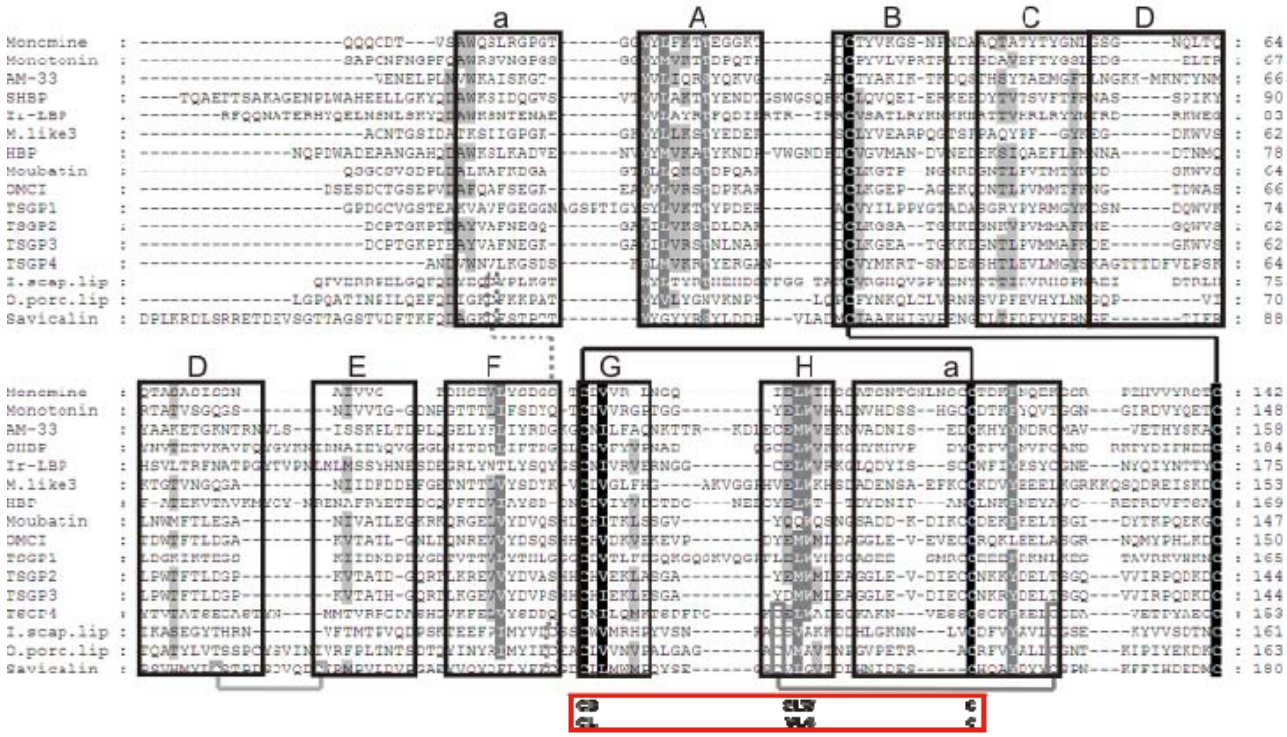


Figure 3.2: Multiple sequence alignments of tick lipocalins. Alignment of savicalin with the following molecules and their accession codes: Monomine (114152936), Monotonin (114152976) and Am-33 (114152974) from the soft tick, *A. monolakensis;* SHBP (18032205) from the hard tick, *Dermacentor reticulatus*; Ir-LBP (219935277) from the hard tick, *Ixodes ricinus;* M.like3 (Moubatin like 3, 149287030) from the soft tick, *O. parkeri;* HBP (7767032) from the hard tick, *R. appendiculatus;* Moubatin (159945) and OMCI (49409517) from the soft tick, *O. moubata;* TSGPs (TSGP1: 25991387, TSGP2: 25991389, TSGP3: 25991391, TSGP4: 25991438) from the soft tick, *O. savignyi;* O.porc.lip *(O. porcinus* lipocalin, 29779506) from the soft tick, *O. porcinus*; I.scap.lip *(Ixodes scapularis* lipocalin, 241679301) from the hard tick, *I. scapularis*. A secondary structure based on the SWISS model of savicalin is boxed in black as α-helices (a) and β-sheets (A~H). Conserved cysteine residues found in both hard and soft ticks with predicted disulphide bonds are indicated with solid black line. The solid grey line indicates shared TSGP 4 fold disulphide bond. The dotted grey line indicates disulphide bond shared with the *I. scapularis* lipocalin. The light grey line indicates disulphide bond unique only to savicalin. Red rectangular box indicates biogenic amine binding motif compared to CL-VLG-C sequence obtained from savicalin.

Conserved features include two disulphide bonds found in both hard and soft ticks (Cys59-Cys180; Cys132-Cys159) (Mans *et al.,* 2003). A third disulphide bond (Cys145-Cys167) is shared with TSGP4, the serotonin and histamine binding protein from *D. reticulatus*, and an *I. scapularis* sequence, that is characteristic of the leukotriene C4 binding clade of soft ticks (Mans & Ribeiro, 2008a). A fourth disulphide bond is shared with the same *I. scapularis* sequence (Cys35-Cys129). The fifth disulphide bond is unique and links the β strands D and E (Cys96-Cys106) (Fig 3.2). Savicalin lacks the biogenic amine-binding (BAB) motif (CL[L]X(11)VL[G]X(10)C vs CD[VIL]X(7,17)EL[WY]X(11,30)C),

and would therefore not bind biogenic amines (Mans *et al.,* 2008b). In addition, the residues proposed to be involved in leukotriene binding and complement C5 interaction (Mans & Ribeiro, 2008b), are not conserved in savicalin either, suggesting that it will lack these functions as well.

3.4.3 Structural modeling of savicalin

The molecular model obtained presents all secondary structure features associated with lipocalins (Fig 3.3). This includes the eight stranded anti-parallel +1 beta-barrel, the N-terminal helix that closes off the barrel and the C-terminal alpha-helix that packs against the barrel (Flower 2000; Flower *et al.,* 2000; Skerra 2000). This indicated that the overall features of the model fits well with the proposed lipocalin structure and supports the inclusion of savicalin into this family. All cysteine residues are spatially organized to form intact disulphide bonds in the model, supporting the proposed disulphide bond pattern for savicalin (Fig 3.2; Fig 3.3).

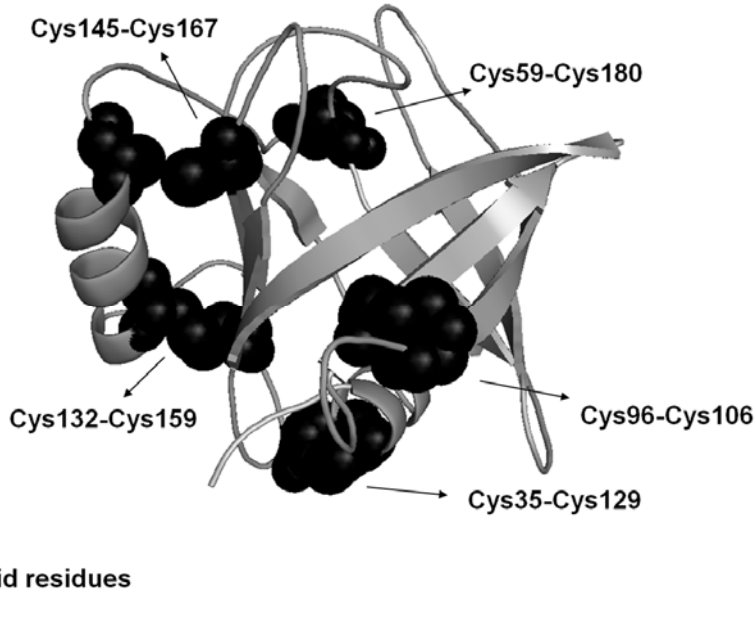
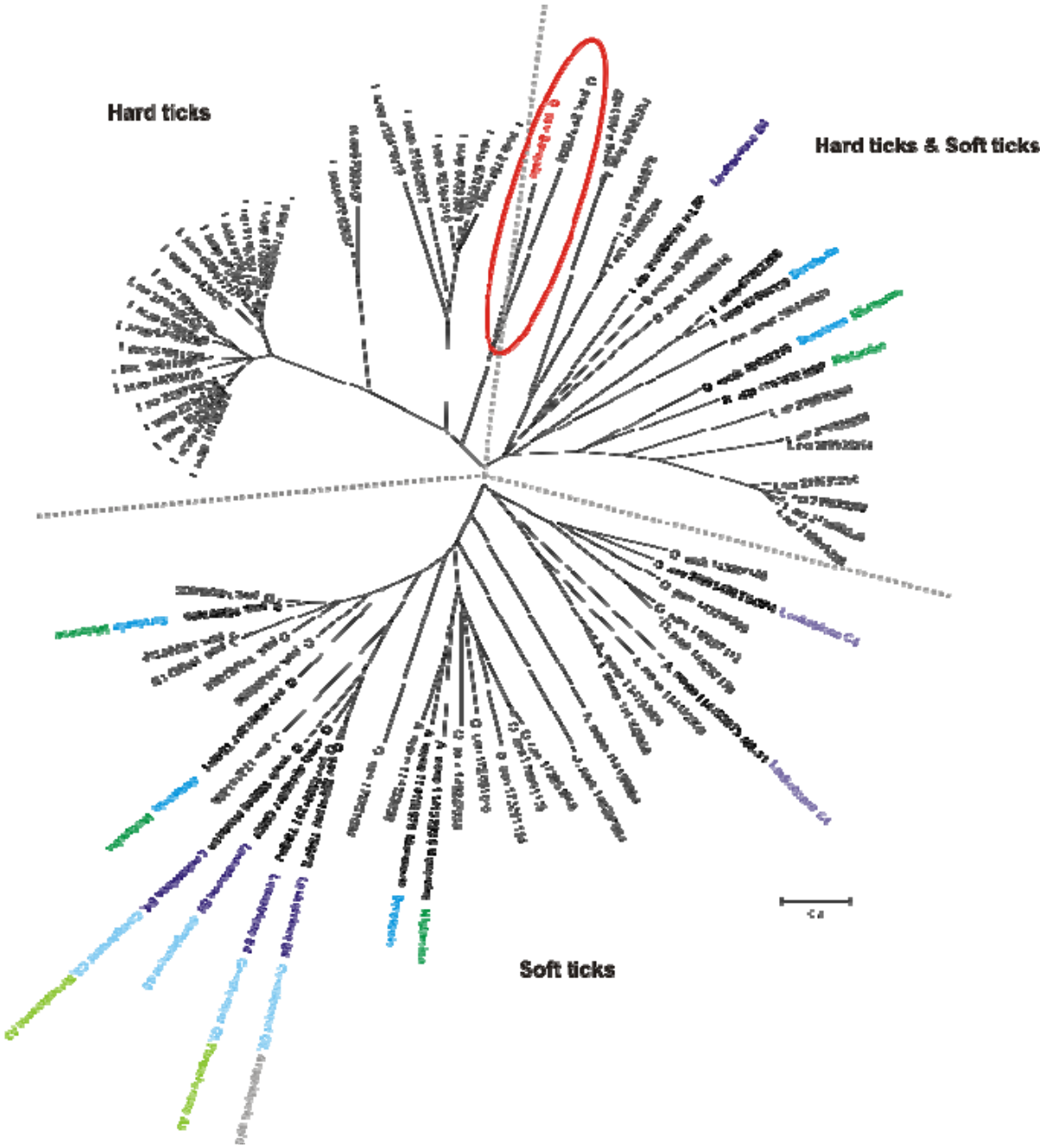


Figure 3.3: Structural modeling of savicalin. Cysteine residues are indicated with space fill spheres with their corresponding disulphide bonds. The root mean square deviation (RMSD) value compared with the modeling template HBP-2 is also indicated.

3.4.4 Phylogenetic analysis

Phylogenetic analysis using the sequence set from the multiple sequence alignments indicated that savicalin does not group with any of the known functionally characterized clades, implying that it will lack these functions (Fig. 3.4).



For figure legend please see following page (Page 68).

Figure 3.4: Phylogenetic analysis of the tick lipocalin family. Lipocalins were retrieved from non-redundant database by BLAST analysis of savicalin. Neighbor joining (NJ) analysis was conducted using MEGA version 4.0. Reliability of the inferred tree was evaluated by bootstrap analysis (100 000 replicates). Sequences are described by a species designation (A. mon: *A. monolakensis*; Am. amer: *A. americanum*; B. micro: *R.* (*Boophilus). microplus*; D. recti: *D. reticulatus*; I. paci: *I. pacificus*; I. scap: *I. scapularis*; I. rici: *Ixodes ricinus*; O. cori: *O. coriaceus*; O. park: *O. parkeri*; O. moub: *O. moubata*; O. porc: *O. porcinus*; O. sav: *O. savignyi*; *R*. app: *R.s appendiculatus*). Savicalin is shown in bold red. Red circle indicate lipocalins that have more than 8 cysteine residues. Grey dotted line divides classes of ticks. Bold black: Functions of lipocalins have been determined experimentally with ligands indicated in different colours (Blue: Serotonin; Green: Histamine; Light purple: Leukotriene C4, Purple: Leukotriene B4; Light blue: Complement C5; Light Green: Thromboxane A2).

Savicalin groups within a clade formed by the three best hits obtained by BLASTP analysis and the translated EST sequence from *O. porcinus*. The support for this clade is quite high, but does not necessarily imply that these proteins are orthologous, as the expected species relationships for *Ornithodoros*, *Argas*, *Ixodes* and *Rhipicephalus* are not recapitulated. It is of interest though that *Ixodes*, *Rhipicephalus* and possibly the *O. porcinus* sequences derive from non-salivary gland tissues and could suggest that their orthologous/ paralogous relationships date back to a split between salivary and non- salivary gland derived sequences.

3.4.5 Tissue expression profile of savicalin

Expression profiling by mRNA level detection showed that savicalin was up-regulated in hemolymph of unfed ticks upon hemocoelic bacterial challenge as well as ten days after feeding (Fig 3.5). Down-regulation occurred, however, 1 day after feeding. In contrast, savicalin was not up-regulated in midgut and ovaries irrespective of bacterial challenge or 1 day after feeding, but seems to be constitutively expressed. It is, however, down-regulated 10 days after feeding. Savicalin was also up-regulated in midgut of unfed ticks. In contrast, no transcription was detected in salivary glands.

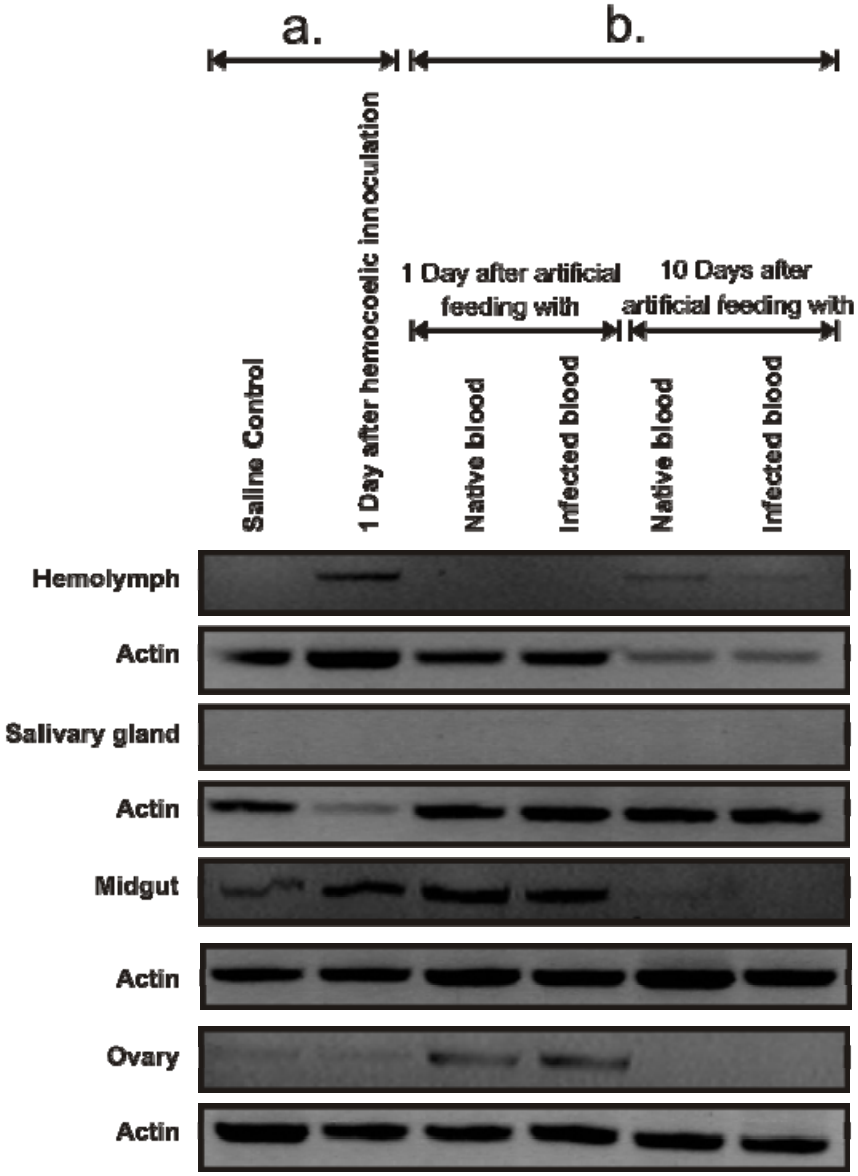


Figure 3.5 Transcriptional profile of savicalin. Tissue distribution was analyzed by RT–PCR in the hemolymph, salivary gland, midgut and ovary of the ticks (a) 1 day after hemocoelic inoculation with heat killed *B. subtilis* or saline and (b) 1 day/10 days after artificial feeding with either native or *B. subtilis* infected blood. Actin is shown as the internal standard

The fact that savicalin is absent from salivary gland, suggests that it does not function at the tick-host interface, as found for other salivary gland derived lipocalins. Expression patterns do, however, indicate that its expression is up-regulated during feeding and this could suggest a role in the post-feeding development of the tick. This could include processes such as blood-digestion,

molting and embryogenesis (Sonenshine, 1991). Orthologs for this lipocalin could also be discovered once more lipocalins are described that are not expressed in the salivary gland. The function for savicalin has not yet been determined, but given its tissue distribution, expression patterns and the fact that it is lipocalin, it would be likely that it can act as a scavenger or transporter of bio-active molecules involved in post-feeding development of soft ticks or have an antimicrobial role.

Chapter 4: Attempted identification of the Gram-positive antibacterial activity in the salivary glands of *O. savignyi*

4.1 Introduction

Reports indicate that ticks have the ability to control infections when challenged with various bacteria (Johns *et al.,* 2000, 2001a). Most of the research on tick innate immunity has focused on the identification of AMPs from hemolymph, fat body, eggs, midgut, but only recently from salivary glands (Table 4.1, 4.2 and Appendices III, IV). From the large number of tick species found worldwide, so far approximately 51 AMPs have been identified from 14 different ixodid and argasid species. These studies have indicated that tick AMPs are either constitutively expressed at very low levels or that their expression is inducible by either bacterial challenge or blood feeding. Most of the identified AMPs are directed at Gram-positive bacteria, however some are effective against Gram-negative bacteria and fungi. Only one report has reported that AMP from *Haemaphysalis longicornis* inhibits the growth of parasites, fungi and Gram-negative bacteria (Tsuji *et al.,* 2007).

Most of the characterized AMPs are defensins. In ticks, defensins are usually small peptides with molecular masses of 3 – 6 kDa. They are arginine-rich, cationic and usually contain 6 – 8 cysteine residues which form the disulphide bridges (Fig 4.1). These bridges play a vital role in stabilization and maintain the tertiary structure known as the “defensin fold” (Ganz, 2003). They range from 67 – 92 amino acids in length and this includes the region which is cleaved when the mature peptide (37- 61 amino acids in length) is secreted. Several species have multiple isoforms, with differential expression seen in different tissues. Analysis of an amino acid alignment of different defensins (full length protein sequences) shows that most align very closely, especially in the mature region (Fig 4.1).

Table 4.1: AMPs from soft ticks

Tick species	AMP name	Primary tissue / source	Antimicrobial activity			References	Genebank Accession code
			G+	G-	Fungi		
O. moubata	Defensin A	MG	+	-	-	Nakajima *et al.*, 2001, 2002	13623797
O. moubata	Defensin B	MG	+	-	-	Nakajima *et al.*, 2001, 2002	13623795
O. moubata	Defensin C	MG	+	-	-	Nakajima *et al.*, 2001, 2002	24210464
O. moubata	Defensin D	FB	+	-	-	Nakajima *et al.*, 2001, 2002	24210462
O. moubata	Rabbit α hemoglobin segment	MG	+	-	-	Nakajima *et al.*, 2003b	

MG: Midgut **+: Activity present**
FB: Fat body **- : Activity absent**

Table 4.2: AMPs from hard ticks

Tick species	AMP name	Primary tissue / source	Antimicrobial activity			References	Genebank Accession code
			G+	G-	Fungi		
A. hebraeum	Peptide 1	HL	+	+	-	Lai *et al.*, 2004a	40888888
A. hebraeum	Peptide 2	HL	+	+	-	Lai *et al.*, 2004a	40888890
A. hebraeum	Hebraein	HL	+	+	+	Lai *et al.*, 2004b	40888892
B. microplus	Bovine α hemoglobin segment	MG	+	-	+	Fogaça *et al.*, 1999	
B. microplus	Defensin	HCs, BME 26	+	-	-	Fogaça *et al.*, 2004, Esteves *et al.*, 2008	28864184
B. microplus	Microplusin	HL, BME26, egg	+	-	+	Esteves *et al.*, 2008, 2009; Fogaça *et al.*, 2004; Silva *et al.*, 2009	28864185
B. microplus	Ixodidin	HCs	+	-	-	Fogaça *et al.*, 2006	32363180
D. variabilis	Varisin	HL	+	+	-	Sonenshine *et al.*, 2002	27902521
H. longicornis	Hlgut-defensin	MG	+	+	-	Zhou *et al.*, 2007	129279020
H. longicornis	Hlsal-defensin	SG	+	+	-	Zhou *et al.*, 2007	62122376
H. longicornis	longicin	MG	+	+	+	Tsuji *et al.*, 2007	62122376
H. longicornis	longicornsin	SG	+	+	+	Lu *et al.*, 2010	186928039
I. ricinus	Defensin	Whole tick body	+	-	-	Rudenko *et al.*, 2007	33114168
I. scapularis	ISAMP	HCs, FB, SG	+	+	-	Pichu *et al.*, 2009	22164302
I. sinensis	Ixosin	SG	+	+	+	Yu *et al.*, 2006	71979959
I. sinensis	Ixosin-B	SG	+	+	+	Liu *et al.*, 2008	155624345

HL: Hemolymph **+: Activity present**
HCs: Hemocytes **- : Activity absent**
MG: Midgut
SG: Salivary gland
FB: Fat body
BME: *B. microplus* embryonic cell line

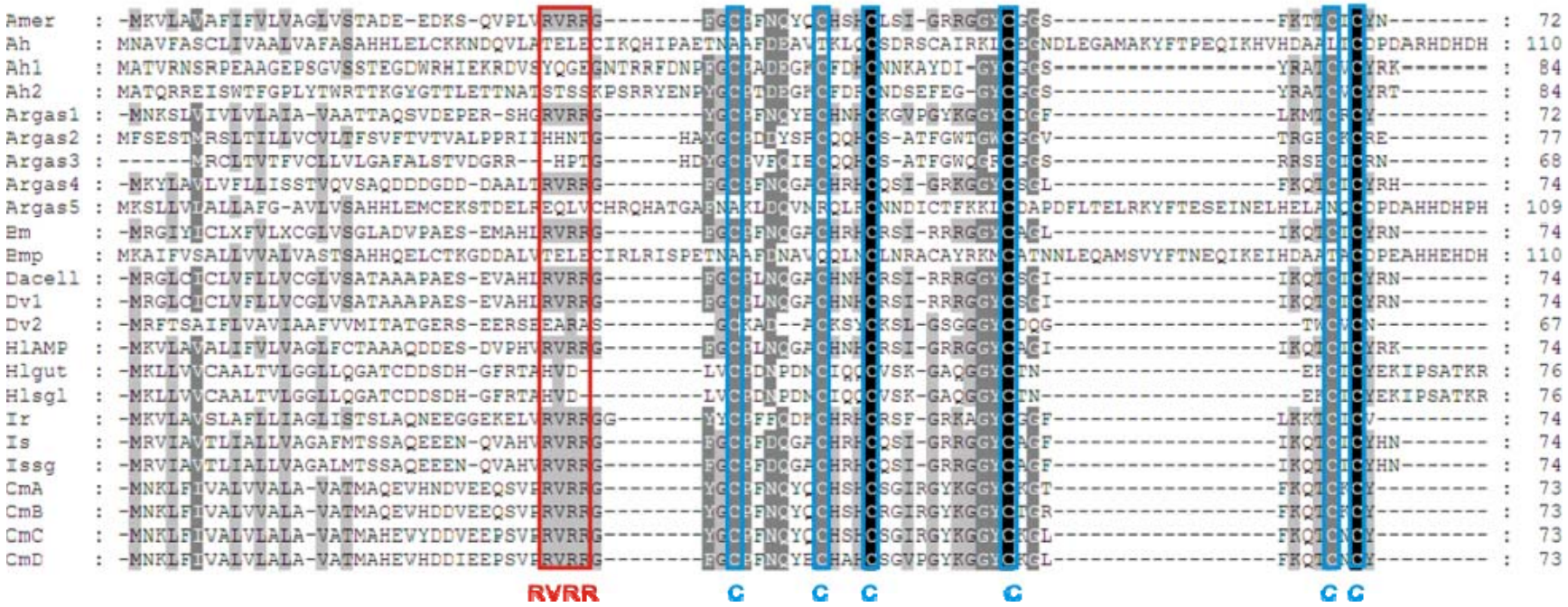


Figure 4.1: Tick defensins and defensin-like sequence alignments. Red indicates the RVRR cleavage site. Blue indicates conserved cysteine residues. Amer (*A. americanum*), Ah (*A. hebraeum*, hebraein), Ah1 and Ah2 (*A. hebraeum*), Argas1, Argas2, Argas3, Argas4, Argas5 (*A. monolakensis*), Bm, Bmp (*R. microplus*), Dacell (*D. andersoni*), Dv1, Dv2 (*D. variabilis*), HlAMP (longicin from *H. longicornis*), Hlgut (gut from *H. longiconis*), Hlsgl (SG from *H. longiconis*), Ir (*I. ricinus*), Is (scapularsin from *I. scapularis*), Issg (SG from *I. scapularis*). OmA/B/C/D (*O. moubata*).

In *O. moubata*, it was observed that defensin isoforms were expressed after bacterial challenge. Defensin A, B and C were expressed mostly in the midgut after 5 days, whereas defensin D was expressed mostly in the fat body (Nakajima *et al.,* 2002, 2003a, c, 2005). After a blood meal *O. moubata* defensin C and D were up-regulated in the midgut. These results suggested that defensins are regulated differently in various tissues via different signaling.

From the NCBI database, 25 tick AMP sequences were identified of which 5 sequences for putative defensin were obtained from salivary gland libraries from *A. monolakensis* (Mans *et al.,* 2008a). Most have a signal peptide with the sequence "RVRR" between the prepro- and mature regions of the peptide as shown in Fig 4.1 (Sonenshine & Hynes, 2008).

In *D. variabilis* the defensin, varisin was up-regulated in hemocytes after challenge of these ticks with *B. burgdorferi* or *B. subtilis*. Anti-*B. burgdorferi* activity increased when chicken lysozyme was added to tick defensin indicating a possible synergism between these two molecules (Johns *et al.,* 2001b). mRNA transcripts of varisin were detected in the midgut and fat body upon microbial challenge (Sonenshine *et al.,* 2005; Ceraul *et al.,* 2007). A second isoform, defensin 2, was recently discovered in the midgut of this species. Expression of varisin increased 35 fold in the midgut of *D. variabilis* after feeding. When *D. variabilis* was challenged with *Rickettsia montanensis*, varisin expression increased 2.6 fold 24 hours post-injection. These findings implied that varisin plays an important role in feeding and protection after microbial challenge (Ceraul *et al.,* 2007).

Hynes *et al.* (2005) identified the gene for a defensin-like peptide (scapularisin) in fed female *I. scapularis* ticks, using a RT-PCR approach. Sequencing analysis revealed a 225 bp open reading frame encoding a 74 amino acid preprodefensin, including the putative 38 amino acid mature peptide. Amino acid sequences of *I. scapularis* and *D. variabilis* were 62.2 % similar for the preprodefensin region. For the mature defensins the similarity was 78.9%, with the six cysteine residues being located in the same relative position (Hynes *et al.,* 2005).Transcriptional profiling indicated expression in the midgut, hemocytes and fat-body, while no evidence of a peptide was found in these tissues.

A cDNA coding for amercin, a defensin-like molecule, was found in the hemocytes, midguts, fat body and salivary glands from the lone star tick, *A. americanum.* The amercin prepropeptide has 60.8 % and 59.5 % similarity with the *I. scapularis* and *D. variabilis* prepropeptides, respectively, whereas the mature amercin peptide has 73.7 % and 71.1 % similarity with the mature peptides of these ticks. Similarity with other tick defensins ranges from 42 % to 71 % (Todd *et al.,* 2007).

In *H. longicornis*, longicin is a small cationic defensin which consists of 6 cysteine residues with a characteristic beta-sheet at the C-terminus. It displays bactericidal and fungicidal properties. The AMP showed a remarkable ability to inhibit the proliferation of merozoites, an erythrocyte blood stage of equine *Babesia equi*. This was the first evidence of a tick defensin that is capable of eradicating an eukaryotic parasite (Tsuji *et al.,* 2007). Recent findings by Rahman *et al.* (2010) showed that longicin and its synthetic partial analog (P4; longicin-derived synthetic analog) displayed antimicrobial, fungicidal and parasiticidal activity. P4 is a cationic peptide with hydrophobic and amphipathic characteristics. Circular dichroism (CD) spectroscopic results have indicated the existence of a beta-sheet and transition to a helical conformation in the presence of membrane-mimicking conditions. Another defensin-like AMP, longicornsin,

was isolated from the salivary glands of the same tick species (Lu *et al.,* 2010). Mature longicornsin is composed of 78 amino acids, contains a C-terminal extension and exerts potent antimicrobial activities against bacteria and fungi (Lu *et al.,* 2010).

In *A. hebraeum*, two non-cationic defensins were identified in the cDNA library prepared from synganglion obtained from fed female ticks (Lai *et al.,* 2004a). The prepropeptides deduced from the cDNA sequences each have 92 amino acid residues. The one AMP, defensin peptide 2, was purified from the hemolymph of fed female ticks and displayed antibacterial activity against Gram-negative and Gram-positive bacteria (Lai *et al.,* 2004a).

Hebraein is an anionic AMP isolated from *A. hebraeum* hemolymph consisting of 102 amino acids with a molecular mass of 11 kDa. This peptide contains 6 cysteine residues and 9 histidine residues in its C terminal domain (Lai *et al.,* 2004b). The secondary structure is α-helical with 4–6 helices except for a very short extension at the C terminus. Such high α-helical content is quite different for most arthropod defensins. The sequence of hebraein shares little similarity with other defensins. Hebraein was found to be up-regulated by blood feeding and to be active against Gram-negative and Gram-positive bacteria as well as fungi (Lai *et al.,* 2004b).

Microplusin, an AMP obtained in hemolymph from *R. microplus*, has a molecular mass of 10.2 kDa. It has the same cysteine motif as hebraein, is active against Gram-positive bacteria and yeast, but does not show antimicrobial activity against Gram-negative bacteria (Fogaça *et al.,* 2004). Recently, microplusin transcripts in the embryonic cell line (BME 26) and eggs of *R. microplus* were detected (Esteves *et al.,* 2008, 2009). Microplusin belongs to a new family of cysteine-rich AMPs containing histidine-rich regions at the N and C termini. The NMR solution structure of microplusin bonded to copper II and iron II has been

described. The structure consists of a single alpha-helical globular domain, which is comprised of 5 alpha-helices: alpha1 (Gly-9 to Arg-21), alpha2 (Glu-27 to Asn-40), alpha3 (Arg-44 to Thr-54), alpha4 (Leu-57 to Tyr-64), and alpha5 (Asn-67 to Cys-80). Both the N and C termini are disordered (Silva *et al.,* 2009; Rezende *et al.,* 2009). Mode of action investigations have shown that microplusin displays a bacteriostatic effect by binding metals and does not permeabilize the bacterial membrane. It was also demonstrated that microplusin affects *M. luteus* respiration, which is a copper-dependent process (Silva *et al.,* 2009).

Potential AMPs have since been identified in numerous studies of salivary gland transcriptomes from soft and hard ticks (Francischetti *et al.,* 2005, 2008a, b; Chmelař *et al.,* 2006; Mans *et al.,* 2008a; Ribeiro *et al.,* 2006). To date only five AMPs have been purified and characterized from the salivary glands of four hard tick species. In a previous study undertaken by a Masters student in the Department of Biochemistry, a defensin-like Gram-positive AMP was isolated from *O. savignyi* hemolymph and its N-terminal sequence determined (Olivier, 2002). This chapter describes the attempted isolation and characterization of the salivary gland Gram-positive antibacterial activity.

4.2 Hypothesis

The salivary gland Gram-positive antibacterial activity is a defensin-like antimicrobial peptide.

4.3 Materials and methods

4.3.1 Flow diagram of methods implemented in this chapter

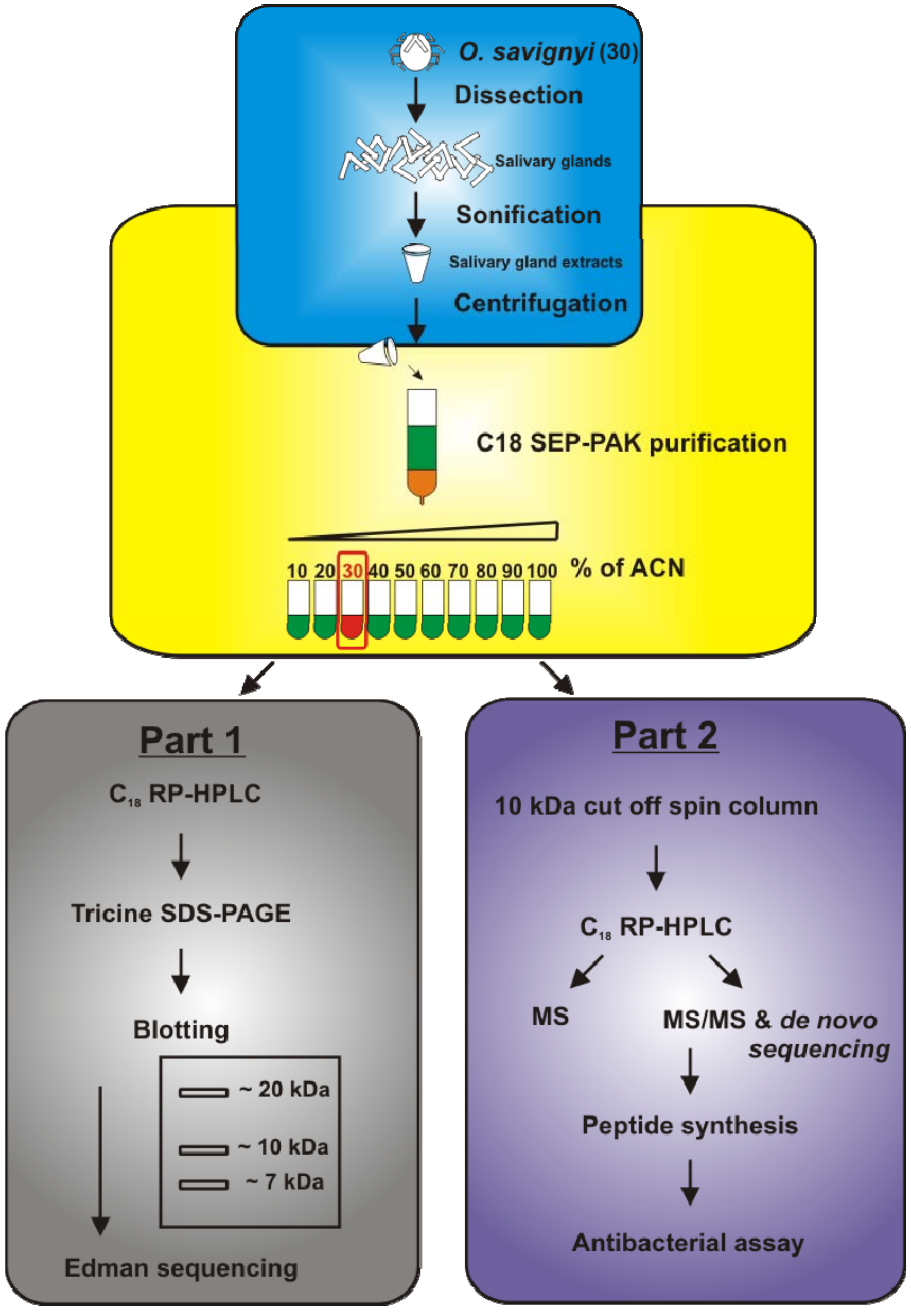


Figure 4.2 Flow diagram of the methods used to identify the Gram-positive antibacterial activity.

4.3.2 Ticks

Ornithodoros savignyi ticks were obtained as described in section 2.3.1

4.3.3 Reagents

All materials were of analytical grade and sterile double distilled deionized water was used in all experiments. Acrylamide, N', N'–methylene bisacrylamide, glycerol, ammonium persulphate, bromophenol blue, 3- Cyclohexylamino- 1-propanesulfonic acid (CAPS), sodium phosphate monobasic (NaH_2PO_4), sodium phosphate dibasic (Na_2HPO_4), Coomassie blue G-250, 4-hydroxy-α-cyano-cinnamic acid, β -mercaptoethanol, sodium dodecyl sulphate (SDS), Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl), TEMED, methanol, ethanol, NaCl, streptomycin, acetonitrile (ACN), trifluoroacetic acid (TFA), ammonium bicarbonate, dithioerythritol (DTE) and iodoacetamide (IAA) were obtained from Sigma Chemical Co. USA. Phosphoric acid was obtained from Merck. Germany. Tryptone and yeast extract were purchased from Oxoid Ltd (Nasingstoke, Hampshire, England). Flamingo^{TM} fluorescent stain solution was obtained from BIORAD, USA and sequencing grade modified porcine trypsin from Promega, USA.

4.3.4 Sample preparation, collection and extraction

Salivary glands from unchallenged ticks were dissected, washed with ice-cold 0.9 % (w/v) NaCl and stored at -70 ^{o}C (Mans *et al.,* 1998). Extracts were prepared by sonication of glands in 0.9 % (w/v) NaCl with a Branson sonifier (Banson Sonic Power Co.), using 3 x 6 pulses at 30 % duty cycles. Tissues were kept on ice throughout the procedure. Sonified products were centrifuged in a microfuge (10 000 *g* for 10 min) and the supernatant was used for further studies.

4.3.5 Antibacterial assay

Bacillus subtilis (ATCC strain 13933) was grown overnight in LB broth. The overnight cultures were subcultured until an OD_{600} ~ 0.5 was reached. Thereafter the culture was diluted in LB broth to obtain an OD_{600} ~ 0.001 and 100 µl was dispensed into sterile 96 - well microtiter plates containing 100 µl of LB broth. To this 10 µl of salivary gland extract or fraction or buffer (growth control) or streptomycin (positive control; final concentration 2 µg/ml) was used. The plates were incubated at 30 ^{o}C for 12 h and bacterial growth was quantified by measurement of optical density at 600 nm.

4.3.6 Purification of the Gram-positive antibacterial activity

In an initial attempt (Part 1, Fig 4.2) a salivary gland extract prepared from 30 ticks was loaded onto a silica C18 SEP-PAK column (pore diameter 60 – 87 Å, purchased from SUPELCO, USA) followed by stepwise elution with an increasing concentration of ACN containing 0.01 % TFA. Fractions were collected and dried under vacuum. Fractions were reconstituted with double distilled deionized sterile water and the protein concentration determined using the Quick Start Bradford Protein Assay Kit (BioRad, USA). Fractions were tested for antibacterial activity against *B. subtilis*. The active fraction was applied to a RP-HPLC C_{18} column (Jupiter 4.6 mm x 25 cm Phenomenex, USA), pre-equilibrated with mobile phase A (0.1 % TFA, 0.1 % ACN). Elution was achieved with a step-gradient (0 – 60 %) with a mobile phase B (0.1 % TFA, 100 % ACN) over 60 min, as set out in Table 4.3.

In part 2, (Fig 4.2) the purification was repeated and the active fraction (eluted with 30 % ACN) from the SEP-PAK C18 was applied to a 10 kDa cut-off spin column (Sigma-Aldrich, Germany). The permeate was subsequently loaded on to a C_{18} RP-HPLC column with elution conditions as set out in Table 4.3.

In order to obtain sufficient material for subsequent analysis the methods described in Part 1 and Part 2 were repeated ten times.

Table 4.3 Flow conditions used during C_{18} RP-HPLC

Time (min)	Flow (ml/min)	% Buffer A	% Buffer B	Duration (min)
0	1	100	0	
5	1	100	0	5
65	1	40	60	60
66	1	5	95	1
67	1	5	95	1
77	1	95	5	10
78	1	95	5	1
83	1	5	95	5
84	1	5	95	1
94	1	95	5	10
95	1	100	0	1
110	1	end		15

Buffer A – 0.1 % TFA, 0.1 % ACN Buffer B – 0.1 %, 100 % ACN

4.3.7 Tricine SDS-PAGE analysis

Fractions were analyzed using a tricine SDS-PAGE system (Schägger & von Jagow, 1987) that is suitable for the resolution of proteins in the range of 1 – 100 kDa. A 16.5 % T, 3 % C separating gel (1 M Tris-HCl, 0.1 % SDS, pH 8.45) and a 4 % T, 3 % C stacking gel (0.75 M Tris-HCl, 0.075 % SDS, pH 8.45) was prepared from acrylamide (48 % acrylamide/ 1.5 % N', N'–methylene bisacrylamide). The cathode buffer consisted of 0.1 M Tris, 0.1 M tricine, 0.1 % SDS, pH 8.25 and the anode buffer was 0.2 M Tris, pH 8.9.

These solutions were degassed for 10 minutes and polymerized by the addition of 50 µl 10 % ammonium persulphate and 5 µl of TEMED. Protein fractions (obtained from section 4.3.6) were diluted 1 : 2 in reducing buffer [0.06 M Tris-HCl, pH 6.8, 2 % SDS (w/v), 0.1 % glycerol (v/v), 0.05 % β-mercaptoethanol (v/v) and 0.025 % bromophenol blue (w/v)] and boiled at 94 ^{o}C for 10 min. Peptide mass markers were used for molecular mass determination. The Hoefer SE 300 miniVE gel system was used, with an initial voltage of 30 V for 1 h followed by 100 V for a further 2 h.

4.3.8 Blotting of protein bands obtained from tricine SDS-PAGE

The gel was transferred to a container containing a mixture of 10 mM CAPS pH 11.0, 10 % methanol (v/v) for 2 min. ImmobilonTM-p^{sq} membranes (polyvinylidene difluoride membrane (PVDF membrane) obtained from Sigma chemical Co. USA) were activated by soaking in 100 % methanol (v/v) for 2 min, and then transferred to the CAPS buffer to disperse excess methanol. A Genie tank blotter was used according to the method of the manufacturer using 10 mM CAPS/ 10 % methanol (v/v) and 6 V for 30 min.

After blotting, the membrane was transferred to a clean glass tray and fixed with 40 % ethanol and 10 % acetic acid for 2 h. The tray was placed on a rocker and gently agitated. FlamingoTM fluorescent stain solution was diluted with distilled deionized filtered water (1: 9) and added to the membrane. The membrane was then visualized under UV light.

4.3.9 Edman sequencing

Protein bands were excised from the membrane and sequenced using Edman degradation at the Research Technologies Branch, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), USA.

4.3.10 MS/MS analysis and *de novo* sequencing

MS/MS analysis was performed, at the Technology Facility, Department of Biology, University of York, UK, using the method described by Shevchenko *et al.* (2001). In-solution tryptic digestion was performed after reduction with tris(2-carboxyethyl)phosphine and alkylation with S-methyl methanethiosulfonate. Sequencing-grade, modified porcine trypsin 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 0.02 μg/μl. Proteins were digested with the addition of 10 μl of trypsin solution, and incubated overnight at 37 ^{o}C.

A 1 μl aliquot of each peptide mixture was applied directly to the ground steel MALDI target plate, followed immediately by an equal volume of a freshly-prepared 5 mg/ml solution of 4-hydroxy-α-cyano-cinnamic acid in 50% aqueous acetonitrile (v/v) containing 0.1% trifluoroacetic acid (v/v).

Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800-4000. 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 algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a S/N threshold of 2.

For each fraction the ten strongest peaks of interest, with a S/N greater than 30, were selected for MS/MS fragmentation. Fragmentation was performed in LIFT mode without the introduction of a collision gas. The default calibration was used for MS/MS spectra, which were baseline-subtracted and smoothed (Savitsky-Golay, width 0.15 m/z, cycles 4); monoisotopic peak detection used a SNAP algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) with a minimum S/N of 6. Bruker flexAnalysis software (version 3.0) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra.

Tandem mass spectral data were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version 2.1), through the Bruker BioTools interface (version 3.2). Search criteria included: Enzyme, Trypsin; Fixed modifications, Methylthio (C); Variable modifications, Oxidation (M); Peptide tolerance, 250 ppm; MS/MS tolerance, 0.5 Da; Instrument,

MALDI-TOF-TOF (The version and size of the database can be obtained from the Mascot result page.).

Prior to DeNovo sequencing tandem MS/MS spectra were reprocessed through flexAnalysis (version 3.0) to generate peak lists containing only the 50 most intense peaks. DeNovo sequencing was performed using the BioTools interface (version 3.2). All sequences generated were submitted via BioTools (version 3.2) to the Washington University BLAST 2.0 server1, where MS-BLAST searches were run against the nr95_clean database. Actin sequences obtained from Mascot were searched against the NCBI database using PSI-BLAST.

4.3.11 Anti-bacterial analysis of synthetic actin fragments

A tick actin fragment (TAF sequence: SYELPDGQVITIGNER, MW: 1790.93 Da; pI: 4.14) shown in Table 4.5 and Actin5C [A5C sequence from *D. melanogaster*: SSSSLEKSYELPDGQVI, MW: 1838.97 Da; pI: 4.14 (Verleyen *et al.,* 2006)] were synthesized by GenScript Corporation, New Jersey, USA (>85 % purity). Both peptides were solubilized in 1 ml dddH_2O to prepare a stock solution of 2 mg/ ml.

The antibacterial assay was based on the method followed by Nakajima *et al.* (2003c). *Bacillus subtilis* was grown overnight in LB broth at 37 ^{o}C with shaking at 250 rpm. Overnight bacterial cultures were diluted in LB broth and subculture to obtain an OD_{600} ~ 0.001 and 90 μl dispensed into sterile 96 well sterile microtiter plates. Two-fold dilution series of the synthetic peptides (100 – 0.1 μg/ml) were prepared in dddH_2O and 10 μl added to bacterial suspensions. The plates were incubated at 37 ^{o}C for 24h. For the growth control, 10 μl of dddH_2O was added instead of peptide and for the positive control, 10 μl of streptomycin (final concentration of 2 μg/ml) was used.

4.4 Results and discussion

4.4.1 Purification of the Gram-positive antimicrobial activity and identification using N-terminal sequencing

To identify the Gram-positive antibacterial activity, salivary gland extracts prepared from unchallenged ticks were subjected to a SEP-PAK C18 column. Elution was performed with various concentrations of ACN. Only the fraction eluted with 30 % ACN (v/v) exhibited inhibitory activity. SDS-PAGE analysis of SEP-PAK fractions showed that the 30 % fraction was free from most high molecular mass proteins present in other fractions (Fig 4.3A).

The 30 % fraction was subjected to C_{18} RP-HPLC (Fig 4.3B). Gram-positive antibacterial activity was only found in the C18-fraction with a retention time of 37~38 min. Further purification of the active fraction by SDS-PAGE was performed and the protein components subjected to Edman sequencing (Fig 4.3C).

SDS-PAGE analysis showed that the active fraction contained three components of approximately 20 kDa, 10 kDa and 7 kDa (Fig 4.3C) with N-terminal sequences of LNVRCNNPHTA, XQPGCLERPFEFK and YIPEYLLRPFFK, respectively. BLAST searching of the NCBI database with the N-terminal sequence for the 20 kDa band identified the molecule as savignin (*e*-value of 1e-65). Savignin is a thrombin inhibitor of 12 430.4 Da that was previously isolated from *O. savignyi* (Nienaber, 1999). In a previous study, savignin showed anomalous behavior during SDS-PAGE analysis by showing lower eletrophoretic mobility than expected and hence a higher apparent molecular mass (Nienaber, 1999; Cheng, 2004). The other two components (~10 kDa and ~ 7 kDa) have high *e*-values with no significant match.

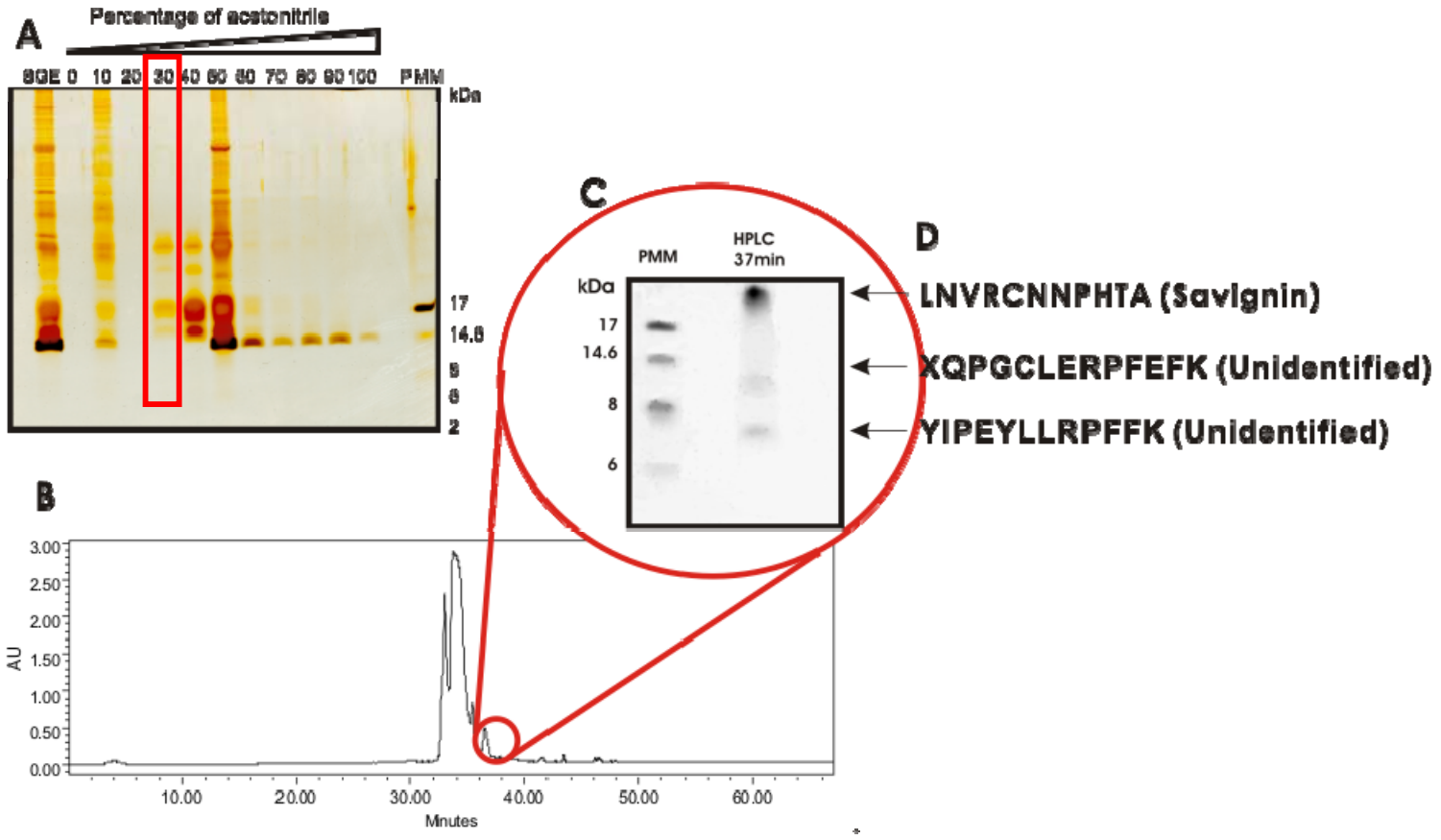


Figure 4.3 Purification of the Gram-positive antibacterial activity from salivary gland extracts and identification using N-terminal sequencing. A. Tricine SDS-PAGE gel of fractions after SEP-PAK C18 purification. B. C_{18} RP-HPLC chromatogram of the SEP-PAK 30 % fraction. Red circle indicates where anti-Gram positive activity was found. C. Tricine SDS-PAGE analysis of the C_{18} RP-HPLC fraction (retention time 37 min). Arrows indicate N-terminal sequence of bands. PMM: Peptide mass markers.

4.4.2 Purification of the Gram-positive antibacterial activity and identification using MS

To rule out the possibility of savignin participating in the antimicrobial activity, the purification was repeated and 10 kDa cut-off spin columns were introduced as an additional step before C_{18} RP-HPLC. MS was used to determine the purity and molecular mass of the components in the active fraction (Fig 4.4A).

The Gram-positive inhibitory activity was eluted at a retention time of 39 ~ 40 min. This retention differs from that observed in Fig 4.3B. This shift was due to HPLC maintenance (replacement of new pumps). The shift in retention time was also observed for lysozyme, which was used as an internal standard. Again, active fractions were pooled and freeze dried. MS analysis of the active fraction indicated various components ranging from 997.23 ~ 7182.97 Da (Fig 4.4B, C). However, the predominant components in the fraction ranged from 997.23 ~ 2039.54 Da (Fig 4.4B). Introduction of the 10 kDa cut-off filter had effectively removed savignin (20 kDa) and the 10 kDa component from the active fraction (Fig 4.3), excluding these proteins from being responsible for the observed Gram-positive antibacterial activity.

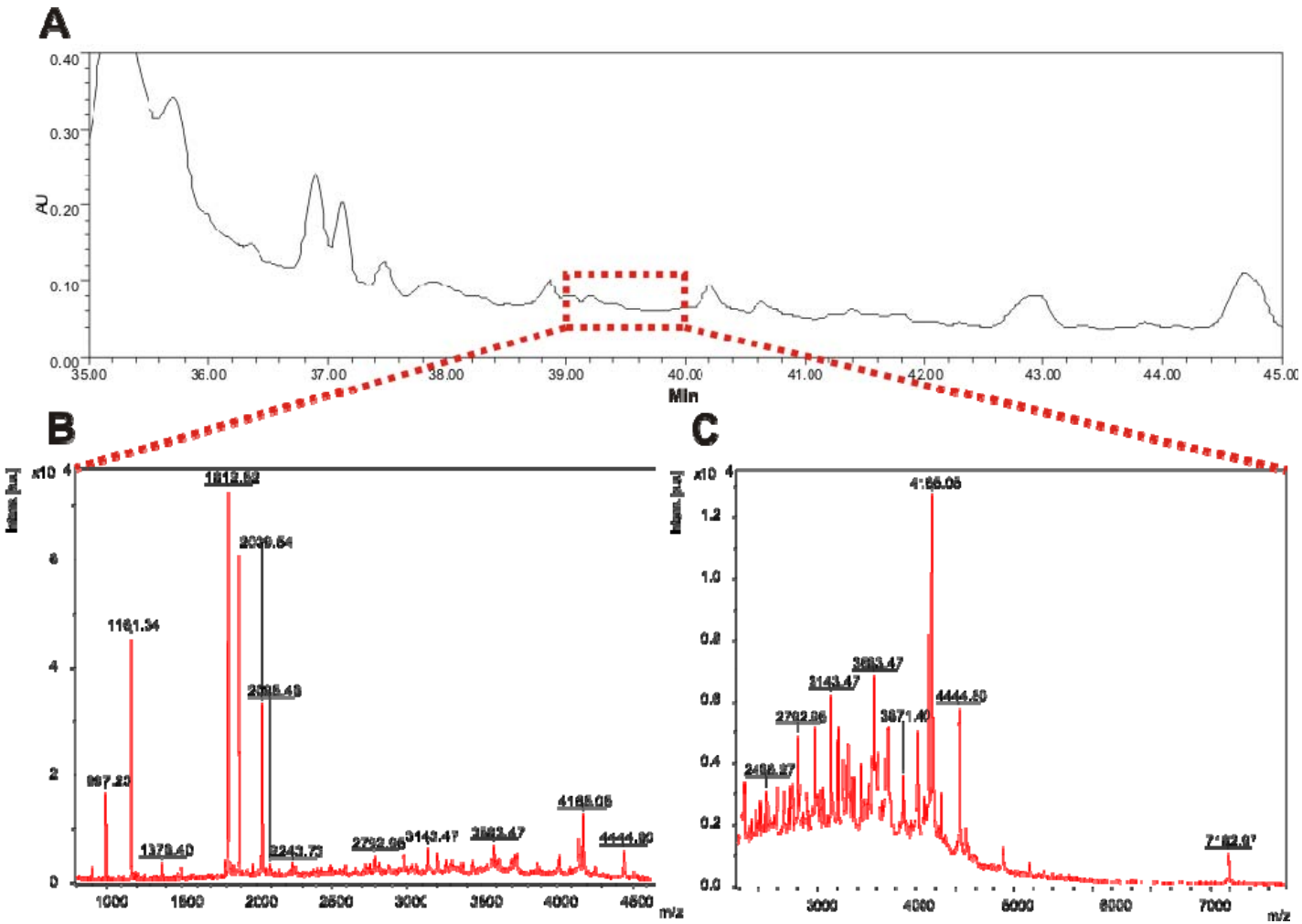


Figure 4.4 Purification of the Gram-positive antibacterial activity from salivary gland extracts and identification using MS analysis. A. C18 RP-HPLC chromatogram of the 10 kDa ultrafiltration permeate. B & C. Mass spectra of the active C18 fraction.

4.4.3 MS/MS ion search and *de novo* sequencing of the active fraction

In order to identify the components observed in the active fraction from C_{18} RP-HPLC (Fig 4.4A), the collected fraction was freeze-dried, trypsinized and subjected to MS/MS analysis. The MS/MS data obtained for three of the tryptic peptides (Fig. 4.5) were found to match with actin (Table 4.4).

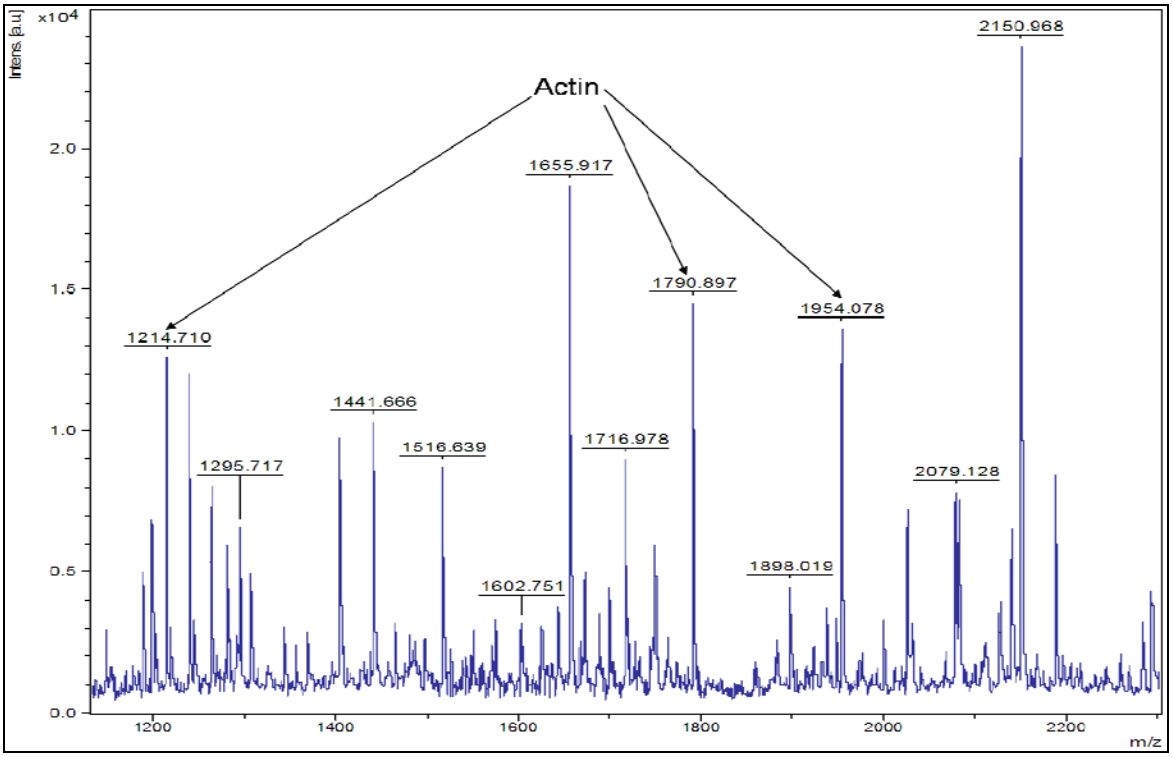


Figure 4.5 MS spectrum showing the three peptides whose MS/MS ion spectra match with actin.

Table 4.4: MS/MS ion search result of active fraction

Hit	Protein[a]	Accession number	Peptide sequence[b]	Observed Mass (M^+H^+)/ Da	Theoretical Mass (M)/ Da	Mowse score[c]	Expect score
1	Actin	1703149	R.SVFPSIVGRPR.H	1214.7100	1213.6931	32	4.5
2	Actin	1703149	K.SYELPDGQVITIGNER.F	1790.8974	1789.8846	81	5.5e-05
3	Actin	1703149	R.VAPEEHPVLLTEAPINPK.S	1954.0782	1953.0571	90	5.2e-06

[a] Protein from fungus *Ajellomyces capsulatus* to which the peptide sequence was matched with MASCOT

[b] Sequence information obtained for matched peptide from tandem mass spectrometry as determined by searching with MASCOT

[c] Mowse scores greater than 67 are considered significant

To increase the chances of identification of the other components in the active fraction, *de novo* sequences were derived from the MS/MS spectra data (Appendix V). Searching of the database with these did not reveal significant matches to defensins or other known antimicrobials.

Actin sequences obtained from the MASCOT search were used to search the NCBI database using PSI-BLAST and results are shown in Table 4.5.

Table 4.5 PSI-BLAST search against NCBI database (actin sequence information)

Sequence of fungus, *Ajellomyces capsulatus*	Hits	Accession #	*e values*[a]
SVFPSIVGRPR	Actin, *Amblyomma americanum*	196476734	1e-04
	Actin, *Rhipicephalus* (*Boophilus*) *microplus*	32330667	1e-04
	Actin, *Haemaphysalis longicornis*	157779728	1e-04
	Actin, *Haemaphysalis longicornis*	32423714	1e-04
	Actin, *Ixodes persulcatus*	164472819	1e-04
	Actin, *Ixodes ricinus*	59894747	1e-04
	Actin, *Ornithodoros moubata*	45269081	1e-04
	Actin, *Ornithodoros moubata*	77539277	1e-04
	Actin, *Rhipicephalus appendiculatus*	32423716	1e-04
VAPEEHPVLLTEAPINPK	Actin, *Amblyomma americanum*	196476734	4e-11
	Actin, *Boophilus microplus*	32330667	4e-11
	Actin, *Haemaphysalis longicornis*	157779728	4e-11
	Actin, *Haemaphysalis longicornis*	32423714	4e-11
	Actin, *Ixodes persulcatus*	164472819	4e-11
	Actin, *Ornithodoros moubata*	45269081	4e-11
	Actin, *Ornithodoros moubata*	77539277	4e-11
	Actin, *Rhipicephalus appendiculatus*	32423716	4e-11
SYELPDGQVITIGNER	Actin, *Boophilus microplus*	32330667	2e-10
	Beta-actin, *Boophilus microplus*	41618682	2e-10
	Beta-actin, *Boophilus microplus*	41618678	2e-10
	Actin, *Haemaphysalis longicornis*	157779728	2e-10
	Actin, *Ixodes persulcatus*	164472819	2e-10
	Actin, *Ixodes ricinus*	59894747	2e-10
	Actin, *Ornithodoros moubata*	45269081	2e-10
	Actin, *Ornithodoros moubata*	77539277	2e-10
	Actin, *Rhipicephalus appendiculatus*	32423716	2e-10
	Actin, *Haemaphysalis longicornis*	32423714	8e-09
	Actin, *Ixodes scapularis*	16902904	2e-08

[a] *e values* ranged from 4e-11 to 1e-04 are considered significant

Hits are indicated with their accession numbers and *e values* were filtered for ticks.

The PSI-BLAST search matched the actin sequences obtained from the MASCOT search to actin from both hard and soft ticks. In the soft tick, *O. moubata,* full length actin has a molecular mass of 41 837.84 Da (Horigane *et al.,* 2007). However, both MS and SDS-PAGE analysis of the active fraction did not indicate the presence of full length actin. These results suggest that the three peptides that match with actin are most probably derived from shorter tick actin fragments (997.23 – 7182.97 Da). It cannot be excluded that the observed tick actin fragments are produced by proteolytic degradation of actin during the isolation process. However, precautions were taken to limit degradation of proteins during the isolation process.

4.4.4 Testing of actin-derived peptides for Gram-positive antibacterial activity

To date, actin fragments have not been reported to be associated with antimicrobial activity. Fogaça *et al.* (1999) and Nakajima *et al.* (2003b) have shown that ticks are able to generate antimicrobial fragments from host hemoglobin using proteases.

In the arthropods, *D. melanogaster* and *A. gambiae*, it has been shown that full length actin is up-regulated in hemolymph after infection (Paskewitz & Shi, 2005; Vierstraete *et al.,* 2004a, b). In the starfish, *Asterias rubens*, two actin fragments (1.8 – 2.0 kDa) were found in a celomocyte extract showing antimicrobial activity (Li *et al.,* 2010; Maltseva *et al.,* 2004, 2007). However, Maltseva *et al.* (2004, 2007) did not confirm that the observed actin-derived fragments were responsible for the observed antimicrobial activity and remains as yet to be further investigated.

Vierstaete *et al*. (2004) observed an increase in the expression of actin (actin5C) in the hemolymph of immune challenged *Drosophila* larvae. In a later study by Verleyen *et a*l. (2006), an actin fragment (SSSSLEKSYELPDGQVI) derived from

the precursor actin5C was detected in the hemolymph of challenged fruitflies by means of 2D- nanoLC MS/MS. In this study, one of three partial actin fragment sequences (SYELPDGQVITIGNER, Table 4.5) is similar to the sequence derived from actin5C. For this reason, both peptides were synthesized and tested for their effect on the growth of *B. subtilis*.

No inhibition of *B. subtilis* growth was detected in the presence of both peptides when tested at various concentrations up to 100 μg/ml. However, it cannot be excluded that the other two actin fragments for which the partial sequences are available (SVFPSIVGRPR and VAPEEHPVLLTEAPINPK) are not responsible for the observed Gram-positive antibacterial activity.

Chapter 5: Concluding discussion

Over the past few years, investigations in the field of tick innate immunity have increased considerably, but there still remains much to be done in order to understand the mechanisms and molecules involved. Knowledge obtained by these studies will lead to better control strategies against ticks as well as diseases they transmit. The current ideas regarding tick innate immunity were outlined in the introduction to this thesis. Survival of the tick in nature is dependent on its awareness and response to pathogens. Ticks utilize a defense mechanism similar to the innate immune system of vertebrates to protect themselves from pathogens (Johns *et al.,* 2000; 2001a, b; Nakajima *et al.,* 2001; 2002; 2003a; Sonenshine & Hynes, 2008). Information obtained from structure-function investigations of the molecules involved in tick innate immunity, may also be useful for the development of novel antimicrobial drugs or anti-tick vaccines.

A number of AMPs have been described from ticks including defensin from the hemolymph of *O. savignyi* (Olivier, 2002). No work has however, been done on the PRRs molecules from this tick species. The aim of the current study was therefore to investigate potential immunoprotective molecules that recognize and bind to Gram-negative bacterial surfaces. Chapter 2 describes the attempts to identify such molecules, while Chapter 3 describes the molecular characterization of a potential binding protein identified in Chapter 2. Due to numerous reports on the presence of potential AMPs secreted during feeding, the antimicrobial activity from the salivary glands of *O. savignyi* was investigated in Chapter 4.

Two high molecular mass HMM PRRs (Mr > 250 kDa) were identified from hemolymph, based on the ability to selectively elute tick-derived proteins from *E. coli* affinity beads using high ionic strength conditions (0.5 M NaCl). Previously, chaotropic and extreme pH conditions were used to elute surface binding

proteins from Gram- positive bacteria (Zhu *et al.,* 2005). These conditions were not suitable in the present study as it caused disruption of the Gram-negative bacteria. The high ionic strength method developed in the present study proved to be more effective and could in future be used to identify PRRs that recognize Gram-negative bacteria in other tick species. However, this method has some disadvantages, in that low molecular mass proteins from bacterial origin were also extracted. This could mask tick-derived proteins with similar molecular masses. Future studies may improve the current methods by using LPS rather than whole bacteria as affinity beads. This will eliminate bacterial background and allow bound proteins to be characterized without bacterial protein interferences (Mitsuzawa *et al.,* 2006). This strategy is based on biotinylated-LPS and the affinity of biotin which associates with streptavidin-resin and forms a hemolymph protein-LPS-biotin-streptavidin-resin complex (Fig 5.1).

The HMM proteins identified in this study are noteworthy. It is not known whether they are polymers composed of monomers or synthesized as one large protein. These monomers could be stored in the hemocytes and covalently linked in plasma following a challenge. Such a large protein was identified in the horseshoe crab and was shown to be composed of two monomers, coagulin and proxin. These molecules make up a complex coagulation network of non-covalent head-to-tail homopolymers (x-coagulin-coagulin-x). These polymers act in synergy with hemolymph plasma to trap, bind and immobilize microbes (Isakova & Armstrong, 2003). Alternatively, coagulin binds to proxin on the hemocytes by means of the action of a transglutaminase (TGase) (Osaki & Kawabata, 2004). It has been suggested that these polymers play a crucial role in the defense mechanism of the horseshoe crab (Armstrong & Armstrong, 2003; Matsuda *et al.,* 2007).

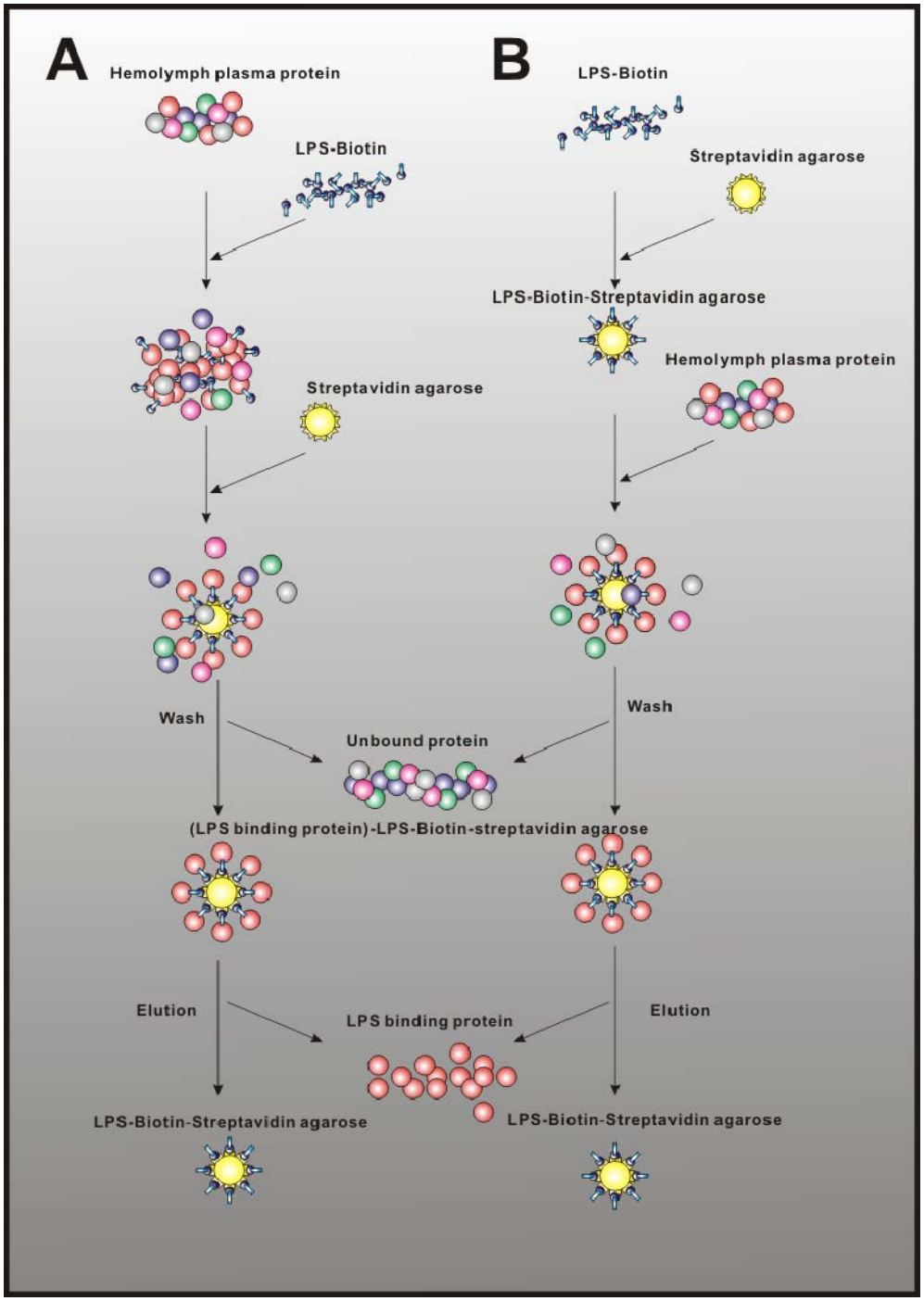


Figure 5.1 Schematic representation of the LPS binding strategy. A. Hemolymph plasma binds to LPS-biotin followed by subsequent binding to streptavidin B. Preparation of LPS-biotin-streptavidin beads prior to binding.

This might explain the absence of the HMM proteins when hemocyte extracts were analyzed. Future experiments could consider ways to activate hemocyte contents before analysis, thereby inducing the formation of HMM complexes. Alternatively, the HMM protein could be expressed in fat body tissue and secreted into the hemolymph. Future studies that use antibodies generated against the HMM proteins could resolve this.

The HMM proteins were further characterized using tryptic digestion followed by tandem MS/MS and *de novo* sequencing. While this yielded high quality ion spectra data, no homologous proteins were identified from the sequence database. This is most probably due to the current paucity in sequence data for tick hemolymph proteins. It is a well recognized current problem that the limited number of tick protein sequences available in databases is a drawback to the identification of tick proteins using a proteomics approach (Madden *et al.,* 2002; Oleaga *et al.,* 2007). The number of tick sequences in Genebank is however rapidly growing and MS/MS ion spectra data and *de novo* sequences may be archived and used to search databases in the future (Blackburn & Goshe, 2009; Shevchenko *et al.,* 2009). However until then, *de novo* sequences may be employed for the design of degenerate primers for cloning of the genes of the corresponding proteins by a PCR-based approach (Lingner *et al.,* 1997; Shevchenko *et al.,* 2001).

In the case of Protein X, a number of hits showed a similar peptide (WTDYYDRM) which had a low degeneracy. Due to its recurrence, we considered the possibility that this peptide would be a true sequence of Protein X and the degenerate primer designed from this sequence was used in an attempt to identify this molecule using cDNA derived from hemocytes. This strategy did not yield the expected sequence for Protein X, but rather a novel lipocalin-like sequence designated as savicalin which is described in Chapter 3.

The discovery of savicalin in hemocytes seems to be serendipitous because its expression profiles seem to correlate with responses towards bacterial challenge, even though it seems to be completely unrelated to the HMM proteins. However, it might not be surprising that savicalin could be involved in antimicrobial responses, because the primary function of hemocytes is related to the innate immune system of arthropods (Schlenke *et al.,* 2007). It can as such be expected that most hemocyte-derived proteins that are not involved in house-keeping functions will have some function related to the arthropod immune system.

Perhaps more surprising is the fact that savicalin is the first non-salivary gland lipocalin described in ticks. Lipocalins are the most abundant proteins found in tick salivary glands, in terms of transcript numbers, protein expression levels and numbers of family members (Mans *et al.,* 2008a, b). To date more than 300 lipocalin sequences (Table 3.1; 3.2 and Appendix I; II) have been deposited into the sequence databases and most have been indicated to have been derived from salivary glands (Mans *et al.*, 2008 a, b). This could suggest, perhaps erroneously, that tick lipocalins are limited to salivary glands and tick feeding.

However, a number of lipocalins have been identified in whole-body derived EST libraries from *R.* (*Boophilus*) *microplus* and *O. porcinus*, and have the potential to function in other facets of tick biology (Mans *et al.,* 2008a). In addition, salivary gland-derived lipocalin transcripts were also detected in midguts from the hard tick *I. ricinus* (Beaufays *et al.,* 2008a, b). However, the study described in this thesis is the first to assign a lipocalin-like molecule, savicalin, uniquely to non-salivary gland derived tissues and this includes hemocytes, midgut and ovaries of the soft tick *O. savignyi*. This provides evidence that tick lipocalins can also function in a non-feeding capacity in ticks.

Ticks are obligate blood-feeding parasites that interact with their hosts mainly at the feeding site. Soft ticks such as *O. savignyi* feed multiple times imbibing

relatively small amounts of blood that is utilized for laying small batches of eggs as well as for development and molting (Sonenshine, 1991; Mans & Neitz, 2004a). During feeding a salivary gland derived cocktail of bio-active proteins is secreted into the feeding site and this suppresses the host's immune and hemostatic defenses. Soft ticks may secrete up to 200 different proteins during the feeding process, all of which belong to various protein families (Mans *et al.,* 2008a; Francischetti *et al.,* 2008a, b). Characterization of the protein families found in salivary glands by proteomic methods have become a standard means to identify proteins secreted during feeding that are involved in the regulation of the host's defense mechanisms (Ribeiro & Francischetti, 2003; Francischetti *et al.,* 2009).

The presence of a signal peptide is indicative of the secretory nature of a salivary gland protein and implicates it as being functional at the feeding site in the host (Valenzuela *et al.,* 2002; Ribeiro *et al.,* 2006; Mans *et al.,* 2008a). Signal peptides also target secretory proteins to the secretory granules where proteins are stored as aggregated masses until needed (Mans *et al.,* 2001). These proteins do not have a house-keeping function (i.e: functions different to the tick-host interaction). A basic assumption is thus that salivary gland derived proteins are limited to the salivary gland and will not be present in other tissues. The absence of proteins in tissues other than the salivary gland is thus also used as evidence for potential roles in feeding (Stutzer *et al.,* 2008). It was, however, indicated that most protein families present in salivary glands are related to much larger families generally found in arthropods and that salivary gland proteins probably derived from these families with house-keeping function during the evolution of hematophagy in ticks (Mans & Neitz, 2004a; Mans *et al.,* 2008a). As such, protein family members should also exist in non- salivary gland tissues. Exon - intron gene structure similarities suggested that tick salivary gland-derived lipocalins are evolutionary related to lazarillo sequences from insects and it was therefore suggested that

such non-salivary gland derived lipocalins should also exist in ticks (Mans & Neitz, 2004a, b).

Homology modeling supports the inclusion of savicalin into the lipocalin family. The model and multiple alignments indicate the presence of five disulphide bonds. Two conserved disulphide bonds are found in hard and soft tick lipocalins. A third disulphide bond is shared with the TSGP4-clade of the leukotriene C4 binding soft tick lipocalins, while a fourth is shared with a lipocalin from the hard tick *I. scapularis*. The fifth disulphide bond is unique and links strands D-E in the beta-barrel lipocalin fold. Phylogenetic analysis showed that savicalin is a distant relative of salivary gland-derived lipocalins, but groups within a clade that is possibly non-salivary gland derived. It lacks the biogenic amine-binding motif associated with tick histamine and serotonin binding proteins. Expression profiles indicated that savicalin is found in hemocytes, midguts and ovaries, but not in the salivary glands. Up-regulation occurs in hemocytes after bacterial challenge and in midguts and ovaries after feeding. Given its tissue distribution and up-regulation of expression, it is possible that this lipocalin functions either in tick development following feeding or in an antimicrobial capacity.

There are similarities between hemocytes, midgut and ovaries that might explain the distribution of savicalin in these tissues. Note that antibodies against lipocalin-like proteins may cross-react with hemocytes, midgut and ovaries (Magnarelli *et al.,* 1991; Cristofoletti *et al.,* 2005; Wang *et al.,* 2007). This implies that hemocytes, midgut and ovaries share common antigenic determinants or that the same or similar proteins are expressed in these organs. It has also been shown that the number of hemocytes (nongranular cells and granule-scant basophilic granular cells) increases during ecdysis of fed nymphs and that basophilic granular cells increase in fed adult ticks and nymphs (Kadota *et al.,* 2003). Up- and down-regulation of savicalin could thus be closely related to

hemocyte numbers during ecdysis. Molecules known to influence tick development and ecdysis, include ecdysone and juvenile hormone.

Savicalin could also act as an antimicrobial by scavenging siderophores, as found for the mammalian lipocalin NGAL (Alpízar-Alpízar *et al.,* 2009). Given its up-regulation in hemocytes upon bacterial challenge, savicalin might also play a critical role in the defence against harmful pathogens. In this regard, it should be noted that ecdysone and juvenile hormone influence the innate immune system of insects and could be potential ligands for savicalin (Meister & Richards, 1996; Figueiredo *et al.,* 2006; Flatt *et al.,* 2008).

The innate immune responses associated with hemocytes and hemolymph function primarily to protect the tick against foreign invasions that gain access to the tick via breaches in the integument, genital pore or from the gut by crossing the midgut barrier. A significant amount of fluid in the form of blood is ingested during the feeding process, which is eventually digested in the midgut. Argasid ticks digest blood over a prolonged period (Sonenshine, 1991), which makes the midgut a potential ground for bacterial growth and invasion. To counteract this, ticks can secrete AMPs into the lumen of the gut (Nakajima e*t al.,* 2005). It is, however, known that salivary gland contents can be ingested with the blood-meal and has been suggested as a possible mechanism to maintain the fluidity in the gut after feeding (Bowman & Sauer, 2004). Secretion of AMPs in the saliva, could therefore serve to “sterilize” the feeding site and the ingested blood-meal from any microbial contamination. Ingestion of such AMPs with the blood-meal could also help to maintain the sterility after feeding has been completed.

This rationale prompted us to look into the potential for AMPs in the salivary glands (Chapter 4). Gram-positive antimicrobial activity was detected and purified using reversed-phase chromatography. Three potential proteins were visualized

using tricine SDS-PAGE of which one was savignin, the anti-thrombin inhibitor previously characterized by Nienaber *et al.* (1999).

Savignin was ruled out as an antimicrobial by introducing 10 kDa cut-off spin columns as an additional purification step. This yielded an antimicrobial fraction with molecular masses ranging from 0.99 ~ 7.182 kDa as determined by MALDI-MS. MS-MS analysis of the active fraction revealed the presence of actin-derived peptide fragments. In arthropods the up-regulation of full-length actin following challenge has been reported (Vierstraete *et al.,* 2004a, b; Paskewitz & Shi, 2005). Actin-derived peptide fragments have been observed in the hemolymph of septically challenged *Drosophila* (Verleyen *et al.,* 2006) as well as in a celomocyte extract of the starfish, *A. rubens* (Maltseva *et al.,* 2007; Li *et al.,* 2010).

The initial focal point of this study was to investigate proteinaceous antimicrobials. Some evidence has shown that insects produce antimicrobial metabolites (Leem *et al.,* 1996, 1999; Chiou *et al.,* 1998). Leem *et al.* (1996) first reported that the fleshfly, *Sarcophaga peregrine,* produces an inducible compound known as N-β-alanyl-5-S-glutathionyl-3,4-dihydroxy-phenylalanine. From larvae of the saw fly, *Acabtholyda parki*, *p*-hydroxycinnamaldehyde, an antimicrobial metabolite was identified (Leem *et al.,* 1999). Another two metabolites β-alanyl-tyrosine and 3-hydroxykynurenine were identified in the hemolymph of grey fleshy fly larvae, *Neobelliera bullata* (Chiou *et al.,* 1998). As such, other compounds or metabolites playing a role in anti-Gram positive activity may have been overlooked. In some cases, these compounds and metabolites could not be detected with a HPLC detector. Thus, more sensitive techniques such as LC-MS may be required for further studies.

This study utilized novel approaches that yielded some interesting results even if they may be deemed to be unconventional. The presence of heretofore unknown

HMM proteins in hemolymph that can bind to microbial surfaces has been detected using a bacterial bead and elution approach. The serendipitous discovery of a non-salivary gland lipocalin implicated in tick innate immunity has suggested novel functions for a very well characterized tick salivary gland family. At this stage one of the actin derived fragments could not be linked to the observed antimicrobial activity. It cannot be excluded that the other fragments are not involved. Their biological functions need to be investigated.

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Appendix

Appendix I: List of putative lipocalins obtained from hard ticks

Gene accession code	Species	name	Expression	Function	Ligand	Reference
196476629	*Amblyomma americanum*	Lipocalin	Salivary gland (Adult female)	Not determined	Not determined	Aljamali, NCBI direct submission 2008 Aljamali *et al.,* 2009
45360102	*Boophilus microplus (Rhipicephalus microplus)*	Lipocalin-like protein (BmLipo)	Salivary gland	Not determined	Not determined	Azzolini, NCBI direct submission
51011424	*Ixodes pacificus*	Putative secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011558	*Ixodes pacificus*	Putative secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011480	*Ixodes pacificus*	Putative secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011422	*Ixodes pacificus*	Truncated secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011604	*Ixodes pacificus*	Putative secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011586	*Ixodes pacificus*	Putative secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011498	*Ixodes pacificus*	Truncated secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011532	*Ixodes pacificus*	Putative secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011410	*Ixodes pacificus*	Putative secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
51011416	*Ixodes pacificus*	Putative secreted histamine binding protein	Salivary gland	Not determined	Not determined	Francischetti *et al.*,2005
160423587	*Ixodes ricinus*	IR24H	Salivary gland	Not determined	Not determined	Chmelař *et al.*,2008
160423404	*Ixodes ricinus*	IR4D	Salivary gland	Not determined	Not determined	Chmelař *et al.*,2008
160423666	*Ixodes ricinus*	IR7D	Salivary gland	Not determined	Not determined	Chmelař *et al.*,2008
219935266	*Ixodes ricinus*	LIR1	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935268	*Ixodes ricinus*	LIR2	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935270	*Ixodes ricinus*	LIR3	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935272	*Ixodes ricinus*	LIR4	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935274	*Ixodes ricinus*	LIR5	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935278	*Ixodes ricinus*	LIR7	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935280	*Ixodes ricinus*	LIR8	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935284	*Ixodes ricinus*	LIR9	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935288	*Ixodes ricinus*	LIR10	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935290	*Ixodes ricinus*	LIR11	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935292	*Ixodes ricinus*	LIR12	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935294	*Ixodes ricinus*	LIR13	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
219935296	*Ixodes ricinus*	LIR14	Salivary gland	Not determined	Not determined	Beaufays *et al.*,2008a
22164270	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
22164268	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083743	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083682	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083741	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006

22164278	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083455	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083457	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083713	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083333	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083659	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083417	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083755	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro et al., 2006
67083587	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083589	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083709	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
22164320	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083431	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083637	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083407	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
15428292	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083485	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083717	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083719	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083721	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
15428310	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
15428302	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
22164276	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083547	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
15428298	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083725	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083439	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083737	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
22164318	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083769	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083623	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083669	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083537	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083178	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083238	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
22164316	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083315	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083222	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083405	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083619	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
56159943	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083262	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083611	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083627	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083651	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083684	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083686	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083749	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
15428306	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083653	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083735	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006
67083284	*Ixodes scapularis*	IXOSC	Salivary gland	Not determined	Not determined	Ribeiro *et al.*,2006

Appendix II: List of putative lipocalins obtained from soft ticks

Gene accession code	Species	name	Expression	Function	Ligand	Reference
114153299	*Argas monolakensis*	AM-1124	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114153281	*Argas monolakensis*	AM-925	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114153165	*Argas monolakensis*	AM-304	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114153123	*Argas monolakensis*	AM-249	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114153089	*Argas monolakensis*	AM-209	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114153071	*Argas monolakensis*	AM-194	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114153281	*Argas monolakensis*	AM-925	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114153053	*Argas monolakensis*	AM-180	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114153035	*Argas monolakensis*	AM-118	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114152997	*Argas monolakensis*	AM-53	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114152995	*Argas monolakensis*	AM-51	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114152993	*Argas monolakensis*	AM-50	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114152989	*Argas monolakensis*	AM-47	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114152981	*Argas monolakensis*	AM-41	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114152959	*Argas monolakensis*	AM-26B	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
114152957	*Argas monolakensis*	AM-26A	Salivary gland (Adult female)	Not determined	Not determined	Mans *et al.*, 2008a
172051078	*Ornithodoros coriaceus*	Salivary lipocalin (OC-15)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051154	*Ornithodoros coriaceus*	Salivary lipocalin (OC-43)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051146	*Ornithodoros coriaceus*	Salivary lipocalin (OC-41)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051090	*Ornithodoros coriaceus*	Salivary lipocalin (OC-2)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051116	*Ornithodoros coriaceus*	Salivary lipocalin (OC-3)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051210	*Ornithodoros coriaceus*	Salivary lipocalin (OC-62)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051236	*Ornithodoros coriaceus*	Salivary lipocalin (OC-80)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051084	*Ornithodoros coriaceus*	Moubatin-like lipocalin (OC-169)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051218	*Ornithodoros coriaceus*	Salivary lipocalin (OC-677)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051166	*Ornithodoros coriaceus*	Salivary secreted lipocalin (OC-477)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051204	*Ornithodoros coriaceus*	Salivary lipocalin (OC-6)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051222	*Ornithodoros coriaceus*	Salivary lipocalin (OC-7)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051234	*Ornithodoros coriaceus*	Salivary lipocalin (OC-8)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051114	*Ornithodoros coriaceus*	Salivary lipocalin (OC-29)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b

172051112	*Ornithodoros coriaceus*	Salivary lipocalin (OC-28)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051206	*Ornithodoros coriaceus*	Moubatin-like lipocalin (OC-60)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051168	*Ornithodoros coriaceus*	Salivary lipocalin (OC-481)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
172051062	*Ornithodoros coriaceus*	Salivary lipocalin (OC-128)	Salivary gland	Not determined	Not determined	Fracischetti *et al.*, 2008b
149287076	*Ornithodoros parkeri*	Salivary lipocalin (OP-468)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149286978	*Ornithodoros parkeri*	Salivary lipocalin (OP-168	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287102	*Ornithodoros parkeri*	Salivary lipocalin (OP-509)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149286916	*Ornithodoros parkeri*	Salivary lipocalin (OP-113)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287038	*Ornithodoros parkeri*	Salivary lipocalin (OP-316)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287088	*Ornithodoros parkeri*	Salivary secreted lipocalin (OP-49)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287092	*Ornithodoros parkeri*	Salivary lipocalin (OP-49a)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287008	*Ornithodoros parkeri*	Salivary lipocalin (OP-24)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287084	*Ornithodoros parkeri*	Salivary lipocalin (OP-485)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149286896	*Ornithodoros parkeri*	Moubatin homologue 1 (OP-1)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287032	*Ornithodoros parkeri*	Short salivary moubatin (OP-30)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149286962	*Ornithodoros parkeri*	Moubatin homologue 6 (OP-15)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149286972	*Ornithodoros parkeri*	Salivary lipocalin (OP-16)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287118	*Ornithodoros parkeri*	Moubatin homologue 5 variant (OP-55a)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287116	*Ornithodoros parkeri*	Moubatin homologue 5 (OP-55)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287000	*Ornithodoros parkeri*	Moubatin 1 homolog 2 (OP-2)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
147287126	*Ornithodoros parkeri*	Moubatin homolog 4 (OP-6)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287112	*Ornithodoros parkeri*	Salivary secreted lipocalin (OP-540)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149287170	*Ornithodoros parkeri*	Moubatin homolog 7 (OP-88)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
149286990	*Ornithodoros parkeri*	Truncated salivary lipocalin (OP-177)	Salivary gland	Not determined	Not determined	Francischetti *et al.*, 2008a
29779506	*Ornithodoros porcinus porcinus*	-	Total ticks	Not determined	Not determined	Neilan, 2003 NCBI direct submission

Appendix III: Overview of AMPs from soft ticks

Tick species	AMP name	Primary tissue source (s)	Antimicrobial activity			References	Genebank Accession code
			G+	G-	Fungi		
A. monolakensis	AM-135	SG	**N/D**	**N/D**	**N/D**	Mans *et al.*, 2008a	114153040
A. monolakensis	AM-383	SG	**N/D**	**N/D**	**N/D**	Mans *et al.*, 2008a	114153200
A. monolakensis	AM-1173	SG	**N/D**	**N/D**	**N/D**	Mans *et al.*, 2008a	114153302
A. monolakensis	AM-1246	SG	**N/D**	**N/D**	**N/D**	Mans *et al.*, 2008a	114153306
A. monolakensis	AM-189	SG	**N/D**	**N/D**	**N/D**	Mans *et al.*, 2008a	114153066
O. coriaceus	OC-32	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008b	172051122
O. coriaceus	OC-31	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008b	172051120
O. coriaceus	OC-33	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008b	172051124
O. coriaceus	OC-696	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008b	172051220
O. coriaceus	OC-487	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008b	172051172
O. parkeri	OP-104	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008a	149286902
O. parkeri	OP-129	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008a	149286938
O. parkeri	OP-New-613	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008a	149287206
O. parkeri	OP-336	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2008a	149287050
O. savignyi	Fraction 50%	HL	**+**	**-**	**N/D**	Unpublished	Unpublished
O. savignyi	Fraction 30%	SG	**+**	**+**	**N/D**	Unpublished	Unpublished

+: Activity present
-: Activity absent
N/D: Not determined
SG: Salivary gland
HL: Hemolymph

Appendix IV: Overview of AMPs from hard ticks

Tick species	AMP name	Primary tissue source (s)	Antimicrobial activity			References	Genebank Accession code
			G+	G-	Fungi		
A. americanum	Amercin	HC	**N/D**	**N/D**	**N/D**	Todd *et al.*, 2007	114438982
I. pacificus	Neuropeptide -like protein (nlp-31)	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2005	51011444
I. pacificus	Neuropeptide -like protein (nlp-31)	SG	**N/D**	**N/D**	**N/D**	Francischetti *et al.*, 2005	51011404
I. ricinus	Contig 921	SG	**N/D**	**N/D**	**N/D**	Chmelař *et al.*, 2008	160423978
I. ricinus	Contig 969	SG	**N/D**	**N/D**	**N/D**	Chmelař *et al.*, 2008	160424079
I. ricinus	Contig 279	SG	**N/D**	**N/D**	**N/D**	Chmelař *et al.*, 2008	
I. ricinus	Contig 494	SG	**N/D**	**N/D**	**N/D**	Chmelař *et al.*, 2008	
I. ricinus	Contig 935	SG	**N/D**	**N/D**	**N/D**	Chmelař *et al.*, 2008	
I. scapularis	56159955	SG	**N/D**	**N/D**	**N/D**	Ribeiro *et al.*, 2006	56159955
I. scapularis	56159959	SG	**N/D**	**N/D**	**N/D**	Ribeiro *et al.*, 2006	56159959
I. scapularis	IS-18-24-clu340	SG	**N/D**	**N/D**	**N/D**	Ribeiro *et al.*, 2006	67083170
I. scapularis	ISJ-ISPL_P10_H6	SG	**N/D**	**N/D**	**N/D**	Ribeiro *et al.*, 2006	67083433
I. scapularis	50363174	SG	**N/D**	**N/D**	**N/D**	Ribeiro *et al.*, 2006	50363174

N/D: Not determined
HC: Hemocytes
SG: Salivary gland

Appendix V: *De novo* sequence results of active fraction

Hit	Score[a]	Accession number	Peptide sequence[b]	Protein name[c]
1	71	46447343	682 LMZYTPMELR 691	Hypothetical protein.//:sptrembl|Q6MAG6|Q6MAG6_PARUW. Hypothetical protein.//:trembl|BX908798|BX908798_1709 product: "unknown protein"; Parachlamydia-related symbiont UWE25, complete genome
2	68		529 YALSPNALFPR 539	AgCP1586 (Fragment)
3	64	44355455	732 ETDYFFNZRZE 742	"unknown"; Environmental sequence IBEA_CTG_2107665, whole genome shotgun sequence.
4	64	29747767	859 EDYFFNZR 866	"4921510J17Rik protein"; Mus musculus RIKEN cDNA 4921510J17 gene, mRNA (cDNA clone MGC:58741 IMAGE:6770929), complete cds.
5	64	51859290	859 EDYFFNZR 866	"Unknown (protein for MGC:93934)"; *Rattus norvegicus* cDNA clone MGC:93934 IMAGE:7113453, complete cds
6	63	259683019	753 ETDFYFNZ 760	"Hypothetical protein CBG20942"; *Caenorhabditis briggsae* contig cb25.fpc4206 from assembly cb25.agp8
7	63	24636146	753 ETDFYFNZ 760	Serpentine receptor, class e (epsilon) protein 52"; *Caenorhabditis elegans* cosmid C50E10, complete sequence.
8	63	24636146	753 ETDFYFNZ 760	"Hypothetical protein C50E10.5"; *Caenorhabditis elegans* cosmid C50E10, complete sequence. //:pironly|T33499|T33499 hypothetical protein C50E10.5 - Caenorhabditis elegans//:gp|AF098988|14916287. Hypothetical protein C50E10.5 [*Caenorhabditis elegans*]
9	59	15042158	899 SSFZRFFN 906	"048R"; *Chilo iridescent virus* complete genome. //:gp|AF303741|15042201 048R [*Chilo iridescent virus*]

[a] Score (high scoring pair, HSP) as determined by BLAST, using sequences obtained from *de novo* sequencing. Matches higher than 64 are considered significant

[b] Peptide sequence of the *de novo* data. The numbers give the residues to which the query sequence matched to the protein sequence in the database

[c] Identity of the proteins to which the *de novo* sequences were matched