Isolation and characterization of antibacterial peptides from the hemolymph of the soft tick, *Ornithodoros savignyi*

by

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CONTENTS

LIST OF ABBREVIATIONS

A .....Adenosine
Aedes aegypti.....*A. aegypti*
Ala or A .....Alanine
Arg or R.....Arginine
Asn or N.....Asparagine
Asp or D.....Aspartic acid
ATP.....Adenosine triphosphate
ATZ .....Anilinothiazolinone

BAEE.....N-α-benzoyl-L-arginine ethyl ester
B. cereus .....*Bacillus cereus*
BLAST.....Basic local alignment search tool
bp .....Base pair
BSA .....Bovine Serum Albumin
B. subtilis .....*Bacillus subtilis*

C.....Cytosine
°C.....Degrees Celsius
cDNA.....Complementary DNA
Cif.....Cecropia immunoresponsive factor
C-terminal.....Carboxyl terminal
Cys or C.....Cysteine

DEPC.....Diethyl pyrocarbonate
Dif.....*Drosophila* immunoresponsive factor
DMF.....Dimethylformamide
DNA.....Deoxyribonucleic acid
DNAse .....Deoxyribonuclease
dNTP.....Deoxynucleotide triphosphate
ds.....Double stranded
DTT .....Dithiotreitol

D. variabilis .....*Dermacentor variabilis*

E. coli .....*Escherichia coli*

EDTA.....Ethanol diamine tetra-acetic acid

E. faecalis .....*Enterococcus faecalis*

EtBr.....Ethidium Bromide

G .....Guanidine

Gln or Q.....Glutamine

Glu or E .....Glutamic acid

Gly or G.....Glycine

H. cecropia .....*Hyalophora cecropia*

His or H .....Histidine

HPLC.....High performance liquid chromatography

Hsp.....Heat shock protein

I.....Inosine

Ile or I .....Isoleucine

IPTG .....Isopropyl-β-D-thiogalactopyranoside

K .....Lysine

kDa.....Kilo dalton

K. pneumoniae.....*Klebsiella pneumoniae*

LB .....Luria-Berthani

Leu or L .....Leucine

Lys or K.....Lysine

M.....Molar

mM.....Millimolar

µM .....Micromolar

MALDI.....Matrix Assisted Laser Desorption Ionization

Met or M.....Methionine

mg .....Milligram
MH.....Mueller-Hinton
min .....Minutes
M. luteus .....*Micrococcus luteus*
MOPS .....Morpholinopropanesulfonic acid
mRNA.....Messenger RNA

NCBI.....National Center for Biotechnology Information
ng .....Nanogram
N-terminal.....Amino terminal
NTP.....Nucleotide triphosphate
dNTP.....Deoxynucleotide triphosphate

O. moubata .....*Ornithodoros moubata*
O. savignyi.....*Ornithodoros savignyi*
OD .....Optical density

P. aeruginosa .....*Pseudomonas aeruginosa*
PAGE.....Polyacrylamide gel electrophoresis
PBS .....Phosphate buffered saline
PCR.....Polymerase chain reaction
PITC .....Phenylisothiocyanate
P. mirabilis .....*Proteus mirabilis*
Phe or F.....Phenylalanine
PMSF.....Phenylmethylsulfonyl fluoride
Pro or P .....Proline
P. terranovae .....*Phormia terranovae*
PTH.....Phenylthiohydantoin

RACE.....Random amplification of cDNA ends
RNA.....Ribonucleic acid
RNAse .....Ribonuclease
RP-HPLC.....Reversed phase HPLC
rRNA .....Ribosomal RNA

RT .....Reverse transcription

RT-PCR .....Reverse transcription PCR

SDS .....Sodium dodecyl sulfate

SE-HPLC .....Size exclusion HPLC

Ser or S .....Serine

S. peregrina .....*Sarcophaga peregrina*

ss .....Single stranded

Staph. aureus .....*Staphylococcus aureus*

T .....Thymidine

TAE .....Tris-acetate EDTA buffer

Taq .....*Thermus aquaticus*

TE .....Tris-EDTA buffer

TEMED .....N',N',N,N,-Tetramethyl-ethylene diamine

Thr or T .....Threonine

T_m .....Melting temperature

TOF .....Time of flight

Tricine .....N-tris (hydroxymethyl) methylglycine

Tris .....Tris (hydroxymethyl) aminomethane

Trp or W .....Tryptophan

T. tridentatus .....*Tachypleus tridentatus*

Tyr or Y .....Tyrosine

U .....Units

UV .....Ultraviolet

Val or V .....Valine

X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Z. atratus .....*Zophobas atratus*

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Chapter 1
General introduction

1.1 The biology of ticks

Ticks are obligate blood sucking arthropods found in most regions of the world. They are members of the subclass Acari, the major subclass of the Arachnida, and the approximately 850 species are divided into two major subfamilies. The first family is the *Argasidae* or "soft ticks", so called because of their flexible, leathery skin/cuticle (Fig 1.1). The second family is the *Ixodidae* or "hard ticks", so called because of their sclerotized dorsal scutal plate (Fig 1.2). A third family, the *Nuttalliellidae*, contains only one species (Sonenshine, 1991).

Ticks are regarded as pests since they have been found to transmit more vector-borne diseases than any other vector in the United States of America (Nakajima *et al.*, 2001). They can transmit a very wide variety of pathogenic organisms, including fungi, viruses, rickettsiae, bacteria and protozoa. Due to the widespread prevalence of ticks, these pathogens infect not only mammals, but also birds, reptiles and amphibians. Ticks are also of major economic importance, since they are responsible for major stock losses. In addition to the tick mediated pathogenic infections, ticks may cause lethal paralysis or severe toxemia as a result of their bite. Since ixodid ticks consume a large quantity of blood, the resultant blood loss could negatively influence the condition of the animal. The weakened condition could also lead to the increased susceptibility to other illnesses (Sonenshine, 1991).

Tick life stages are similar to the paurometabulous pattern found in other arachnids and many insects, i.e., the immature stages resemble the adult forms. Hard ticks have three active developmental stages: larva, nymph and adult. Soft ticks have two or more nymphal stages, depending on the species, feeding success and other variables, resulting in a much longer developmental cycle (Sonenshine, 1991). Two differences between

larvae/nymphs and adults ticks include the fact that larvae have six, instead of eight legs, and that larvae and nymphs do not possess genital openings.

1.1.1 Argasidae

The tough, leathery cuticle of Argasid ticks is highly folded (Fig 1.1) and enables it to expand during feeding, allowing the ticks to consume their blood meal rapidly. Argasid ticks can consume 5-10 times their own body weight, usually in less than an hour. After this single feeding period, the ticks drop from the host (Sonenshine, 1991).

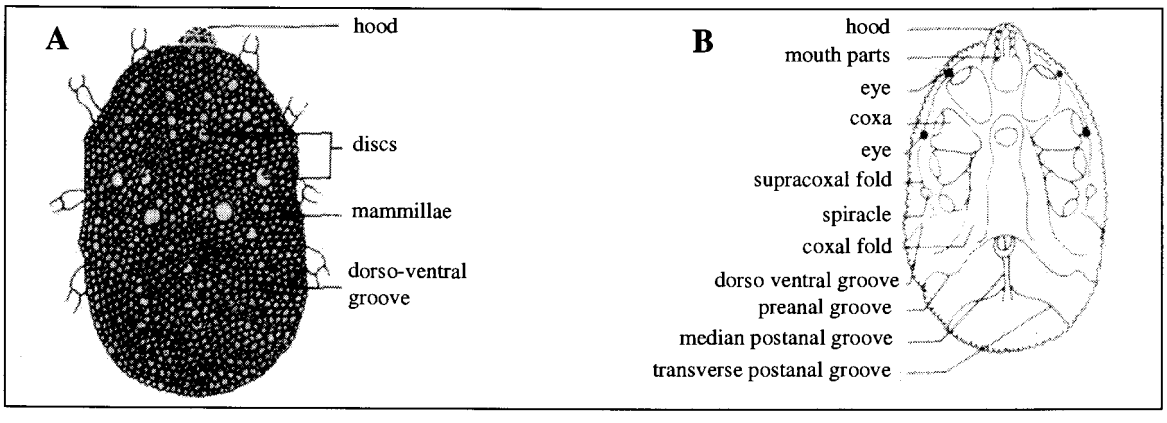

Figure 1.1 The (A) dorsal and (B) ventral view of a generalized argasid tick (Sonenshine, 1991).

1.1.2 Ixodidae

The tough, sclerotized dorsal plate, the scutum of Ixodid ticks (Fig 1.2), serves as the site for attachment of many major muscle groups. The remainder of the cuticle is expansible and fresh cuticle synthesis during blood feeding increases the cuticle size dramatically. Since the ixodid ticks must remain attached during cuticle growth, they are slow feeders, feeding time varying from a few days to several weeks (Sonenshine, 1991).

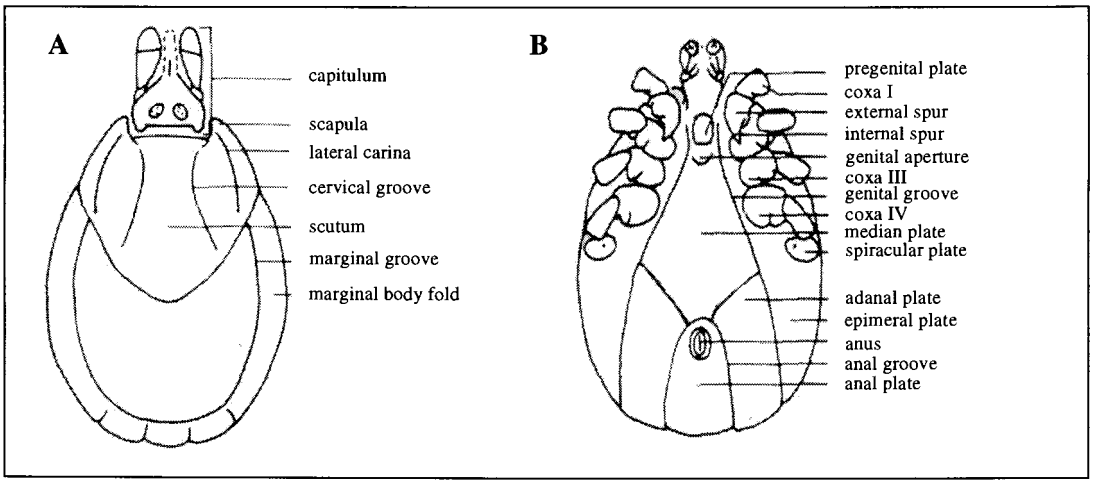

Figure 1.2 (A) The dorsal view of a general female and (B) the ventral view of a general male ixodid tick (Sonenshine, 1991).

1.1.3 Nuttalliellidae

At present there is little data about these ticks. Only one species has been described in this family to date, *Nutalliellidae namaqua*. The nuttalliellidae incorporate characteristics of both argasid and ixodid ticks in their external physiology. The females have a pseudoscutum, resembling the scutum in outline, but without the smooth appearance of the sclerotized plate in ixodid ticks, while the cuticle is extremely convoluted. The palps show only three segments (instead of four for the other families), and the leg segments articulate by means of a unique ball and socket joint (Sonenshine, 1991).

1.1.4 Tick fat-body structure and function

The functional role of the invertebrate fat body has been compared to that of the vertebrate liver. It is the organ that stores food reserves, metabolizes hormones and other essential messenger molecules and detoxifies wastes or harmful compounds. It also plays an important part in the synthesis and secretion of an egg yolk protein precursor vitellogenin. It was found that the fat body of other invertebrates is also involved in the synthesis of defense proteins, such as antimicrobial peptides (Gillespie *et al.*, 1997), although these studies have not been performed in ticks.

In contrast to insects, where the fat body is a discrete organ located in the abdominal region, the fat body of ticks is a diffuse organ. It consists of highly dispersed strands of cells clinging to branches of the tracheal system and other internal organs. The fat body is divided in two parts i.e. the central and peripheral regions. The peripheral region stains positive for glycogen, suggesting a storage function, although this region is highly active following female tick feeding and mating. The central region has, however, been implicated in the biosynthesis of several molecules. Fat body cells consist of one basic cell type, trophocytes, while subdivisions of these cells show functional differentiation. In unfed adult ticks, the cells show dense inclusions, indicative of storage, although some synthesis of lipid, glycogen and protein may occur. In fed mated female ticks the cells increase in size, the cytoplasm is filled with rough endoplasmic reticulum and the ultrastructure of the fat body cells appears consistent with intense protein synthesis (Sonenshine, 1991).

1.1.5 Hemolymph composition and function

The hemolymph of ticks is a complex tissue, and is not only involved with the transportation of hemocytes, hormone messengers, nutrients and waste products, but also maintains a osmotically balanced internal environment, acts as support for various body tissues and as a medium for the transfer of mechanical energy in the form of hydrostatic pressures. Haemocyanin is absent from the hemolymph and it is thus hypothesized that the hemolymph does not play an important role in oxygen transport (Binnington and Obenchain, 1982).

The greatest change in the protein concentration of the hemolymph occurs in mated females during feeding and directly following detachment from the host. The increase following detachment was found to coincide with the synthesis and release of vitellogenin by the fat body. The protein concentration decreases to pre-fed levels following oviposition. No protein concentration profiling studies before and after bacterial infection has been performed to date. The main carbohydrate present in hemolymph is glucose (Binnington and Obenchain, 1982).

1.1.6 *Ornithodoros savignyi* as a model for study

O. savignyi (Fig 1.3), also known as the sand tampan, is a sand-dwelling soft tick. It belongs to the family Argasidae and is endemic to the Northwestern Cape area of South Africa, Egypt, Zimbabwe, India, and Central Africa (Evans, 1929). A close relative of *O. savignyi*, *O. moubata*, is very similar in outer appearance, but they can be differentiated by the presence of two pairs of eyes situated in the supracoxal fold of *O. savignyi* which are absent in *O. moubata* (Howell *et al.*, 1978).

O. savignyi feeding activity is not limited to a specific season, and when not attached to a host the ticks usually are dormant underground in the shade of trees, bushes and stones. Complaints from farmers indicate that they are more frequently being found inside cattle pens and in the surrounding water troughs (Howell *et al.*, 1978). Both *O. savignyi* and *O. moubata* can be found living in huts and coming out at night to feed on the human occupants (Alexander, 1984). Acaricide treatment of host animals is not very effective at controlling this tick, as it feeds quickly (15-30 min) and contact with the host is therefore minimal. The ticks also avoid surface contact with ground treated with acaricides (Evans, 1929).

O. savignyi has not been proven to be responsible for the transmission of pathogens, but it is suspected that they can transmit *Treponema duttoni*, causing relapsing fever in man. *O. moubata* normally transmit this pathogen, but relapsing fever still occurs in areas endemic to *O. savignyi*, but not *O. moubata* (Evans, 1929). It is known that *O. savignyi* secretes a toxin when feeding, leading to heart failure and even death of livestock (Howell *et al.*, 1975; Mans *et al.*, 2001).

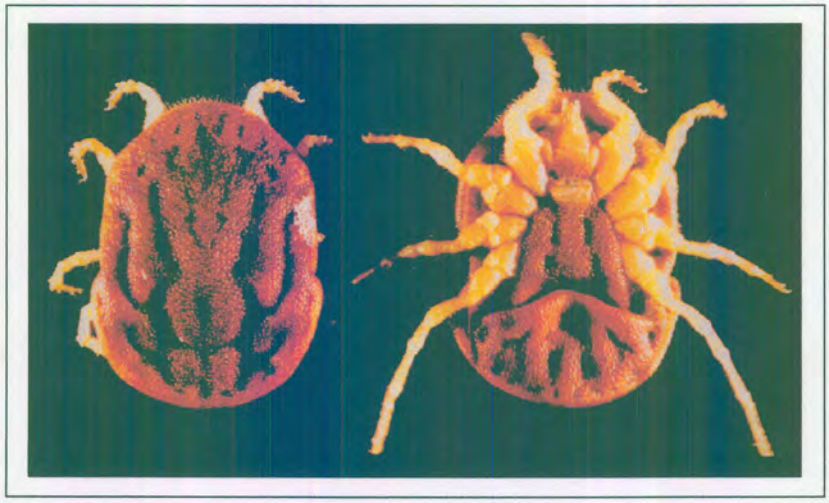

Figure 1.3 The dorsal (left) and ventral (right) view of a female *O. savignyi* tick (Mans, B.J., personal communication).

Very little is known about the defense mechanisms of *O. savignyi*. Since these ticks exhibit a very selective and low incidence of pathogen transmission, the investigation of the immune system of *O. savignyi* is important in explaining this phenomenon. Incapacitating this defense mechanism might increase the susceptibility of the ticks to infections, leading to illness or death. This could prove to be an important component in tick control.

1.2 The invertebrate immune system

A generalized immune response can be defined as an inducible defense mechanism that allows the discrimination between self and non-self. An immune system is also capable of showing specific memory in response to a second challenge with the same foreign cell or molecule. This memory response can be manifested as an enhanced reaction – such as an increased level of circulating antibody or cellular cytotoxic activity – or as a diminished response, in which case there is a decreased antibody or cellular reactivity (Marchalonis and Schluter, 1990).

The immune response of invertebrates is not adaptable, and does not have the rearranging genes for the mammalian immunoglobulin superfamily. The diverse lymphocyte lineages

of vertebrates are also not present, thus there is no clonal selection for recognition of different antigenic determinants, while a major histocompatibility complex is also lacking. Therefore, the immune system of invertebrates more closely resembles the vertebrate innate immune system. The system is rapid and relatively non-specific while the responses are both cellular and humoral (Gillespie *et al.*, 1997).

The invertebrate immune system is composed of several separate elements working in synergy. Firstly, the outer shell (integument) and the gut are the primary, physical barriers against infection (Gillespie *et al.*, 1997). Secondly, invasion leads to hemocytic activation and coordination, leading to phagocytosis (Bayne and Ryan, 1990; Brehelin, 1978; Gillespie *et al.*, 1997), encapsulation of the invading microorganisms (Gillespie *et al.*, 1997), nodule formation (Gillespie *et al.*, 1997) and the recognition of bacterial membrane carbohydrate groups by lectins (Mullainadhan and Renwrantz, 1986; Minnick *et al.*, 1986). Thirdly, several humoral processes are activated concurrently: (a) the induced synthesis of antimicrobial peptides and proteins primarily by the fat body (Gillespie *et al.*, 1997), (b) the activation of the phenoloxidase and melanization reactions (Bayne and Ryan, 1990; Boman and Hultmark, 1987; Soderhall, 1994), and (c) the activation of the hemolymph coagulation cascades (Bohn and Barwig, 1984; Iwanaga, 1993; Muta and Iwanaga, 1996; Rowley, 1977).

In invertebrates the synthesis and release of antimicrobial peptides in response to a bacterial challenge is a crucial process, and since this is also the main subject of this study, this aspect of the invertebrate immune response will be extensively discussed (Section 1.2.1).

1.2.1 Antimicrobial peptides

Unlike vertebrate humoral immunity, there is no evidence of clonal selection or memory in invertebrates. Neither is highly specific antibodies produced in response to microbial infection. Independent studies by three authors (Glaser, 1918; Paillot, 1920; Metalnikow, 1920), showed that the vaccination of insects with attenuated bacterial cultures conferred resistance to subsequent injections of virulent bacteria, even lethal doses. Most important

about these studies was that they showed the appearance of a potent antibacterial activity in the cell-free hemolymph when this resistance was induced. Similar experiments showed that no antibacterial activity was present before induction, the antibacterial activity was maximal after several hours and several stimuli could induce the antibacterial activity. However, the nature of these antibacterial factors remained elusive (Hultmark, 1993).

Lysozyme-like activity was found in several insects, and it was claimed that this constituted 100% of the humoral reaction (Mohrig and Messner, 1968). However, a poor correlation was found between lysozyme activity and induced humoral reaction against *Pseudomonas aeruginosa* (Hultmark *et al.*, 1980).

The breakthrough occurred in 1980, when three inducible antibacterial peptides were isolated from the hemolymph of immune challenged giant silk moths, *Hyalophora cecropia*. One of the peptides isolated was similar to lysozyme, but the other two were novel peptides displaying bacteriolytic ability. These peptides were called cecropins and attacins (Hultmark *et al.*, 1980).

Since then a large variety of peptide classes have been identified and characterized from both vertebrates and invertebrates. Their antimicrobial peptides can be classified in four main groups: α-helical peptides, β-sheet peptides, proline rich peptides and glycine rich peptides.

1.2.1.1 α-Helical peptides

As the name indicates, the secondary structure of the α-helical peptide is composed mostly of α-helices. The peptides belonging to this class includes the cecropins, magainins, melittin and temporins.

1.2.1.1.1 Cecropins

The cecropins were first isolated from the pupae of the giant silk moth, *H. cecropia* (Hultmark *et al.*, 1980), and have been isolated from various other species, including the

intestine of the pig (Lee *et al.*, 1989). Insect cecropins are small, cysteine free peptides 35-39 residues long, active against Gram-positive and Gram-negative bacteria, but are apparently inactive against eukaryotic cells, presumably due to the presence of cholestrol (Christensen *et al.*, 1988). The peptides are cationic with pI values ranging from 8.2 - 9.6. The C-terminal is amidated and the whole structure is a helix-bend-helix motif (Fig 1.13). The two helices are not homologous and the differences are important for the function of the peptides. The N-terminal helix has a positive charge and is amphipathic. The C-terminal on the other hand, is either less charged or neutral, and is more hydrophobic (Cociancich *et al.*, 1994).

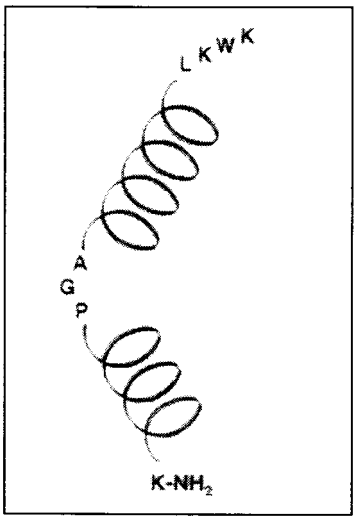

Figure 1.4 The schematic representation of the three dimensional structure of cecropin A from *H. cecropia* (Cociancich *et al.*, 1994).

The amino acid sequence alignment of cecropin sequences from various organisms is displayed in Table 1.1 to indicate the high number of conserved residues in the N-terminal region, compared to the low number of conserved residues in the C-terminal region of the peptides.

Table 1.1 Peptide sequence alignment of 19 cecropin sequences (18 invertebrate and one mammalian). Bold letters indicate conserved residues. Asterisk indicates amidated C-terminal. Dashes included to optimize alignment (Cociancich *et al.*, 1994).

Species	Peptide sequence
Hyalophora cecropia D	-**W**--NPF**K**EL**EK**V**GQ**RV**RD**AVISAGPAV**A**TVAQATALAK*
Antherea pernyi D	-**W**--NPF**K**EL**ER**A**GQ**RV**RD**AIISAGPAV**A**TVAQATALAK*
Manduca sexta B-2	-**W**--NPF**K**EL**ER**A**GQ**RV**RD**AVISAAPAV**A**TVGQAAAIAR*
Manduca sexta B-3	-**W**--NPF**K**EL**ER**A**GQ**RV**RD**AIISAGPAV**A**TVGQAAAIAR*
Manduca sexta B-4	-**W**--NPF**K**EL**ER**A**GQ**RV**RD**AIISAAPAV**A**TVGQAAAIAR*
Manduca sexta B-5P	-**W**--NPF**K**EL**ER**A**GQ**RV**RD**AVITSAAAV**A**TVGQAAAIAR*
Hyalophora cecropia A	K**W**--KLF**K**KI**EK**V**GQ**NI**RD**GIIKAGPAV**A**VVGQATQIAK*
Hyalophora cecropia B	K**W**--KVF**K**KI**EK**M**G**RNI**R**NGIVKAGPAI**A**VLGEAKAL*
Antherea pernyi B	K**W**--KIF**K**KI**EK**V**G**RNI**R**NGIIKAGPAV**A**VLGEAKAL*
Bombyx mori CMIV	R**W**--KIF**K**KI**EK**V**GQ**NI**RD**GIVKAGPAV**A**VVGQAATI*
Bombyx mori A	R**W**--KLF**K**KI**EK**V**G**RNI**RD**GLIKAGPAI**A**VIGQAKSL*
Bombyx mori B	R**W**--KIF**K**KI**EK**M**G**RNI**RD**GIVKAGPAIEVLGSAKAI*
Drosophila melanogaster A	G**W**LKKIG**K**KI**ER**V**GQ**HT**RD**ATI-QGLGI**A**QQAANVAATAR*
Drosophila melanogaster B	G**W**LRKIG**K**KI**ER**I**GQ**HT**RD**ASI-QVLGI**A**QQAANVAATAR*
Sarcophaga peregrina IA	G**W**LKKIG**K**KI**ER**V**GQ**HT**RD**ATI-QGLGI**A**QQAANVAATAR*
Sarcophaga peregrina IB	G**W**LKKIG**K**KI**ER**V**GQ**HT**RD**ATI-QVIGV**A**QQAANVAATAR*
Sarcophaga peregrina IC	G**W**LRKIG**K**KI**ER**V**GQ**HT**RD**ATI-QVLGI**A**QQAANVAATAR*
Sarcophaga peregrina ID	G**W**IRDFG**K**RI**ER**V**GQ**HT**RD**ATI-QTIAV**A**QQAANVAATLK*
Porcine P1	S**W**LSKTA**K**KL**EN**SAK-K**R**---ISEGIAI**A**IQGG-----PR

It has been shown that cecropin A and B from *H. cecropia* exist as a random coil structure in dilute buffer solutions, but assume a helical conformation in a hydrophobic environment (Steiner, 1982).

Hybrids of different cecropin peptides, for example the hybrid of *H. cecropia* A(1-11)D(12-37), can be made to increase the efficiency of the peptides. The hybrid mentioned above shows increased activity relative to both cecropin A and D. This is due to the presence of a more helical structure, a more hydrophobic C-terminal relative to cecropin A and a more basic N-terminal relative to cecropin D. The use of synthetic analogues of natural peptides in order to increase biological activity and efficiency is a growing practice in the design of new antibiotic therapies. A hybrid between cecropin A (CA) and melittin (M) displays a higher antibacterial activity, while the hybrid CA(1-

13)M(1-13) has hemolytic activity and lyses *Plasmodium falciparum* more efficiently than either cecropin A or B (Blondelle and Houghten, 1992).

Cecropins are not only restricted to insects, since this class of peptides has also been isolated from pig intestine (Lee *et al.*, 1989). This discovery could mean that the distribution of these antibacterial peptides is greater than previously thought and they could also play a more pronounced part in immune systems. Cecropin D from *H. cecropia* was found to modulate various biological functions in murine hematopoietic cells such as lymphocyte DNA synthesis and antibody production of B-cells, while having no lymphocyte cytotoxic effects (Blondelle and Houghten, 1992). Two cecropin analogues were found to exhibit anti-tumor activity (Moore *et al.*, 1994), while cecropin A was found to inhibit the replication of human immunodeficiency virus 1 (Wachinger *et al.*, 1998).

1.2.1.1.2 Magainins

The magainin class of peptides was first isolated from the skin of the amphibian *Xenopus laevis*. Magainins are small, basic peptides, 21-24 residues long with a very broad range of specificity. They are active against Gram-positive and Gram-negative bacteria, fungi and protozoans like malaria, but display negligible hemolytic activity (Tytler *et al.*, 1995). The magainins form amphipathic α-helices of sufficient length to cross the lipid membrane bilayer (Duclohier, 1994) and they have been classified as class L (lytic) amphipathic α-helices, because of their secondary structure similarities to class L peptides from bee and wasp venom (Tytler *et al.*, 1993) (Fig 1.11). In water, magainin 2 assumes a temperature-insensitive random coil structure, but it reverts to the amphipathic α-helix in a hydrophobic environment (Tytler *et al.*, 1995).

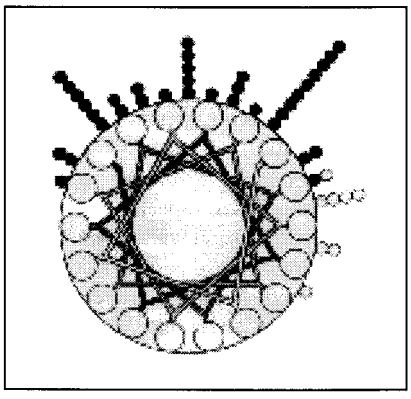

Figure 1.5 Helical wheel distribution of positively charged (•) and negatively charged (o) amino acid residues in class L amphipathic helixes (Tytler *et al.*, 1995).

Magainin 2 and analogues have been found to display anticancer activity. One of the big advantages in using these peptides to control cancer is the fact that novel or multidrug resistance to the peptides do not take place as it does with conventional chemotherapeutic compounds such as platinum complexes or anthracyclins. Two magainin analogues, (magainin A and G) designed to have enhanced α-helical formation compared to natural magainin peptides, were found to consistently inhibit the growth of six cancer cell lines (Ohsaki *et al.*, 1992). The peptides also showed additive antitumor effects when combined with the conventional chemotherapeutic drugs. It was hypothesized that the enhanced expression of phosphatidylserine on the surface of tumor cells compared to normal cells could create a target for magainin activity (Zasloff *et al.*, 1993).

1.2.1.1.3 Temporins

Temporins were isolated from the skin of the European red frog, *Rana temporaria* and are active against Gram-positive and Gram-negative bacteria. They are cysteine free, have an amidated C-terminal and are the shortest antibacterial peptides isolated so far, containing 10-13 residues. The structure of the temporins is much like the structure of peptides present in the venoms of wasps, probably being an amphipathic α-helix. The proposed mechanism is the formation of membrane spanning channels, but since the minimum length for membrane spanning is 23 residues, the temporins could only form the membrane spanning channels if they assume N-terminal tail-to-tail dimers. Data

collected is consistent with an activity inside the outer membrane. The temporins do not display hemolytic activity (Simmaco *et al.*, 1996). The amino acid sequence alignment of various temporin sequences is displayed in Table 1.2 to indicate the high level of homology between the different temporin peptides.

Table 1.2 Amino acid sequence alignment of temporin sequences. Swissprot accession numbers are included (source National Center for Bioinformatics (NCBI, http://www.ncbi.nlm.nih.gov)). Bold letters indicate conserved residues, italic letters indicate residues conserved in chemical nature. Dashes are included to optimize the alignment.

Peptides	Peptide sequence
Temporin A (P56917)	*FL***P***LI***G***R***V***L***S***G***I***LA***
Temporin C (P56918)	*LL***P***IL***G***N***L***L***N***G***L***LA***
Temporin D (P56919)	*LL***P***IV***G***N***L***L***N***S***L***LA***
Temporin E (P56920)	*VL***P***II***G***N***L***L***N***S***L***LA***
Temporin F (P56921)	*FL***P***LI***G***K***V***L***S***G***I***LA***
Temporin K(P56922)	*LS***P**---*NL***L***K***S***L***LA***
Temporin L (P56923)	*LL***P**---*NL***L***K***S***L***LA***

1.2.1.2 β-Sheet peptides

β-Sheet peptides, containing a substantial degree of β-sheet structure, occur in a diverse variety of organisms, including plants, insects, mammals and crustaceans.

1.2.1.2.1 Defensins

Defensins are the most conserved and ubiquitous class of antimicrobial peptides, occurring in vertebrates (Hancock and Lehrer, 1998), plants (Broekaert *et al.*, 1995), insects (Hoffman and Hetru, 1992), ticks (Nakajima *et al.*, 2001, Johns *et al.*, 2001) and crustaceans (Hubert *et al.*, 1996). The first insect defensins were isolated from *S. peregrina* (Matsuyama and Natori, 1988) and the name insect defensin was coined due to sequence similarities to the mammalian defensins. Insect defensins are small cationic peptides, 34 - 43 residues long with pI values in the range of 8.0 – 8.5 and are active against Gram-positive bacteria. The peptides are inactive against Gram-negative and eukaryotic cells. The major structural homology between all defensins is the presence of

six cysteine residues involved in three intramolecular disulfide bridges. There are three domains apparent in the structure of the insect defensin: a N-terminal loop showing flexibility, a central amphipathic α-helix and a C-terminal antiparallel β-sheet. The α-helix is stabilized by two disulfide bridges to one of the strands of the β-sheet and the N-terminal loop is linked to the other strand of the β-sheet via one disulfide bridge (Fig 1.12). The peptides display the same global folding in aqueous and organic solvents.

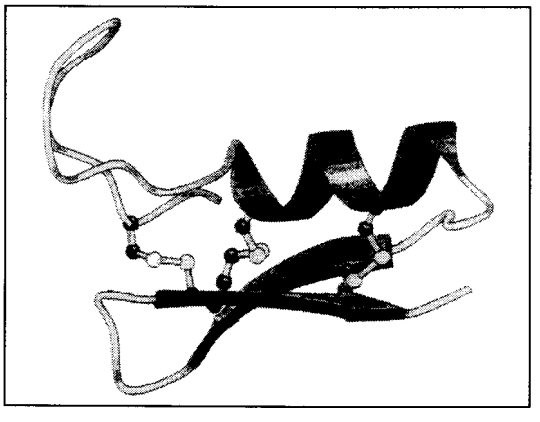

Figure 1.6 The structure of defensin A from *Phormia terranovae* (Cociancich *et al.*, 1994).

The insect defensin is related to mammalian defensins, but the structure is markedly different. The mammalian defensin does not contain a α-helix, but is composed of three antiparallel β-sheets stabilized by three intramolecular disulfide bridges (Hoffman and Hetru, 1992). Insect defensins are synthesized as larger precursor molecules known as preprodefensins. These molecules contain a 29-residue prosegment, in addition to a 23-residue signal peptide and the mature peptides (Cociancich *et al.*, 1994). Studies have shown that the global folding of defensins in methanol does not differ significantly from the folding in water.

Structural studies done on several insect peptides have shown that defensins display similarities with two peptides that are not involved in the inducible antibacterial response. These two peptides are royalisin, a 53-residue antibacterial peptide present in the royal

jelly of honeybees. The other peptide is charybdotoxin, a 37-residue toxin from scorpion venom glands. Charybdotoxin is a K^+ channel blocker and is active against eukaryotic cells (Hoffman and Hetru, 1992). The amino acid sequence alignment of various defensin sequences is displayed in Table 1.3 to indicate the conservation of the six cysteine residues.

Table 1.3 Amino acid sequence alignment of defensins from various invertebrates. Bold letters indicate conserved residues. Dashes are included to optimize alignment. Accession numbers provided indicate sequences obtained from the National Center for Bioinformatics (^{N} indicates source is Genbank; ^{S} indicates source is Swissprot) (Hoffman and Hetru, 1992; Taguchi *et al.*, 1998; Cociancich *et al.*, 1994; Nakajima *et al.*, 2001).

Species	Peptide sequence
Phormia terranova A	-ATCDLL----SGTGINHSACAAHCLLR-GNRGGYCNG--KGVCVCRN
Phormia terranova B	-ATCDLL----SGTGINHSACAAHCLLR-GNRGGYCNR--KGVCVCRN
Sarcophaga peregrina	-ATCDLL----SGTGINHSACAAHCLLR-GNRGGYCNG--KAVCVCRN
Eristalis tenax	-ATCDLL----SFLNVNHAACAAHCLSK-GYRGGYCDG--KKVCNCR
Aedes aegypti A (AAB35392)^N	-ATCDLL----SGFGVGDSACAAHCIARR-NRGGYCNA-KKVCVCRN
Aedes aegypti B (P81602)^S	-ATCDLL----SGFGVGDSACAAHCIAR-GNRGGYCNSQ-KVCVCRN
Aedes aegypti C (P81603)^S	-ATCDLL----SGFGVGDSACAAHCIAR-GNRGGYCNS-KKVCVCRN
Zophobas atratus A	-FTCDVLGFEIAGTKLNSAACGAHCLAL-GRRGGYCNS--KSVCVCR
Zophobas atratus B	-FTCDVLGFEIAGTKLNSAACGAHCLAL-GRTGGYCNS--KSVCVCR
Formica rufa	-FTCDLL----SGAGVDHSACAAHCILR-GKTGGRCNSD-R-VCVCRA
Aeschna cyanea	GFGCPL----------DQMQCHRHCQTITGRSGGYCSGPLKLTCTCYR
Leiurus quinquestriatus	GFGCPL----------NQGACHRHCRSIRRR-GGYCAGFFKQTCTCYRN
Ornithodoros moubata A	GYGCPF----------NQYQCHSHCSGIRGYKGGYCKGTFKQTCKCY
Ornithodoros moubata B	GYGCPF----------NQYQCHSHCRGIRGYKGGYCTGRFKQTCKCY

1.2.1.2.2 Tachyplesin and polyphemusin

Two new related classes of antibacterial peptides have recently been isolated from the small granules in the hemocytes of the horseshoe crab, *Tachypleus tridentatus*. These peptides are called tachyplesins and polyphemusins and are novel peptides found only in crustaceans up to date (Nakamura *et al.*, 1988). The peptides display activity against Gram positive and Gram negative bacteria as well as fungi and certain viruses such as extracellular HIV-1 (Hancock and Lehrer, 1998). Their structures are rigid antiparallel β-

sheets with two intramolecular disulfide bonds. The peptides also bind strongly to bacterial lipopolysaccharide (Matsuzaki *et al.*, 1991). The amino acid sequence alignment of various tachyplesins and polyphemusins sequences is displayed in Table 1.4 to indicate the high homology between the different peptides.

Table 1.4 Amino acid sequence alignment of tachyplesins and polyphemusins. Bold letters indicate conserved residues and italic letters indicate residues conserved in chemical nature. Dashes are included to optimize alignment (Miyata *et al.*, 1989)

Species	Amino acid sequence
Tachypleus tridentatus tachyplesin I	-*K***WCFRVCY***RG**I***CYR***R**CR
Carcinoscorpius rotundicauda tachyplesin I	-*K***WCFRVCY***RG**I***CYR***R**CR
Tachypleus gigas tachyplesin I	-*K***WCFRVCY***RG**I***CYR***R**CR
Tachypleus tridentatus tachyplesin II	-*R***WCFRVCY***RG**I***CYR***K**CR
Tachypleus gigas tachyplesin III	-*K***WCFRVCY***RG**I***CYR***K**CR
Tachypkeus tridentatus polyphemusin I	R*R***WCFRVCY***RG**F***CYR***K**CR
Tachypkeus tridentatus polyphemusin II	R*R***WCFRVCY***K**G**F***CYR***K**CR

1.2.1.2.3 Big defensin

Big defensins are 8 kDa peptides, containing 79 amino acid residues, isolated from the large and small granules of the hemocytes of the horseshoe crab, *T. tridentatus*. The peptides are active against Gram-positive bacteria, Gram-negative bacteria and fungi and shows LPS-binding activity. The C-terminal region, composed of 37-residues, is hydrophilic and resembles the insect defensins. The disulfide bond arrangement in this defensin domain is similar to that of defensins from bovine neutrophils. This region displays potent activity against Gram-negative bacteria and fungi. The highly hydrophobic N-terminal region of the big defensins shows no similarity to any other antibacterial domains, and it displays potent activity against Gram-positive bacteria. Thus, the molecule seems to be a functional chimera. The LPS-binding activity has, however, been shown to be facilitated by the whole molecule (Iwanaga *et al.*, 1998).

1.2.1.3 Glycine-rich peptides

Several large polypeptides isolated from immune insects have a higher than normal percentage of glycine residues. These polypeptides are active against Gram-negative, and in some cases, against Gram-positive bacteria.

1.2.1.3.1 Attacin

The first glycine rich peptide to be isolated was attacin, a bacteriostatic polypeptide, isolated from the pupae of the giant silk moth, *H. cecropia* (Hultmark *et al.*, 1980). Six attacins have been isolated from *H. cecropia*, the sizes ranging from 184 - 186 residues. Four of the attacins are acidic, and two are neutral or slightly basic. Attacins have two 60-residue G-domains (Figure 1.4). Attacin is synthesized as a product of two genes, one for basic and one for acidic attacin. The cleavage of a signal peptide and a prosequence from the preproattacin, forms the respective isoforms (Cociancich *et al.*, 1994). The attacin polypeptide is active on the outer membrane of Gram-negative bacteria. It is bacteriostatic, causing an alteration in the permeability properties of the outer membrane. This improves the access of lysozyme and cecropins to their targets in the cell wall and cytoplasmic membrane respectively. The precise mechanism of this inhibition is not known, but it is hypothesized that attacin pre-translationally inhibits the synthesis of several outer membrane proteins involved in a regulatory system. This result suggests the presence of a novel regulatory system for outer membrane assembly (Carlsson *et al.*, 1991).

1.2.1.3.2 Sarcotoxin

Sarcotoxins, first isolated from *S. peregrina*, are related to attacins in that they contain two G-domains of roughly 60 residues. The two G-domains are well conserved between attacins and sarcotoxins. In addition, sarcotoxins also contain a P-domain, similar to that of the proline-rich peptides (Figure 1.4; Hultmark, 1993).

1.2.1.3.3 Diptericin

Diptericins can be considered to be chimaeric molecules, since they contain both proline-rich and glycine-rich regions. The proline-rich region is homologous to the P-domain in

apidaecins and abaecins. The glycine-rich region is homologous to the G-domain in attacins and sarcotoxins (Fig 1.13; Hultmark, 1993).

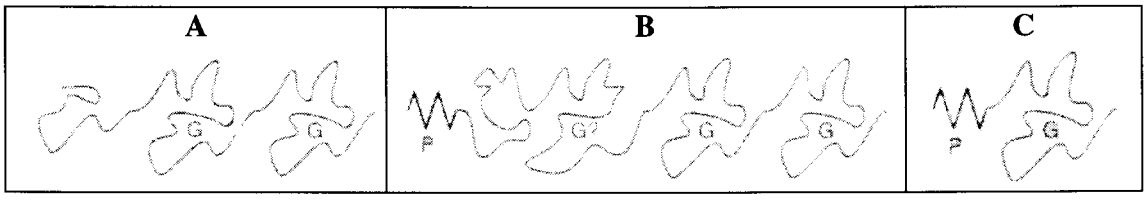

Figure 1.7 The schematic representation of three glycine-rich antibacterial peptides. The peptides are (A) attacin, (B) sarcotoxin II and (C) diptericin. (Adapted from (Hultmark, 1993).

1.2.1.3.4 Hymenoptaecin

Hymenoptaecins, glycine-rich polypeptides from immune honeybees are active against Gram-positive and Gram-negative bacteria. They are 93-residue polypeptides and show some sequence homology with diptericin. Their mechanism of bacteriolysis is through sequential permeabilization of the outer and inner bacterial membranes (Cociancich *et al.*, 1994).

1.2.1.3.5 Gloverin

Gloverins are glycine rich, basic proteins (pI 8.5) of 130 residues, and are active against Gram negative bacteria. They were isolated from the immune hemolymph of *H. cecropia* pupae and have a structure that displays a helix-bend-helix motif. Their mode of action is through the binding to LPS in the bacterial envelope, followed by the inhibition of synthesis of vital outer membrane proteins. This causes an increased permeability of the outer bacterial membrane (Axen *et al.*, 1997).

1.2.1.3.6 Coleopterecin

Coleopterecins are strongly basic, 74-residue glycine-rich polypeptides isolated from the tenebrionid beetle (*Zophobas atratus*). They are active against Gram-negative bacteria and have no apparent sequence similarity with other glycine-rich polypeptides (Cociancich *et al.*, 1994).

1.2.1.4 Proline-rich peptides

Several small proline-rich peptides have been isolated from several insect species. The peptides are active against Gram-negative bacteria and a unique feature of some of these peptides is the presence of O-glycosylation (Cociancich *et al.*, 1994).

1.2.1.4.1 Drosocin

Drosocins are 19-residue peptides, with one third of their residues made up of proline in three pro-arg-pro repeats. O-glycosylation is found at Thr11 in the form of an N-acetylgalactosamine-galactose disaccharide. The absence of this disaccharide greatly decreases the effectivity of the peptides. Drosocins are active at relatively low concentrations, and while the mechanism of action of drosocins has not been studied, preliminary data suggests that the action of the peptides is not via channel or pore formation (Cociancich *et al.*, 1994).

1.2.1.4.2 Abaecin and apidaecin

Abaecins and apidaecins are peptides isolated from immune honeybees. Apidaecins, the most prominent component of the immune system of the honeybee, are synthesized as precursor molecules containing up to 12 copies of the apidaecin peptide. Abaecins also contain a site for glycosylation, but it is not known whether glycosylation of the peptides occur. Both molecules contain a region called a P-domain (Figure 1.5) which also appears in other molecules like diptericin and sarcotoxin II (Cociancich *et al.*, 1994). The amino acid sequence alignment of various abaecin and apidaecin sequences is displayed in Table 1.5 to indicate the homology between the different peptides.

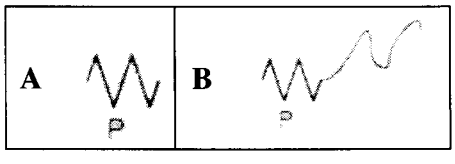

Figure 1.8 Schematic representation of the structures of (A) apidaecin and (B) abaecin from honeybees, *Apis mellifera*. The presence of a conserved P-domain in both peptides is indicated (Hultmark, 1993).

Table 1.5 Amino acid sequence alignment of abaecins and apidaecins. Bold letters indicate conserved residues, italic letters indicate residues conserved in chemical nature. Dashes are included to optimize alignment.

Species	Peptide sequence
Bombus pascuorum abaecin	*F***VP***Y*-**N**P**P**R**PG**Q*S**K*****PFP***S***FPG**H**GPFNPKI**Q**WP**—**Y**PLPNPGH
Apis mellifera abaecin	*Y***VP***L*P**N**V**P**Q**PG**R-*R***PFP***T***FPG**Q**GPFNPKIKWP**QG**Y**------
Apis mellifera apidaecin Ia	EAKPEAKP**GNNRP***V***YIP**Q**PRPPHPR***I***
Apis mellifera apidaecin II	--------**GNNRP***I***YIP**Q**PRPPHPR***L***
Bombus pascuorum apidaecin	--------**GN-RP***V***YIPPPRPPHPR***L***

1.2.1.4.3 Formaecin

Formaecins are 16 residue proline-rich cationic peptides isolated from the ant *Myrmecia gulosa*. The peptides contain O-glycosylation in the form of N-acetyl-hexosamine linked to Thr-11 and were found to display activity only against several strains of *Escherichia coli* (Mackintosh *et al.*, 1998).

1.2.2 Lysozyme

Lysozymes are ubiquitous antibacterial enzymes occurring in vertebrate and invertebrate species. The lysozymes from invertebrates are all similar to the vertebrate lysozymes of the c (chicken) type. They are part of the inducible immune system of insects, but recent evidence also underline their importance in the digestive process of several insects.

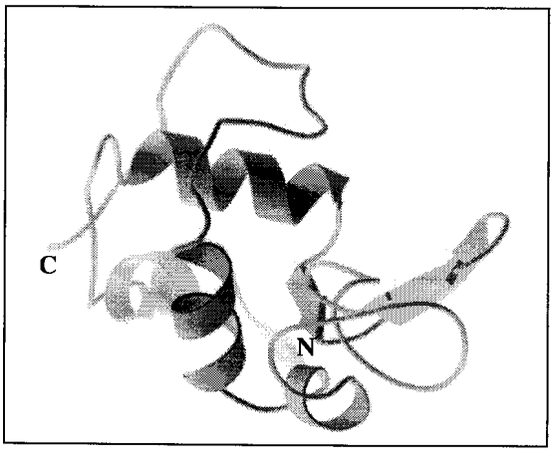

Figure 1.9 Schematic representation of the structure of chicken egg white lysozyme (E.C. 3.2.1.17) obtained by x-ray crystallography. The amino-terminal (N) and the carboxyl-terminal (C) are indicated. (Source: Brookhaven Protein Database id number 6LYZ).

The enzymatic action of lysozymes is the hydrolysis of the β-1,4 linked glycosidic bond of the peptidoglycans that generally reinforce the cell wall of bacteria (Ito *et al.*, 1999). The molecular masses of invertebrate lysozymes are largely similar, with 14 kDa for lysozyme from the gut of the tick *O. moubata* (Kopácek *et al.*, 1999), 15.5 ± 1 kDa for lysozyme from the European starfish *Asteria rubens* (Jollès and Jollès, 1975), 13 kDa for lysozymes from the bivalve *Tapes japonica*, conch *Lunella coronata* and earthworm *Eisenia foetida* (Ito *et al.*, 1999). The pI values of invertebrate lysozymes are also largely similar, being basic at ± pH 10 (Kopácek *et al.*, 1999).

Lysozyme is usually constitutively expressed, and this might be to serve as an initiator for other antibacterial factors as the products of lysozyme action are potent inducers of antibacterial peptide synthesis and release. Lysozyme activity can, however, be induced to levels higher than baseline levels in the event of bacterial infection. The main site for lysozyme synthesis is the fat body, although other tissues can also be involved, notably the hemocytes and pericardial cells. Lysozyme has also been found in the gut of several invertebrates and in the salivary glands of *Drosophila melanogaster* and mosquitoes

(Hultmark, 1996). It was also recently isolated from the gut of the soft tick, *O. moubata* (Kopácek *et al.*, 1999).

The exact role of lysozyme is still unclear. It is generally acknowledged to be involved in immunity, though few bacteria are susceptible to lysozyme action under physiological conditions. It is believed that lysozyme more often than not acts synergistically with other components of the immune response. *E. coli*, normally immune to lysozyme action, becomes susceptible when grown in the presence of attacin. There is also a strong synergism between lysozyme and cecropin, and lysozyme was found to make *E. coli* susceptible to defensin, a peptide it is normally immune to (Jolles, 1996). It is also thought that lysozyme removes bacterial cell wall debris after the action of other bactericidal components.

The presence of lysozyme in the gut of several insects suggests that lysozyme may also have adapted to a digestive role. These lysozymes have acidic iso-electric points and have low pH-optimum values. Lysozyme does not play a digestive role in all insects though. When lysozyme is expressed in the gut, it could play a role in sterilizing the gut contents (Jolles, 1996). In the case of ticks, e.g. *O. moubata*, it was found that expression of lysozyme increased following the acquisition of a blood meal. Since digestion in ticks occurs intracellularly and the contents of one blood meal could be stored in the gut for months or years, the microbial content of the gut must be eliminated to protect the tick (Kopácek *et al.*, 1999). The simultaneous up-regulation of the expression of defensin (Nakajima *et al.*, 2001) and lysozyme (Kopácek *et al.*, 1999) in the gut of *O. moubata* following the blood meal acquisition could be an indication of this protective function, in light of the synergy between defensin and lysozyme.

1.2.3 Induction of antibacterial activity

The tissues involved in the production of these induced insect antibacterial factors are varied. A subset of the hemocytes contributes to the production of these factors. However, the main site for production is not the hemocytes, but the fat body. This is an adipose tissue with a similar metabolic function as the mammalian liver. A study done on

the larvae of the silk moth, *Bombyx mori*, shows that abrasion of the outer epithelial layers induces the localized production of antibacterial factors in the underlying epidermal cells. Accumulation of these factors also take place in the wounded area (Hultmark, 1993).

Since the tissues mentioned, especially the fat body, do not usually come in active contact with the bacteria, a signal must be transmitted from the infected site to the fat body to induce a response. The system is also not adaptive, and it is reasonable to assume that there are a number of fixed recognition molecules specific for microbial epitopes, like the peptidoglycan fragments from Gram-positive bacteria, LPS molecules from Gram-negative bacteria and 1,3-β-glycans from fungal cell walls. It is possible that factors released from tissue after injury could also induce production of antimicrobial peptides (Hultmark, 1993).

The induction of transcription of the antibacterial genes has been studied for several organisms, including *H. cecropia*, *D. melanogaster* and *S. peregrina*. It was noted that several insect immune genes share an upstream motif that bear some homology with the binding site of the transcription factor NF-κB. This is a mammalian transcription factor that regulates the immune and acute-phase responses. In 1992 the cecropia immunoresponsive factor (Cif) was purified. This is a 65 kDa nuclear factor that binds specifically to the κB-like site (Sun and Faye, 1992). A similar factor, named *Drosophila* immunoresponsive factor (Dif), was found in *D. melanogaster*. Cif was found in nuclear and cytoplasmic extracts of the fat body of *H. cecropia* after induction of the immune response by known inducing factors. The homology of Cif and Dif to NF-κB was confirmed when antibodies directed to NF-κB was found to also inhibit the binding of Cif. These factors also competed for binding to the same DNA sequences as NF-κB (Hultmark, 1993). Studies in *Drosophila* showed that the expression of the antifungal peptide, drosomycin, was regulated by the dorsoventral regulatory gene cassette also known as the Tl pathway (composed of spätzle/Toll/cactus). The expression of antibacterial peptides in *Drosophila* is regulated by the activity of either a pathway involving the immune-deficiency (*imd*) gene, or by the combined activation of both the

imd and Toll pathways (Lemaitre *et al.*, 1997). A similar pathway to the Toll/spätzle pathway has recently been found to regulate the vertebrate innate immune response (Schnare *et al.*, 2001; Takeda and Akira, 2001; Akira *et al.*, 2001).

The studies in *Drosophila* indicate that the induction of antimicrobial peptides is not just a broad, non-specific event, but the peptides can be differentially expressed in response to different activators (Lemaitre *et al.*, 1997).

1.2.4 Biosynthesis of antibacterial peptides

Most antibacterial peptides are synthesized as prepropeptides. Some peptides, like cecropin and apidaecin are constitutively excreted and processed outside the cell. The role of the pro-segment could be to inactivate the peptides until they are needed. Targeting in the propeptides can also be complicated, since in defensins some targeting information is locked in the mature peptide itself, and not just in the pro-segment. The pre-pro-segments are cleaved before the peptides are secreted, making them biologically active (Hwang and Vogel, 1998).

The storage of peptides can be problematic, since some peptides could be harmful to the organism itself, especially at high concentrations. One way of containing the peptides is storage in granules. The granules contain a polyanionic matrix consisting mainly of chondroitin sulfate. There could be electrostatic interactions between the chondroitin sulfate and the cationic peptides, and it thus acts as a neutralizing agent (Hwang and Vogel, 1998). The orientation of the transmembrane voltage is also important for storage. Some peptides cannot exert any activity against the host membranes because the polarity of the voltage is reversed, and thus the peptides are compartmentalized in the cytoplasm without cytotoxic effects. The pro-segments may also play a role in protecting the host cells, since it is believed that the acidic pro-segment of defensins play a role in protecting the secretory cells from the cytotoxicity of the mature peptides (Hwang and Vogel, 1998).

Several peptides undergo post-translational modifications, such as amidation of the C-terminal. The usual method for amidation requires the presence of a glycine at the C-terminal end. The α-carbon and carboxyl groups of the glycine residue are cleaved off. This leaves the glycine amino group bound to the C-terminal carboxyl group, forming the C-terminal amide (Ganz, 1994).

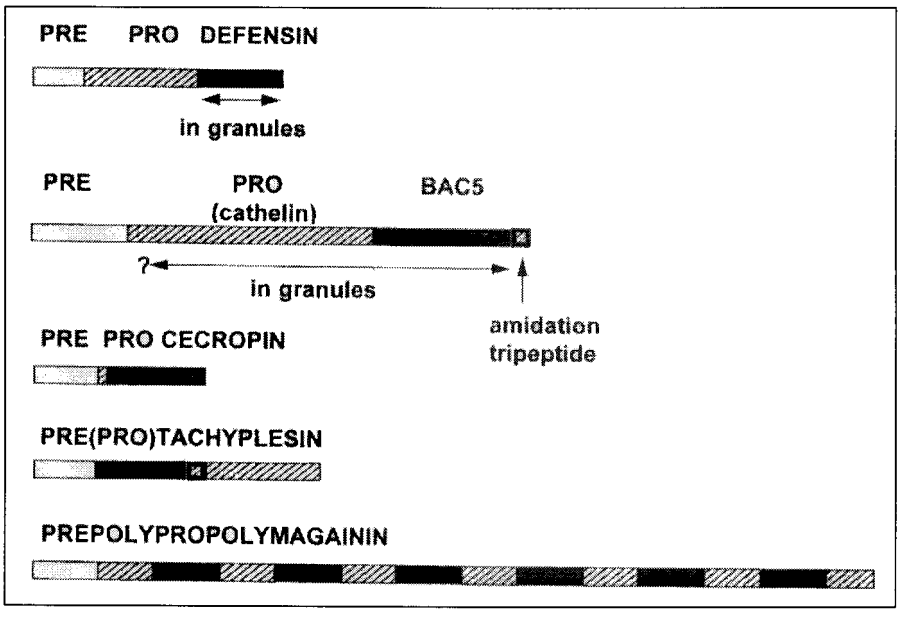

Figure 1.10 Schematic representation of prepropeptides for five families of antimicrobial peptides. Despite the similar sizes of the mature peptides (23-45 residues), the precursors vary substantially, mainly because of the differing structure and function of the propieces. Presegments are in white, anionic prosegments are hatched and mature peptides in black. Amidation sites are marked by squares (Ganz, 1994).

1.2.5 Mechanism of antibacterial action

Many studies have focused on the exact mechanism of action of the antibacterial peptides. Since the chemical structure of the two main structural motifs of lytic peptides, α-helices and β-sheets, are so different, more than one mechanism was predicted for the different classes. Sequence analysis showed that most antibacterial peptides have an amphiphilic structure with hydrophobic and positively charged surfaces. The strong positive charges suggest binding to negative charges, while the hydrophobic surfaces

suggest binding to membranes. The action of antimicrobial peptides on membranes was hypothesized to be the main reason for activity. This hypothesis was strengthened by the finding that D-enantiomers of four helical peptides displayed identical activities to the L-enantiomers. This precludes any stereospecific binding to membrane proteins or effector molecules and suggests a direct binding of peptides to the membrane surface (Shai, 1995, Hwang and Vogel, 1998). The increase in positive charges of a peptide increased the antibacterial action, while the increase in hydrophobicity increased both the hemolytic and antibacterial activity. Thus, hemolysis and antibacterial action seems to be mediated through different mechanisms (Hwang and Vogel, 1998).

1.2.5.1 Bacterial membrane composition

It has been suggested that the difference in the lipid-composition between eukaryotic and bacterial cells play a major role in the selectivity of most antimicrobial peptides for bacterial cells. Bacterial cell membranes have a high anionic-to-zwitterionic ration versus eukaryotic cells which have a high zwitterionic lipid composition (Hwang and Vogel, 1998), mostly phosphatidylcholine (Ojcius and Young, 1991). Both the peptidoglycan and lipopolysaccharide structures, surrounding Gram-positive and Gram-negative bacteria respectively, also display a high negative charge (Hwang and Vogel, 1998). It was found that cancerous eukaryotic cells express anionic phospholipids on the outer surface, and this might explain why some antibacterial peptides can lyze cancer cells, but not normal eukaryotic cells (Shai, 1995).

1.2.5.2 Predominantly α-helix containing peptides:

Amphipathic α-helices are formed when the hydrophobicity of the residues varies with a period of between 3 and 4 residues in the helix (Ojcius and Young, 1991. One side of the helix would thus be polar, and the other side hydrophobic. Although these helices have similar structural features, the difference in activity is quite marked. Some amphipathic helices show cytolytic, hemolytic and antibacterial properties, while others only display some of these properties. The difference can be seen between the amphipathic helices melittin and magainin. Melittin is hemolytic (Georghiou *et al.*, 1981; Dempsey, 1990; Wachinger *et al.*, 1998) and antibacterial (Wachinger *et al.*, 1998), while the magainins

are antibacterial (Duclohier, 1994), non-hemolytic (Duclohier, 1994) and only display cytolytic activity against some cancer cell lines (Baker*et al.*, 1993; Ohsaki *et al.*, 1992). The organizational states of α-helices interacting with lipid membranes are displayed in Fig 1.11.

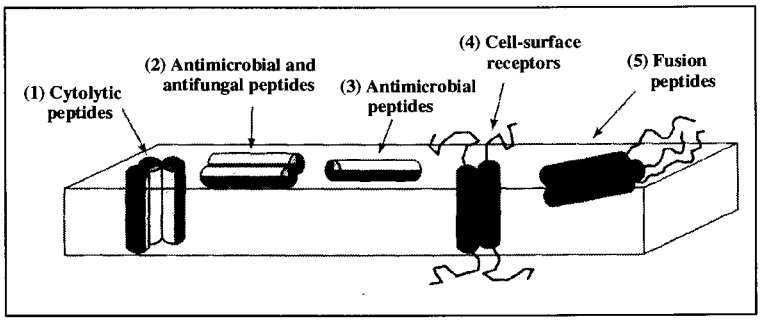

Figure 1.11 Organizational states of membrane interacting α-helices. Membrane traversing amphipathic helices can aggregate, with hydrophilic surfaces on the interior and the hydrophobic surfaces interacting with the membrane lipids (1). An amphipathic α-helix can lie on the surface of the membrane as a bundle (2) or as monomers (3). A hydrophobic segment can traverse the membrane and specifically self-associate or co-assemble with a counterpart segment (4) or may interact when slightly buried within the membrane (5) (Shai, 1995).

The exact molecular mechanisms of interaction between antibacterial peptides and lipid membranes were analyzed with nuclear magnetic resonance (NMR) and H^3-exchange techniques (Table 1.6).

Table1.6 Membrane interaction properties of various peptides (adapted from Shai, 1995).

Peptide	Source	Membrane-bound state	Biological function	Mode of action
Cecropin A	Silk moth	Not known	Antibacterial	Not known
Cecropin B	Silk worm	Surface; assemble at high [peptide]/[lipid]	Antibacterial	Carpet-like[a]
Cecropin P	Pig	Surface; no assembly	Antibacterial	Carpet-like[a]
Dermaseptin S	Frog	Surface; aggregates at high [peptide]/[lipid]	Antibacterial	Carpet-like[a]
Dermaseptin B	Frog	Surface; aggregates at low [peptide]/[lipid]	Antibacterial, antifungal	Carpet-like[a]
Magainin	Frog	Surface; aggregates at high [peptide]/[lipid]	Antibacterial, anti-fungal, cytotoxic to cancer cells	Totroidal [b]
Melittin	Bee	Surface / Transmembrane?	Antibacterial, cytotoxic	Non-barrel-stave [c]

[a] See Figure 1.12

[b] Ludtke *et al.*, 1996

[c] Sharon *et al.*, 1999

Due to their amphipathic nature, the barrel-stave mechanism for the formation of ion channels permeating membranes was initially proposed for the antibacterial action of α-helical peptides (Fig 1.12).

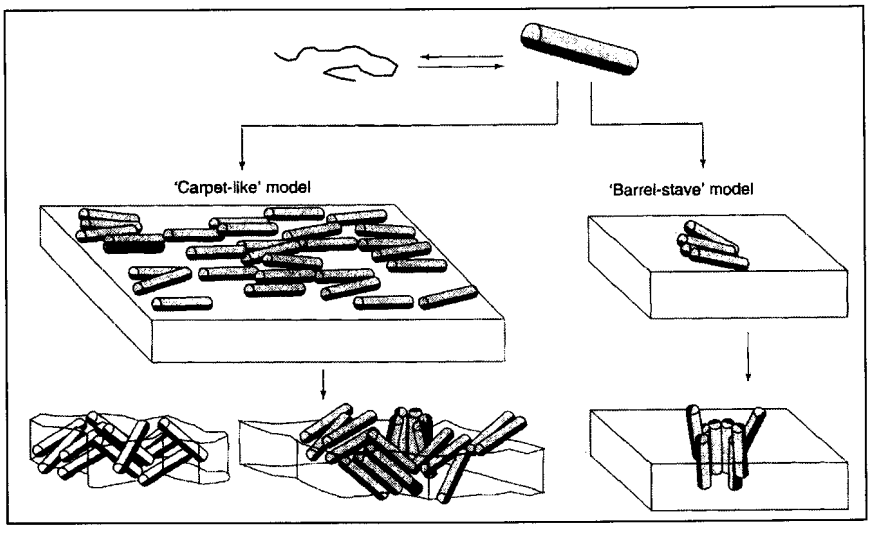

Figure 1.12 Schematic representation of the carpet-like (left) and the barrel-stave model (right) of the interaction of antibacterial peptides with lipid bilayers. In the carpet-like model the peptides are bound to the surface with their hydrophobic surfaces facing the membrane and their hydrophilic surfaces facing the solvent. When a threshold concentration of peptide monomers is reached, the membrane breaks into pieces. At this stage a transmembrane pore might be formed by a small fraction of the peptide. The barrel-stave mechanism proposes the aggregation of peptide monomers followed by insertion into the membrane. The hydrophobic surfaces of the peptides are in contact with the membrane core, while the hydrophilic surfaces line the inside of the stable membrane pore (Shai, 1995).

Recent studies using, among others, circular dicroism (CD) and NMR have, however, provided no evidence for the barrel-stave mechanism. The data suggests a two part mechanism: (1) the peptides bind parallel to the surface of the membrane, not penetrating deeply, (2) the aggregation of peptides upon reaching a threshold concentration, leading to the formation of a membrane pore. The peptides are highly unlikely to remain in the monomeric form in the hydrophobic core due to energetically unfavourable binding of the hydrophobic membrane core with the charged surface of the peptides. The charged surfaces of the peptides stay in electrostatic contact with the polar surface of the membrane. This mechanism was called the carpet-like method of membrane association (Shai, 1995). This mechanism would correlate with the flip-flop of phospholipids from the inner to the outer leaflet of the membrane observed in other studies. The inner and

outer membranes would thus become continuous as a result of the disruption of the membrane by the peptide aggregates. This would lead to the formation of large pores and the leakage of large cellular components (Hwang and Vogel, 1998). The shape of the pores can also be described as toroidal, a model for the interaction of magainins with the lipid bilayer (Fig 1.13, (Ludtke *et al.*, 1996).

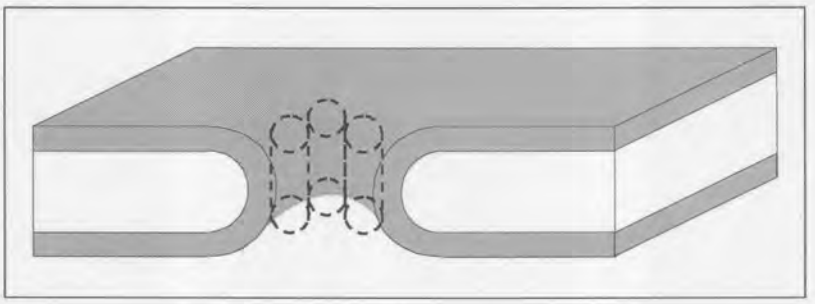

Figure 1.13 Membrane cross-sections for the toroidal model of the formation of membrane pores by aggregation of magainins. Peptides remain adsorbed to the head groups of the lipid membrane. This model would allow exchange of lipids from the inner to the outer leaflet of the bilayer. Shaded areas indicate polar heads of the membrane phospholipids, white areas indicate the hydrophobic core of the bilayer. Barrels indicate peptide monomers (adapted from Ludtke *et al.*, 1996).

Further studies showed that a lower peptide concentration might be needed to cause bacterial cell death through the dissipation of the gradient across the cell membrane, negating the formation of large, stable, multimeric membrane pores. The parallel binding of peptides on the surface in low concentrations could lead to the local disruption of phospholipid packing, leading to the disruption of the electrochemical gradient. Mammalian cells might be better adjusted to the prevention of these disruptions and the presence of cholestrol might provide an additional stabilizing effect. This mechanism is known as self promoted uptake (Hwang and Vogel, 1998).

Conflicting studies do not support for the carpet-like or the barrel-stave models. These studies propose that the peptides remain adsorbed parallel to the surface of the membrane until the peptides transport across the membrane, acquiring their targets inside the cell.

Possible intracellular mechanisms might include (1) stimulation of autocatalytic enzymes, (2) interference with bacterial DNA and/or protein synthesis and (3) general binding to and inhibition of cellular nucleic acids. It has been confirmed that cationic peptides can interact with nucleic acids, making this a credible alternative. It was also hypothesized that α-helical peptides binding to the outer surface of lipid bilayers could flip inwards, carrying lipids with them and would create brief disruptions in permeability mechanism (Wu *et al.*, 1999). The formation of toroidal disruptions (Fig 1.13) is also hypothesized. The peptides remain bound to the outer layer of the lipid membrane, and the inner and outer leaflets of the membrane join to form a continuous membrane.

1.2.5.3 β-sheet containing peptides

The mechanism of activity in the β-sheet peptides has not been extensively studied. The study of helical peptides are more convenient since their orientation is easier to ascertain than that of β-sheet containing peptides (Heller *et al.*, 1998).

An amphiphilic β-strand is predicted when the hydrophobicity of the residues varies with a period of two residues (Ojcius and Young, 1991). The target for the amphiphilic β-sheet containing peptides are also believed to be the plasma membrane, as tachyplesin was found to adsorb to the membrane surface, although the mechanism of interaction is still unknown (Matsuzaki *et al.*, 1991). It has been found that the β-sheet peptides produce pores in membranes, and that the D-enantiomers were as active as L-enantiomers, suggesting that the membrane bilayer is the main target of activity (Heller *et al.*, 1998).

Calculations indicate that should these peptides interact through a barrel-stave mechanism, a minimum of 8 amphiphilic β-strands must be present. Thus, either 8 monomers containing one β-sheet or less monomers with multiple β-sheets could satisfy this requirement (Ojcius and Young, 1991). Studies with synthetic peptides show a high incidence of intermolecular hydrogen-bonding associated with membrane binding of β-sheet peptides, leading to the stabilization of the aggregate and native structure (Ojcius and Young, 1991). The aggregation of β-sheet peptides prior to membrane binding was

also discovered. Peptides chemically dimerized prior to membrane binding display greater activity than monomers (Hwang and Vogel, 1998). These findings suggest that some β-sheet peptides associate prior to membrane binding, and that the aggregate is inserted in membranes to form pores, rather than monomer association and aggregation on the membrane surface. The inability of β-sheet peptides to form stabilizing intramolecular disulfide bonds leads to the abolishment of structure and function, showing the importance of these bonds (Wu and Hancock, 1999).

In a study on protegrin, it was found that the binding of the peptide parallel to the surface of the membrane produced a membrane thinning effect. Once the peptide reaches a specific peptide-to-lipid ratio, it becomes energetically more favourable to insert across the membrane rather than remain bound to the surface. This insertion, presumably leading to the formation of a pore, leads to the relaxation of the membrane back to the thickness of the unperturbed bilayer. The data suggests either a barrel-stave type interaction, or a toroidal type interaction with the membrane bilayer as in shown Fig 1.13 (Heller *et al.*, 2000). The range of activities of the β-sheet peptides show that non-helical structures are important elements in membrane interaction and deserve more study (Kagan *et al.*, 1990).

1.2.6 Evasion of the invertebrate humoral immune response

Bacterial resistance to antibacterial peptides, which is more complicated than resistance against non-protein antibiotics, has two mechanisms of action, identified as passive and active resistance. Passive resistance is aimed at reducing the effectiveness of antimicrobial peptides through changes to the microorganism itself e.g. the composition of the membrane phospholipids could be adjusted and changed to decrease the effectivity of peptide binding to the membrane. Active resistance is the mechanism whereby the pathogen attempts to interfere with peptide action through the release of specific proteases that degrade bacteriolytic peptides. The proteinase of *Bacillus thuringiensis* is defined as an immune inhibitor of type A, and selectively degrades cecropins and attacins. The extracellular *Pseudomonas aeruginosa* proteinase specifically degrades cecropin-like peptides induced in lepidopteran insects (Jarosz, 1997).

1.2.7 Natural insect flora

Like other animals, insects are infected by three different kinds of organisms: *(a)* harmless, non-pathogenic organisms, *(b)* occasional pathogens and *(c)* obligate pathogens. Representative bacteria from each group have been assayed for sensitivity to endogenous antibacterial peptides, and it was found that the peptides were only active against non-pathogenic bacteria and against some pathogens (Steinhaus, 1967). Thus the main function of the antibacterial peptides appear to be the control of the natural flora an insect might carry. In insects living in decomposing organic matter, like flies and cockroaches, this could be very important. Thus through evolution some bacteria have appeared against which insects have no defense. Many insects are also dependent on intracellular symbiotic bacteria, and it is important that the immune system is compatible with these symbionts (Boman and Hultmark, 1987). A consequence of the inactivity of the invertebrate immune system against non-pathogenic bacteria is that some invertebrates, especially blood sucking species, might become vectors of diseases. Examples include the transmission of the *Plasmodium* parasites by mosquitoes, *Leschmania* parasites by sandflies, *Trypanosoma* parasites by tsetse flies and the bubonic plague by fleas (Harrow *et al.*, 1991).

1.3 Aims of the study

To investigate the selective and low incidence of pathogen transmission by *O. savignyi*, the characterization of the antimicrobial activity and antimicrobial factors in the hemolymph of the tick is vital.

The characterization of the antimicrobial activity is described in Chapter 2. The isolation of the antimicrobial factors, followed by their sequence and mass characterization are discussed in Chapter 3. To investigate the presence of lysozyme in *O. savignyi*, a degenerate primer was designed based on homologous regions in a multiple sequence alignment. This molecular biological characterization of lysozyme is described in Chapter 4.

Chapter 2

Characterization of the antibacterial activity in the hemolymph of the soft tick *Ornithodoros savignyi*

2.1 Introduction

The presence of antibacterial factors has been determined in most eukaryotic orders (Section 1.2.1). By characterization of the antibacterial activity prior to isolation of the antibacterial factors, the putative identity of the factors can be deduced. Thus, when the activity profile shows an exclusively anti-Gram-positive response mediated by proteins with high temperature stability, we hypothesised that the factors could be members of the defensin class of antibacterial factors.

The elucidation of the induction profile of the antibacterial response could indicate whether or not an immunization step needs to be included in the isolation protocol. Thus, the need for induction and optimum collection time of hemolymph will be known. This could increase the yield and range of antibacterial factors isolated. If the induction profile indicates the constitutive expression of the antibacterial factors, this would indicate that an induction step is unnecessary and would lead to a simplified and less time-consuming isolation protocol.

The antibacterial activity can be characterized by evaluating the following factors: (1) the induction profile of the antibacterial factors, including the optimal crude sample collection time and preference for Gram-positive or Gram-negative bacterial immunization, (2) the activity spectrum against diverse bacterial species, both Gram-negative and Gram-positive, (3) the temperature stability of the antibacterial factors and (4) the protein dependence of the activity determined by proteolytic degradation of proteins in crude sample and determining the reduction in activity.

Several methodologies involved in the characterization of the antibacterial activity in organisms are very important to facilitate meaningful results. Three important facets that should be addressed are: the antibacterial assay used, the induction of the antibacterial response (if necessary) and the optimal extraction of hemolymph/body

tissue from invertebrate organisms. The methodologies used in this study is discussed in some detail below.

2.1.1 The antibacterial assay

The most important tool during the classification and analysis of the antibacterial activity in an organism is undoubtedly a sensitive, reproducible antibacterial assay. The assay needs to be adaptable to allow determination of antibacterial activity against both Gram-positive and Gram-negative bacterial species. It also needs to allow for the detection of low concentrations of antibacterial factors.

Two methods of determining the antibacterial activity in samples are generally used. The first method is a radial diffusion assay where a melted agar or agarose solution is inoculated with a bacterial suspension and upon solidifying, holes are punched in the growth medium. Sample is added into the holes and, following incubation at specified temperatures, the presence of clear zones free of bacterial growth around holes is an indication of the presence of antibacterial factors. The diameter of the clear zones can be measured and standardised relative to the diameter of zones caused by the known concentration of a pure antibacterial factor e.g. cecropin B (Chalk *et al.*, 1994).

The second method is a liquid phase assay (broth microdilution assay). The method was developed by Lehrer that allows for the determination of antibacterial activity in liquid growth medium performed in microtiter plates. A decrease in light dispersion at approximately 600 nm indicates an inhibition of antibacterial growth (http://www.cmdr.ubc.ca/bobh/MIC.htm).

2.1.2 The induction of antimicrobial activity

In some organisms the expression of antibacterial factors is constitutive. This facilitates a less cumbersome isolation process, since the time-consuming induction process can be circumvented. This does, however, lead to lower yields, since the induction of the response in such organisms does always cause an increase in the level of antibacterial response and concentration of the antibacterial factors. The study on the scorpion, *Leiurus quinquestriatus*, shows the constitutive expression of defensin peptides in unchallenged individuals. Since there is considerable danger inherent in handling these organisms during bacterial challenge, the use of unchallenged

hemolymph is preferred (Cociancich *et al.*, 1993). In the soft tick *O. moubata*, the presence of two defensin isomers could be determined in unimmunized individuals and the isolation of these peptides was facilitated without bacterial challenge (Nakajima *et al.*, 2001). In the hemolymph of the tobacco budworm *Heliothis viriscens* (Lamberty *et al.*, 1999) and the bumblebee *Bombus pascuorum* (Rees *et al.*, 1997), low levels of antibacterial activity could be detected in the hemolymph of individuals prior to bacterial challenge, but the levels were too low to allow for the elimination of the immunization step.

The methods for the induction of antibacterial activity are varied, but follow the same trend. The organisms are immobilised and bacterial cells are administered internally in order to make contact with the hemolymph and other organs. The bacterial cultures used for induction are grown to stationary phase and the cells collected by centrifugation and washed. The way the cells are subsequently prepared for immunization differ according to the preference of the researchers, but three general methods can be distinguished. In the first method the bacterial pellet of live centrifuged bacteria is used without further preparation. A needle or thin glass capillary is dipped in the bacterial pellet and administered to the organisms by puncturing or injection (Mackintosh *et al.*, 1998; Charlet *et al.*, 1996; Lamberty *et al.*, 1999). The second method entails the suspension of the bacterial pellet and the subsequent injection of the live bacteria in specific body parts of the organisms (Carlsson *et al.*, 1991; Axen *et al.*, 1997; Hultmark *et al.*, 1980; Lambert *et al.*, 1989; Rees *et al.*, 1997). The third method utilizes the injection of a heat-killed bacterial suspension (Bulet *et al.*, 1991; Hubert *et al.*, 1996; Cociancich *et al.*, 1993).

The induction time of the antibacterial peptides of the different organisms can vary. The collection of the hemolymph is usually performed during the plateau-phase of antibacterial activity. Therefore the collection time does not necessarily reflect the time needed for induction of the antibacterial factors. When the induction time was determined, it was generally found to be swift. In *P. terranovae*, the first transcripts of a defensin gene are apparent 15 min after bacterial challenge, the response reaching a maximum after 8 hours (Hoffman and Hetru, 1992). In *Aedes aegypti* the activity is evident 2-6 hours after bacterial challenge, reaching a maximum between 6 and 24 hours (Chalk *et al.*, 1994), while in *Zophobas atratus* the response is detectable

between 6-12 hours post-challenge, reaching a maximum after about 12 hours (Bulet *et al.*, 1991). The induction in the pupae of *H. cecropia* leads to an anti-Gram-positive response, evident after 6 hours and an anti-Gram-negative response that can be detected after 12 hours. Both these responses reach a maximum after about 18 hours following bacterial challenge (Hultmark *et al.*, 1980). The response can also be long-lived in some organisms, e.g. the response in *Z. atratus* is still detectable 30 days post-challenge.

2.1.3 Hemolymph extraction strategies

The hemolymph extraction protocols for different organisms are mainly dependent on the size of the organisms. Hemolymph extraction in a small organism might be easier to effect by whole body extract preparation, though this would lead to antibacterial factor dilution due to contamination of the sample with non-hemolymph proteins. The use of thin glass capillaries is also an option, and the choice between these methods might depend on personal preference or experimental effectivity. Larger organisms are easier to handle and extraction of hemolymph can be less arduous due to the larger available surface area. The optimum extraction points for these organisms might have to be determined experimentally to facilitate effective extraction and minimal contamination of the sample with non-hemolymph constituents. Collected hemolymph is often pooled in ice-cooled eppendorf tubes containing a protease inhibitor e.g. aprotonin (Bulet *et al.*, 1991; Lambert *et al.*, 1989) or phenylthiourea as inhibitor of the phenoloxidase-mediated melanization cascade (Hultmark*et al.*, 1980). A combination of protease and melanization inhibitors can also be used (Lamberty *et al.*, 1999; Rees *et al.*, 1997). Hemolymph is usually frozen in liquid nitrogen and stored at -70°C.

2.1.3.1 Hemolymph extraction from small invertebrates

Since hemolymph extraction from invertebrates, such as ants, can be difficult due to the small size of the organisms, alternative methods have been developed. A standard method of obtaining crude antibacterial factors from ants involves freezing the ants in liquid nitrogen, followed by the grinding frozen ants in a mortar and pestle to a fine powder. The powder can then be dissolved in a solution used in the initial purification procedures (Mackintosh *et al.*, 1998; Taguchi *et al.*, 1998).

Hemolymph from small invertebrates such as the mosquito, *A. aegypti*, can be extracted by injection of an anticoagulant solution into the thorax with a finely drawn glass capillary. After a specified time the hemolymph/anticoagulant mixture is withdrawn using the glass capillary, accompanied by gentle pressure on the abdomen (Chalk *et al.*, 1994).

2.1.3.2 Hemolymph extraction from larger invertebrates

Extraction of hemolymph from larger invertebrates can be effected by several methods, two of which will be discussed. The first method involves the sectioning of an abdominal appendix, or puncturing the body of pupae. Slight pressure is then exerted on the body of the invertebrate, causing the hemolymph to be expelled. The hemolymph can then be collected and pooled for storage or use (Bulet *et al.*, 1991; Lambert *et al.*, 1989; Lamberty *et al.*, 1999; Astigarraga *et al.*, 1996). The second method involves the puncturing of the abdomen followed by the extraction of hemolymph using a syringe or glass capillary. The hemolymph can be pooled for storage or use (Bulet *et al.*, 1996; Cociancich *et al.*, 1993; Hubert *et al.*, 1996; Rees *et al.*, 1997).

2.1.4 Aim of this study

The aims of this study is the characterization of the antibacterial response in the hemolymph of the soft tick, *O. savignyi* in terms of:

- The presence of antibacterial factors
- The induction profile and induction time-study of the antibacterial activity
- The activity profile of the antibacterial factors
- The heat stability of the antibacterial factors
- The peptide/protein nature of the antibacterial activity

2.2 Materials

Ticks were collected from the North West Province of South Africa by sifting of sand and were kept in sand at room temperature. Yeast extract and tryptone were obtained from Oxoid Ltd., Basingstoke, UK. Nutrient broth agar, NaCl and casein are from Merck, Darmstadt, Germany, sterile 96-well polystyrene plates from Nalge Nunc International, Kamstrup, Denmark and petri-dishes were from Plastpro, South Africa.

Protein concentration assays were performed using the micro-BCA Protein Assay Kit obtained from Pierce, Tattenhall, UK.

2.3 Methods

2.3.1 Bacteria

All bacterial cultures were kindly supplied by the Department of Microbiology and Plant Pathology, University of Pretoria, South Africa except for *Micrococcus luteus* (Boehringer Mannheim, Germany). Gram-negative bacteria used were *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis* and *Pseudomonas aeruginosa*. Gram-positive bacteria used were *Bacillus subtilis*, *Bacillus cereus, Enterococcus faecalis, Staphylococcus aureus* and *Micrococcus luteus*. Bacteria were grown in Luria-Berthani medium (1% (w/v) NaCl, 1% (w/v) Tryptone, 0.5% (w/v) yeast extract in deionized H_2O, pH 7.4, autoclaved) at 30°C and aliquots (in 15% glycerol) were stored at –70°C. A new culture was used for each experiment to minimize chances of contamination or mutation.

2.3.2 Preparation of induction bacterial mixture

Bacterial cultures were grown up in LB-broth overnight at 30°C. Following centrifugation the supernatant was discarded and the pellet re-suspended in sterile deionized H_2O (ddd-H_2O). After three subsequent washing steps the pellet was suspended in 4 ml sterile ddd-H_2O and autoclaved.

2.3.3 Induction of antibacterial activity

The initial method of initiating the antibacterial activity involved the removal of one leg of each tick. The ticks were then submerged in a heat-killed bacterial suspension in water. After a time period, the ticks were removed from the suspension and the hemolymph extracted.

The induction method used during the largest part of the study involved the dorsal immobilisation of ticks on double-sided tape. A thin Pasteur pipette was prepared and 25 µl of the autoclaved bacterial mixture injected in the articulation cuticle between the coxa and the trochanter of the third leg. The ticks were incubated at room temperature and the hemolymph extracted after specific time periods.

2.3.4 Hemolymph extraction

Ticks were dorsally immobilized on double-sided tape. Small nicks were made in the legs and, following gentle pressure on the abdomen, hemolymph was removed with a micropipette (adapted from Astigarraga *et al.*, 1996). The hemolymph, five ticks per sample, was collected in deionized H_2O and immediately frozen in liquid nitrogen and stored at –70°C.

2.3.5 Antibacterial assay

Two different antibacterial assays were used in the study, i.e. a radial diffusion and a broth microdilution assay.

2.3.5.1 Zone inhibition assays

The zone inhibition assay is based on the inhibition of bacterial growth on a solid bacterial growth medium. The presence of clear zones in the solid support indicate the presence of antibacterial activity. Two adaptations of the radial diffusion assay were used. In the first version, bacteria were evenly spread on the surface of a 3% nutrient broth agar plate. Filter paper discs were placed on the surface of the agar plate, and sample was added directly on the filter paper until saturation of the filter paper was reached. Plates were incubated overnight at 30°C. The second version (radial diffusion assay) required the addition of bacteria to the melted autoclaved 3% nutrient broth agar at 55°C and the subsequent preparation of the agar plates. Holes were made in the agar by means of suction, and 4.5 µl of sample was added to the holes. Plates were incubated overnight at 30°C. The presence of clear inhibition zones in the bacterial growth was an indication of the presence of antibacterial activity. The diameter of the inhibition zones was measured.

2.3.5.2 Broth microdilution assay

The antibacterial assay is based on the inhibition of bacterial growth by antibacterial factors in a liquid bacterial growth medium. The method is adapted from the method developed by Lehrer (http://www.cmdr.ubc.ca/bobh/MIC.htm).

Bacteria were grown in LB-broth overnight and diluted with LB-broth to give an OD of 0.2 at 600 nm. A 300 µl solution of PBS (pH 7.4) containing 1% casein was added to each well of a sterile 96-well microtiter plate, and the plate was incubated at room

temperature for 60 minutes under UV-lights. Vacuum dried samples were prepared by the addition of 50 µl deionized water and an incubation at 37°C. The blocking medium was removed and 11 µl of the samples added (negative control: sterile deionize water) to each well followed by 100 µl of the bacterial medium using a reverse pipetting technique. This pipetting technique was used to minimise bubble formation in the growth medium. The absorbance of the microplate was recorded at 620 nm with a SLT 340 ATC scanner (SLT Labinstruments, Austria). The microplate was incubated at 30°C and the antibacterial activity monitored after 3 hours. A decrease in bacterial growth at 620 nm compared to the water control indicated the presence of antibacterial activity. The antibacterial activity is expressed relative to a control (Fig 2.3, 2.4). Relative activity = (Absorbance of negative control – Absorbance of initial negative control) / (Absorbance of sample – Absorbance of initial sample). A relative activity > 1 would be an indication of the presence of antibacterial activity, since the bacterial growth in the sample is lower than the bacterial growth in the negative control.

2.3.6 Protease digestion of hemolymph

The hemolymph from ticks treated with heat-killed *E. coli* was centrifuged at 10 000 g for 10 min. The sample preparation for the trypsin and proteinase K digestion of the hemolymph is given in Table 2.1. Trypsin (8 U[a]/mg hemolymph) and proteinase K (6 U[b]/mg hemolymph) were added to the hemolymph samples and the digestion allowed to proceed for 18 hours at 37°C. The residual antibacterial activity of the trypsin and proteinase K digested hemolymph was determined by using the broth microdilution assay with *B. subtilis*.

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[a] 1 BAEE unit = ΔA_{253} of 0.001 per min with BAEE as substrate at pH 7.6 at 25°C.

[b] 1 U = enzyme activity (using hemoglobin as substrate) which liberates Folin-positive amino acids and peptides corresponding to 1 µmol tyrosine per minute.

Table 2.1 Sample preparation for trypsin and proteinase K digestion of hemolymph. The hemolymph was obtained from ticks pre-treated with heat-killed *E. coli*. Trypsin (8 U/mg hemolymph), proteinase K (6 U/mg hemolymph) and a 1 M Tris buffer (pH 8.6) were used. All samples were incubated at 37°C for 18 hours. A + indicates the presence of the reagent in the sample, and 0 indicates the absence of the reagent in the sample.

Samples	Hemolymph (µl)	Trypsin (µl)	Proteinase K (µl)	Tris buffer (µl)	Water (µl)
Trypsin digest sample	+	+	0	+	0
Proteinase K digest sample	+	0	+	+	0
Control 1	0	+	0	+	+
Control 2	0	0	+	+	+
Control 3	+	0	0	+	+
Control 4	+	0	0	0	0

2.4 Results

2.4.1 Determination of the presence of antibacterial factors in crude hemolymph of *O. savignyi*

To determine the presence of antibacterial factors in the hemolymph of ticks not pre-treated with heat-killed bacteria, the hemolymph of five ticks were used in the zone inhibition assay utilizing filter paper discs. In Fig 2.1(A) the clear inhibition zones can be seen around the filter paper discs containing the hemolymph, while the inhibition zones are absent in the presence of the 0.1 M phosphate control (Fig 2.1.B).

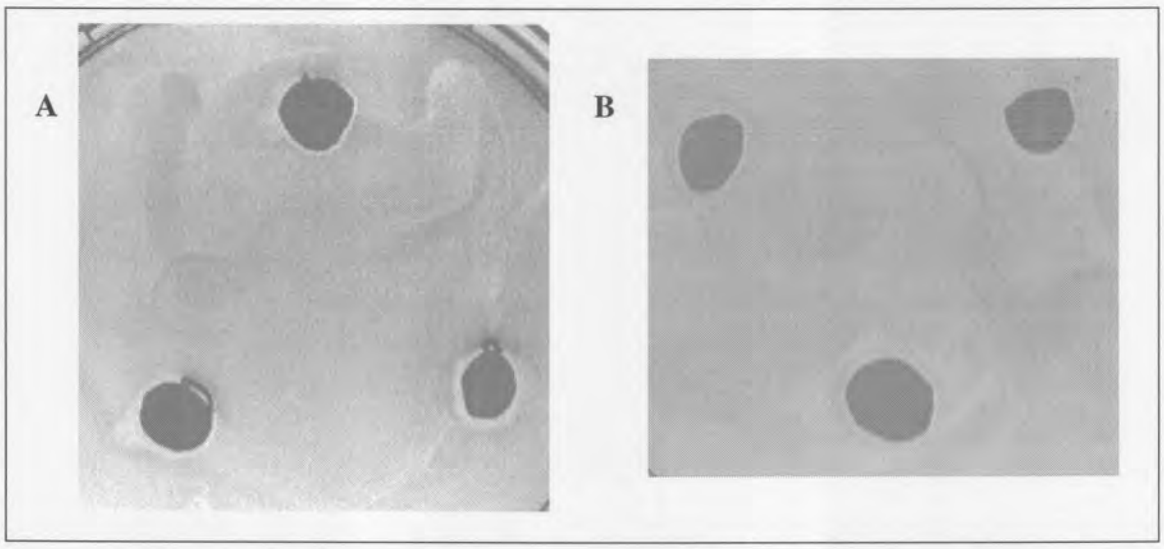

Figure 2.1 Antibacterial activity in untreated tick hemolymph. (**A**) Clear inhibition zones in bacterial growth present in hemolymph and (**B**) absence of clear inhibition zones in 0.1 M phosphate buffer control. Antibacterial activity was determined using a zone inhibition assay using filter paper discs with *B. subtilis* as indicator organism

2.4.2 The induction of antibacterial activity

The effect of pre-treatment of ticks with heat-killed bacteria on antibacterial activity was investigated, since it is known that antibacterial activity can be induced. Activity was initially induced by removing the end of one leg of each tick followed by over-night immersion in a heat-killed bacterial suspension. Gram-positive and Gram-negative bacterial species were tested for their ability to induce activity. The radial diffusion assay was used and the agar was inoculated with the Gram-positive *B. subtilis*. From Fig 2.2 it can be deduced that induction with a Gram-negative bacterial species (Fig 2.2.A) causes a larger level of induction of the antibacterial factors than the induction with a Gram-positive bacterial species (Fig 2.2.B). A low level of background activity can be seen when ticks were incubated in sterile water (Fig 2.2.C). The water control did not lead to inhibition of bacterial growth in any of the three assays.

Figure 2.2 The antibacterial activity in the hemolymph following pre-treatment with different bacterial strains. Ticks were pre-treated with (**A**) heat-killed Gram-negative *E. coli*, (**B**) heat-killed Gram-positive *B. subtilis* and (C) sterile H_2O. Collected hemolymph was centrifuged for 10 min at 10 000 g and the supernatant used in the assay. The antibacterial activity was determined using a radial diffusion assay utilizing holes made in agar and with *B. subtilis* as inoculated bacteria. (A i-iii: hemolymph, iv: water control; B i-iii: hemolymph, iv: water control; C i-ii: hemolymph, iii: water control)

2.4.3 Activity profile of the antibacterial factors against different bacterial species

The bacterial specificity of the antibacterial factors in *O. savignyi* was investigated by assaying for the antibacterial activity of the hemolymph against several different Gram-positive and Gram-negative bacterial species. Ticks were pre-treated with heat-killed *E. coli* and the collected hemolymph was centrifuged for 10 min at 10 000 g. The supernatant was used to determine the presence of antibacterial activity using the broth microdilution assay (Table 2.1). It can clearly be seen from the results in Table 2.1 that the antibacterial factors in the hemolymph do not display any activity against Gram-negative bacteria. Only some Gram-positive species are targeted, the highest activity being directed against *B. subtilis* and *M. luteus*. *B. subtilis* was also the bacteria of choice used during the further characterization and isolation of the antibacterial factors.

Table 2.2 Antibacterial activity spectrum of the hemolymph of *O. savignyi* directed against different bacterial species. The ticks were pre-treated with heat-killed *E. coli* before hemolymph extraction. Hemolymph was centrifuged at 10 000 g for 10 min and the supernatant used to determine the activity with the broth microdilution assay. The level of activity is expressed as the activity relative to that found in *B. subtilis*. (0 = no activity detected, + = low level of activity detected, ++ = high level of activity detected.)

Bacterial species	Gram-positive/negative	Level of activity
Escherichia coli	Negative	0
Klebsiella pneumoniae	Negative	0
Proteus mirabilis	Negative	0
Pseudomonas aeruginosa	Negative	0
Bacillus cereus	Positive	+
Bacillus subtilis	Positive	++
Enterococcus faecalis	Positive	0
Micrococcus luteus	Positive	++
Staphylococcus aureus	Positive	+

2.4.4 Time study of induction of antibacterial activity

The rate of antibacterial activity induction was determined by the pre-treatment of 10 groups of 5 ticks each with heat-killed Gram-negative *E. coli*. The hemolymph of each group was removed at a specific time following bacterial injection. The hemolymph was centrifuged at 10 000 g for 10 min and the antibacterial activity was determined using the broth microdilution assay. The results show that the antibacterial activity increases rapidly, reaching a plateau after one hour. The plateau phase remained constant for four hours after induction. After four hours the activity started to decline, and reached the baseline level 24 hours after induction (Fig 2.3).

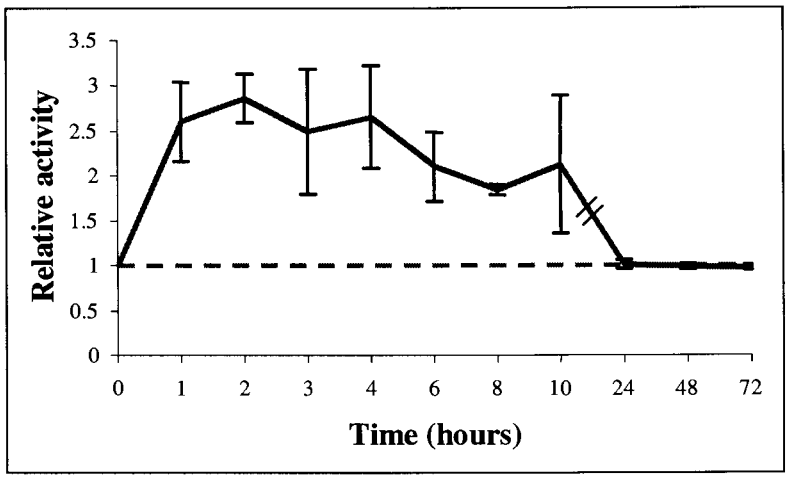

Figure 2.3 Time study of the induction of antibacterial activity. Groups of five ticks each were injected with heat-killed *E. coli* and the hemolymph of each group was removed after a specific time following bacterial challenge. The antibacterial activity of the hemolymph was determined using the broth microdilution assay with *B. subtilis* (solid line). The antibacterial activity in unchallenged hemolymph is also indicated (dashed line). Relative activity = (Absorbance of bacterial growth control – Absorbance of bacterial growth control) / (Absorbance of sample – Absorbance of initial sample).

2.4.5 Heat-stability of the antibacterial factors in the hemolymph of *O. savignyi*

To determine the heat-stability of the antibacterial factors, hemolymph was collected from ticks pre-treated with heat-killed *E. coli*. The hemolymph samples were subjected to increasing temperatures for 10 minutes. After determining the antibacterial activity using the broth microdilution assay with *B. subtilis*, it was found that the antibacterial factors were heat-stable up to 80°C (Fig 2.4).

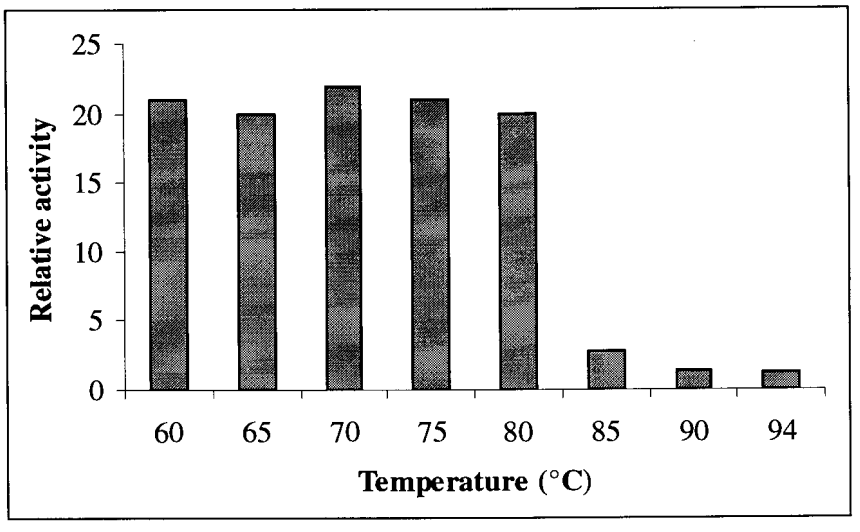

Figure 2.4 The heat stability of the antibacterial factors in the hemolymph of *O. savignyi*. The samples (hemolymph of 5 ticks per group, pre-treated with *E. coli*) were incubated at the specified temperatures for 10 min in a waterbath, centrifuged for 10 min at 10 000 g and the antibacterial activity determined using the broth microdilution assay with *B. subtilis*. Relative activity = (Absorbance of bacterial growth control – Absorbance of bacterial growth control) / (Absorbance of sample – Absorbance of initial sample).

2.4.6 The protein nature of the antibacterial response

The protein nature of the antibacterial response was determined by the proteolytic degradation of crude hemolymph by the proteolytic enzymes trypsin and proteinase K. The hemolymph was incubated with trypsin and proteinase K at 37°C for 18 hours. The controls were included to determine the effect of the Tris, water and the incubation on the antibacterial activity of the undigested hemolymph. The positive control is pure, undiluted hemolymph, included to confirm the presence of antibacterial activity in the hemolymph prior to preparation steps and incubation. The results of the proteolytic treatment of the hemolymph are given in Table 2.2 as the percentage growth of *B. subtilis* relative to the bacterial growth control (water). The controls are labeled as in Table 2.1.

The results show that the enzymes did not completely abolish the antibacterial activity of the hemolymph during the assay period. Proteinase K digestion, when compared to the activity of untreated hemolymph (control 4), degraded a significant percentage of the antibacterial activity (~70%), and presumably the antibacterial factors. The trypsin digestion degraded a less significant fraction of the antibacterial factors (~55%), but

there was still a reduction mediated by the proteolytic action. The results for the trypsin and proteinase K digested hemolymph were adjusted for the action of the proteases on the bacterial growth (control 1 and 2). These results indicate that the antibacterial activity of the hemolymph of *O. savignyi* is, at least partially, dependent on protein molecules.

Table 2.3 The effect of proteolytic treatment of the hemolymph on the antibacterial response. The hemolymph was obtained from ticks pre-treated with heat-killed *E. coli*. Trypsin (22 U/mg hemolymph) and proteinase K (6 U/mg hemolymph) and a 1 M Tris buffer (pH 8.6) were used. All samples were incubated at 37°C for 18 hours. The antibacterial activity of the samples was determined using the broth microdilution assay with *B. subtilis*. The antibacterial activity is expressed as the % bacterial growth relative to the negative control. Values for the trypsin and proteinase K treated samples were adjusted for the presence of the uninhibited enzymes on bacterial growth. Controls are labelled as in Table 2.1.

Samples assayed	**% *B. subtilis* growth**
Trypsin treated sample	64.9
Proteinase K treated sample	31.8
Control 1 (Trypsin + Tris)	80.5
Control 2 (Proteinase K + Tris)	94.8
Control 3 (Hemolymph + Tris)	-19.3
Control 4 (Hemolymph)	-23.6
Negative control (water)	100

2.5 Discussion

This study indicated the presence of antibacterial factors in the hemolymph of the soft tick *O. savignyi*. Other body tissues were also assayed (result not shown), but the hemolymph exhibited the highest antibacterial response, and it presented the most accessible source of antibacterial factors. The identity of the factors involved in the activity has not been confirmed, although preliminary hypotheses could be

constructed based on the activity profile and heat stability of the factors (Table 2.2 and Fig 2.4).

Two different antibacterial assays were used in this study. The first method, the radial diffusion assay, was the least sensitive assay, and also had other disadvantages. The time needed for the visualisation of the antibacterial activity was significant, usually overnight. This negatively affected the studies, since less experiments could be completed daily. The technical skill required for the assay was also a problem, since the assay had to be prepared in a sterile flow cabinet, and great care had to be taken to sustain an appropriate level of sterility. Great care was also required in the handling of the bacterial strains, since a sub-optimal temperature during the agar plate preparation procedure negatively influenced bacterial growth during the assay and could give rise to false negative results.

The broth micro-dilution assay proved to be more sensitive and required less specialised handling. Many samples could be assayed on the same plate, and since the assay time was approximately 4 hours, sequential analyses could be completed daily. A very important step during the assay was the interruption of irreversible, non-specific reactions of the antibacterial components with the negatively charged sides of the polystyrene microplates. The blocking agent in this instance was a solution of casein in PBS, leading to the interaction of the casein to the wells and thus the interaction of the assay components with the wells was minimised. An alternative to casein as blocking agent was the use of bovine serum albumin, but the blocking efficiency of the casein was found to be greater. Polypropylene plates were also an option instead of the polystyrene plates. The polypropylene had a lower non-specific binding potential, but the increase in cost of these plates above the polystyrene plates was an insurmountable obstacle.

The initial analyses of the antibacterial activity in the hemolymph using the radial diffusion assay (Fig 2.1) showed clear zones of inhibited bacterial growth surrounding the filter paper used as the sample transfer medium. The inhibition zones confirmed the presence of antibacterial activity in the hemolymph, a fact supported by the lack of inhibition zones on the negative control plate. The negative control shows some uneven growth surrounding the filter paper, but close analysis shows that this is

merely due to the diffusing water displacing the bacterial colonies directly surrounding the filter paper. The possibility that the diffusion of sample displaced the bacterial colonies of the sample plate can be entertained, but the corresponding zones of the control plate indicate a definite bacterial growth absent on the sample plate. The asymmetrical shape of the inhibition zones on the sample plate is a consequence of the asymmetrical shape of the filter paper used. An additional factor could be the administering of the sample onto an inclined plate, biasing the distribution of the sample on the filter paper.

The induction profile of the antibacterial factors following treatment with Gram-negative and Gram-positive bacteria is not similar, as treatment with Gram-negative bacteria induces a stronger antibacterial response than treatment with Gram-positive bacteria (Fig 2.2). This could be due to factors affecting the distribution of the bacterial membrane components in the hemolymph and tissues of the tick or to the stronger induction potential of the Gram-negative bacterial membrane components.

There was activity present when ticks were treated with sterile water (Fig 2.2) and that could be due to at least three mechanisms. Firstly, the antibacterial activity induction in response to injury has been described in other organisms (Chalk *et al.*, 1994, Boman and Hultmark, 1987), where sterile injury to an organism leads to the release of cellular debris and components that induce the response to counteract any opportunistic infections at the injury site. Secondly, the influx of external bacteria during the injection of the water could also induce a response, and it is possible that the smaller bacterial numbers could lead to a small increase in antibacterial activity. Thirdly, the water and external bacteria could have no discernible effect, but the presence of a constitutive level of antibacterial activity could lead to the small level of antibacterial activity found. Later investigations using the broth microdilution assay showed the absence of a significant constitutive antibacterial response, invalidating the third proposed mechanism.

It was found that the activity is only directed against some Gram-positive bacteria. This result was obtained using both LB-broth and Mueller-Hinton (MH) broth as liquid bacterial growth media (data for MH-broth not shown). It was proposed that MH broth facilitated a more sensitive antibacterial activity (Diamond, G,

Antibacterial Peptide Workshop, SASBMB Congress, January 2000, Grahamstown, South Africa). This disputed increased sensitivity was not evident during analysis of the hemolymph of *O. savignyi*, since only the presence of activity against Gram-positive bacteria was evident. Studies on the antibacterial activity of *O. moubata* also show an exclusively anti-Gram-positive response (Van der Goes van Naters-Yasui *et al.*, 2000). This might suggest that the antibacterial factors in the hemolymph of *O. savignyi* do not target Gram-negative bacteria. The isolation of the defensin peptides (Chapter 3) lends credibility to this result, since these antibacterial factors only display antibacterial activity against Gram-positive bacteria.

The time of induction of the antibacterial factors in *O. savignyi* is relatively rapid, considering that the period used in some other studies is much longer (Fig 2.3). An example of this longer induction time is the synthesis of cecropins and attacins in the pupae of the giant silk moth, *H. cecropia*, where the antibacterial activity can be detected between 10 and 20 hours following bacterial challenge (Hultmark *et al.*, 1980). It was shown in the fleshfly, *Phormia*, that responses to bacterial challenge were swift. Transcripts of *Phormia* defensin were apparent 15 min following bacterial challenge, indicating that the induction of gene synthesis for the antibacterial peptides can be rapid (Hoffman and Hetru, 1992). In the larvae of the large tenebrionid beetle, *Z. atratus*, the antibacterial activity is detectable in the hemolymph 6 hours after bacterial challenge (Bulet *et al.*, 1991). Thus, the appearance of potent antibacterial activity in the hemolymph of *O. savignyi* one hour following heat-killed Gram-negative bacterial challenge is not unique. The exact mechanism of the rapid increase is still unknown, since either the release of stored antibacterial factors, or the rapid synthesis of the factors could be responsible.

The study performed on *H. cecropia* indicates that the antibacterial activity is still present more than 40 hours following bacterial challenge, although the collection of hemolymph in this study was only performed four days after bacterial challenge (Hultmark *et al.*, 1980). The corresponding results in *O. savignyi* indicate that the response is induced within 1 hour and degraded rapidly within 24 hours. This could highlight differences in energy dependence, nutritional availability and strength of the induced response of the organisms.

The protease digestion of *O. savignyi* hemolymph does not totally remove the antibacterial activity, but there is still a significant reduction in the level of activity. This confirms that the antibacterial activity is, at least partially, mediated by protein agents. The presence of protease-inhibiting factors in the hemolymph could be responsible for the incomplete digestion of the protein content of the hemolymph. This hypothesis is supported by the fact that the pure hemolymph was also incubated for the indicated period, but the antibacterial factors were not degraded by the endogenous proteases in the hemolymph. This is confirmed by unpublished results from this laboratory, indicating the absence of protease activity in the hemolymph.

The heat stability of the antibacterial factors (Fig 2.4) may provide information about the structure of the antibacterial factors. Since protease digestion of hemolymph leads to the reduction of the level of antibacterial activity, at least part of the antibacterial response is of proteinaceous nature. The molecular mass of these proteins should be below 20 kDa, since larger proteins are not normally stable when exposed to such high temperatures. The stability could also be enhanced by stabilizing intramolecular interactions e.g. disulfide bonds. A hypothesis can be constructed stating that at least a portion of the antibacterial factors present in the hemolymph is of the invertebrate defensin class of antibacterial peptides. This hypothesis is tested in Chapter 3, which describes the isolation and characterization of the antibacterial factors.

Chapter 3

Isolation and characterization of the antibacterial factors in the hemolymph of the soft tick *Ornithodoros savignyi*

3.1 Introduction

The methods used to isolate specific proteins from a crude sample are varied, and all utilize different properties of the proteins of interest. Some of these properties that can be exploited during the isolation process include the net charge, stability, hydrophobicity and molecular mass of proteins. Techniques that have been developed to specifically target these properties include the use of chromatography, precipitation using various conditions, and electrophoresis.

Detailed knowledge of the procedures and methods used in the purification and characterization of antibacterial proteins in other organisms is essential to facilitate an effective purification of the antibacterial proteins from *O. savignyi*. A large number of invertebrate antibacterial proteins have been isolated to date. The structures and characteristics of these diverse classes of antibacterial proteins differ and this would influence the choice of purification methods of these proteins significantly. The morphological and physiological differences of the different organisms would also play a role in increasing the complexity of the purification procedures and could negate the extrapolation of an optimized purification scheme of a protein from one organism to the proposed purification scheme for a protein from a different organism.

The characterization of the purified proteins is of great importance, since this reveals the identity and physical characteristics of the protein e.g. the molecular mass, pI, amino acid composition and amino acid sequence. The activity profile of the purified antibacterial protein can suggest a possible mechanism of action by comparison with data from known proteins. The morphological distribution of these proteins in the organism and the selective activation of the genes encoding the proteins can suggest a specific role e.g. as part of the immune response.

Purification techniques used in this study are described below.

3.1.1 Protein precipitation techniques

3.1.1.1 Ammonium sulfate precipitation

Salt fractionation of protein mixtures is mediated by the addition of a salt (e.g. $(NH_4)_2SO_4$). The salt ions neutralize the surface charges of the proteins and diminish the effective concentration of the water. This leads to the precipitation of the proteins from the solution. Proteins differ in their ability to be precipitated by a certain concentration of salt. This is related to the number and distribution of charges and of nonionic polar groups on the surface of the protein. The number and distribution of hydrophobic residues exposed by the neutralization of surface charges also play a role.

Ammonium sulfate is the preferred salt used in the salt fractionation of protein mixtures due to its advantages: (1) the molarity at saturation (4.04 M at 20°C) is high enough to cause the precipitation of most proteins, (2) it does not have a large heat of solution, (3) its saturated solution has a density that is not so high as to interfere with the sedimentation of precipitated proteins during centrifugation, (4) its concentrated solutions prevent or limit most bacterial growth, and (5) in solution it protects most proteins from denaturation (Englard and Seifter, 1990).

3.1.1.2 Acid precipitation

Since protein tertiary structure is dependent on, among others, ionic and hydrogen bonds, disruption of these bonds would destabilize the protein structure and could lead to denaturation. Decreasing the pH of a solution using acid, e.g. trifluoroacetic acid (TFA), would lead to the protonation of carboxylic amino acid residues, thus disrupting ionic and hydrogen bonds and increasing the positive charge of the proteins. The destabilization of the protein structure could lead to the exposure of hydrophobic residues and to protein precipitation and denaturation. The concurrent lowering of temperature would favour the hydrophobic interactions and thus precipitation and aggregation. The use of acid precipitation would thus be an effective way of decreasing the complexity of a protein mixture if the protein of interest is not susceptible to acid precipitation.

3.1.2 High Performance Liquid Chromatography (HPLC)

Separation of proteins using chromatography is based on the differential rates of protein migration through the particles of the stationary phase of a column. The objective is thus to optimize conditions and materials in order to maximize the difference in migration between the molecule of interest and the other molecules in the sample (Regnier, 1984). HPLC is an improvement in chromatography techniques due to the greater mechanical strength and smaller size of the sorbent particles and in the increased pressure (higher mobile phase velocity) used during separations, leading to greater sensitivity, higher resolution and better reproducibility of protein separations. Since proteins vary in their size, shape, charge, hydrophobicity and the arrangement of functional groups in their 3-D structure, the use of different chromatographic fractionation modes can be used in concert to isolate a specific protein or peptide. The different chromatographic modes include size-exclusion and reversed-phase chromatography (Chicz and Regnier, 1990).

3.1.2.1 Size-exclusion (SE) HPLC

SE-HPLC is a useful technique employed as an initial fractionation step in order to resolve a complex protein mixture and relies on the fractionation of macromolecules due to size differences between molecules (Hodges and Mant, 1991). The column packing employed in SE is usually macroporous silica coated with organosilanes or organic polymers. The pores in the stationary phase allow smaller proteins and peptides to totally permeate the liquid volume of the particles, and these small molecules elute in a volume of liquid known as the total permeation volume. The proteins too large to permeate the pores of the stationary phase would not be retained in the internal liquid volume of the particles, and would elute in a column void volume equal to the volume of solvent between particles. The proteins with partial access to the internal volume of the particles would thus elute between these two extremes (Chicz and Regnier, 1990).

The nature of the column packing causes most columns to exhibit anionic and hydrophobic properties, resulting in solute/packing interactions that deviate from ideal SE behavior. The addition of salts to the mobile phase is sufficient to minimize these unwanted interactions (Hodges and Mant, 1991). SE-HPLC can also be used to determine the molecular mass of an unknown protein since there is a linear relationship between the

elution volume and the logarithm of the molecular mass of a protein. Using a calibration curve for elution of proteins with known molecular masses, the mass of the unknown protein can be determined if the elution volume is known (Unger, 1984).

3.1.2.2 Reversed-phase (RP) HPLC

RP-HPLC is a very popular method used during the isolation of proteins and peptides. The stationary phase comprises an *n*-alkylsilica based sorbent, with the length of the alkyl chains determined by the nature of the molecules under investigation. Shorter alkyl chain lengths (C_4 to C_8) are used for protein isolation and longer alkyl chain lengths (C_8 to C_{18}) are used for peptide isolation and preparation of peptide and protein samples for amino acid sequence determination (Chicz and Regnier, 1990). The proteins or peptides unfold due to the low pH and presence of organic solvents, adsorb to the stationary phase through hydrophobic interactions, and are eluted from the sorbent using gradients of increasing concentrations of organic solvents. The most common mobile phase system used in RP analysis is the aqueous acetonitrile system containing the ion-pairing reagent TFA. The system has powerful resolving capabilities and the low pH (pH 2.0) suppresses the ionic interactions between the solute and the packing due to the presence of non-derivatized silanols which are negatively charged above ~ pH 4.0 (Hodges and Mant, 1991). RP-HPLC is a powerful technique in protein and peptide isolation, but the conditions of the chromatographic analyses usually disrupt protein tertiary structure, and the subsequent retention of activity is dubious for proteins >10 kDa. This technique is thus usually reserved as a preparative isolation step only for peptides and proteins able to re-attain their native tertiary structure unaided (Chicz and Regnier, 1990).

3.1.3 Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a very useful technique for monitoring the purification process and assessing the protein composition of a sample mixture. The technique can also be used to determine the approximate molecular mass of a purified protein or peptide. The principle of the technique is the migration of proteins and peptides under the influence of an electrical field. A surrounding gel matrix retards the components and results in the separation of protein

components on the basis of molecular mass. The gel matrix is formed by the co-polymerization of the acrylamide and *N,N'*-metylenebisacrylamide monomers. The pore size of the gel matrix is inversely proportional to the concentration of the acrylamide and crosslinking bisacrylamide monomers. The addition of hot SDS to the sample prior to electrophoresis leads to the binding of the SDS to proteins (~1.4 mg SDS/mg protein), negative charges are imparted and their structures become disordered. The concomitant treatment of the sample with a disulfide reducing agent neutralizes secondary structure formation and the proteins all migrate in a manner dependent on their molecular masses alone. Migration rates towards the anode are inversely proportional to the logarithms of the molecular weights of the proteins. This migration behavior is predictable, and the molecular mass of an unknown protein can be deduced from the migration rates of proteins with known molecular masses.

The use of glycine as the trailing ion in the electrophoretic system leads to the resolution of proteins >30 kDa, while the use of tricine would lead to the resolution of proteins <30 kDa. The tricine systems also relies on a smaller pore size in the gel matrix compared to the glycine system, to retard the migration of larger proteins and to increase the resolution of the smaller protein components (Garfin, 1990).

3.1.4 Electrospray ionization mass spectrometry (ESMS)

The technique of mass spectrometry is very useful in obtaining the correct molecular mass of an isolated product and the homogeneity of the product can also be evaluated. The use of the ES ionization source increases the sensitivity and effectivity of the analysis, since the ionization extracts compounds from solution intact and gives rise to multiply charged ions for each compound. Liquid sample is introduced to the ionization source through a needle with an electric potential 2 to 7 kV relative to the surrounding electrodes. Ionized sample droplets form and stepwise droplet breakups decrease droplet size until the diameter of the droplets is small enough for spontaneous ion emission from the droplet surface to occur. The ions are driven into a vacuum due to the electrostatic field, are dried by opposing gas flow and are focussed by dynamic and electrostatic lenses for analysis in the mass spectrometer. As multiply charged ions of higher

molecular mass proteins are formed, the mass spectrum needs to be deconvoluted to obtain the correct molecular mass of the protein/s of interest (Banks Jr. and Whitehouse, 1996).

3.1.5 Matrix assisted laser desorption ionization (MALDI) mass spectrometry

The MALDI-MS system is very versatile and effective for the analysis of proteins and peptides due to the special properties of the technique. Proteins and peptides can quickly be examined without extensive purification, and common biochemical additives do not interfere with the analysis. Useful mass spectra can be obtained from complex mixtures of proteins and peptides, and the total amount of protein required for the analysis is in the range of 1 – 10 pmol. Protein samples are incorporated into crystals of the matrix, a material that can incorporate protein samples into their crystals, eject them intact and ionize them in the same process. Short duration pulses of laser light are directed at the matrix-protein sample mixture, causing a portion of the matrix-protein sample to be volatilized and ionized. The resulting gas phase ions are accelerated in an electrostatic field and directed into a field-free flight tube. The masses of the ions can then be determined using their time of flight, since lighter ions move more quickly down the flight tube than heavier ions. Due to this simplicity, the time of flight (TOF) detector is the most common MALDI-Ms detector in use (Beavis and Chait, 1996).

3.1.6 Amino acid sequence determination

The sequencing of protein and peptides was developed in 1950 (Edman, 1950) and this method is still used widely. The technique involves the coupling of phenylisothiocyanate (PITC) to the N-terminal of a protein/peptide. The N-terminal amino acid is cleaved off and the anilinothiazolinone (ATZ) amino acid is separated from the residual peptide. The ATZ-amino acid is converted to a phenylthiohydantoin (PTH) amino acid and can be identified with an HPLC system. The residual peptide can be subjected to subsequent cycles of degradation to obtain the amino acid sequence. This method is used to obtain the sequences of proteins/peptides in the nmole range (Levy, 1981). To increase the sensitivity of the degradation reaction, an automated modified spinning cup sequenator

was developed. This technique allows the sequencing of proteins/peptides in the subnanomole range (Shively, 1981).

3.1.7 Aims of the study

The aims of this study are the isolation, characterization and identification of the antibacterial components from the hemolymph of *E. coli* treated *O. savignyi* ticks.

3.2 Materials

All materials used were of analytical grade and double-distilled deionized water was used in all experiments. Methanol, acetic acid, tris (tris (hydroxymethyl) aminomethane), tricine (*N*-tris (hydroxymethyl) methylglycine), ammonium peroxide sulfate, TEMED and ammonium sulfate were obtained from Merck, Darmstadt, Germany. Peptide mass marker proteins were purchased from Pharmacia, USA. Acrylamide, N, N'-methylene bisacrylamide and SDS were purchased from BDH Laboratory Supplies Ltd., England. Coomassie Blue was obtained from Bio-Rad Laboratories, USA and the Micro BCA Protein Assay Kit from Pierce, USA. Acetonitrile was purchased from Fluka (Germany). Leupeptin (serine and cysteine protease inhibitor), aprotinin (serine protease inhibitor) and PMSF (serine and cysteine protease inhibitor) were obtained from Boeringer Mannheim GmbH (Germany).

3.3 Methods

3.3.1 Induction of antibacterial response

Induction of the antibacterial response was performed as in Sections 2.3.2 and 2.3.3.

3.3.1 Hemolymph extraction

Hemolymph extraction was performed as in Section 2.3.4.

3.3.2 Antibacterial assay

The broth microdilution assay was used as in Section 2.3.5.2., except where indicated otherwise. To determine the presence of antibacterial fractions during the HPLC isolation steps, *B. subtilis* was used as indicator organism.

3.3.3 Sample preparation

All samples for HPLC were filtered through a 0.22 µm membrane (Millex-GV, Millipore, Bedford, MA, USA) prior to injection.

3.3.3.1 Sample preparation for SE-HPLC

The collected hemolymph was centrifuged in order to remove hemocytes and cellular debris. Following the filtering of the samples using a 0.22 µm syringe filter, the samples were injected onto the SE-HPLC column.

3.3.3.2 Sample preparation for ammonium sulfate precipitation

Ammonium sulfate solutions in increasing concentrations were added to the hemolymph of *E. coli* treated ticks to achieve final concentrations of 20%, 40%, 60% and 80% (w/v) ammonium sulfate respectively. An ultracentrifugation step at 50 000 g for 30 min was performed and the pellets of the samples were analyzed using tricine SDS-PAGE.

3.3.3.3 Sample preparation for acid precipitation

Hemolymph of 30 ticks was added to an ice-cold 0.1% TFA solution. The solution was kept in an ice-bath for 30 min with frequently stirring, followed by an ultracentrifugation step at 50 000g for 30 min.

3.3.4 C_{18} sep-pak chromatography

The supernatant of the acid precipitation step (see 3.3.3.3) was loaded onto a pre-packed bonded silica C_{18} column (500 mg/6 ml, pore diameter 60 – 87 Å, Sigma-Aldrich, Germany). The hemolymph constituents were eluted stepwise from the column with increasing concentrations of acetonitrile containing 0.1% TFA. Fractions were collected, dried under vacuum and stored at 4°C until use.

3.3.5 High Performance Liquid Chromatography (HPLC)

HPLC was performed on a Beckman, System Gold Chromatograph (Beckman) and on a Waters 600 HPLC (Waters) containing a Waters 996 photodiode array detector and using the Millenium[32] chromatography software. Size-exclusion (SE) HPLC was performed

using a TSK-gel G3000 SW_{XL} column (Tosohaas) with a 100 kDa exclusion size. Reversed phase (RP) HPLC was performed using a C_{18} RP-column (Bondclone 10 – C_{18}, 300 x 3.9 mm, pore diameter 300 Å, Phenomenex, CA, USA) and a C_5 RP-column (250 x 4.6 mm, pore diameter 320±40 Å, Phenomenex, CA, USA). All HPLC analyses were performed at a flow speed of 1 ml/min.

3.3.6 Tricine gel electrophoresis

Samples were prepared for electrophoresis under reducing conditions by heat treatment at 94°C for 5 minutes in Laemmli sample buffer (2% SDS, 10% glycerol, 0.05 M Tris pH 6.8, 5% mercapto-ethanol). Sample buffer prepared for non-reducing conditions did not contain mercapto-ethanol.

The method (Schägger and Von Jaggow, 1987) facilitates the separation and resolution of low molecular mass proteins (<30 kDa). An acrylamide, N, N'-methylene bisacrylamide stock solution (49.5% T, 3% C) as well as a buffer stock solution (3 M Tris, 0.3% SDS, pH 8.45) were used to prepare a 4% stacking gel, a 10% spacer gel and a 16% separating gel (pH 8.45). The solution was polymerized with the addition of 0.015% ammonium persulfate and 0.1% TEMED. For the anode electrophoresis buffer, 0.2 M Tris (pH 8.9) was used and for the cathode electrophoresis buffer, 0.1 M Tris, 0.1M Tricine, 0.1% SDS (pH 8.45). A constant voltage of 30 V was maintained until the samples were concentrated in the stacking buffer. Thereafter, the voltage was adjusted to 70 V and kept constant for the remainder of the separation period.

Gels were stained with Coomassie blue (0.1% (w/v) Coomassie Brilliant Blue R-250, 40% methanol, 10% acetic acid) for several hours and destained with a large excess of 40% methanol containing 10% acetic acid.

3.3.7 Electrospray mass spectrometry (ESMS)

Electrospray mass spectrometry was performed by Dr. M.J. van der Merwe (Department of Biochemistry, University of Stellenbosch). Samples were dissolved in 50% formic acid. Electrospray mass spectrometry was performed using a Micromass Quattro triple

quadropole mass spectrometer equipped with an electrospray ionization source. The capillary voltage applied in the positive mode was 3.5 kV, the source temperature 80°C and cone voltage 70V with the skimmer lens set at 5V. All other lenses were tuned for maximum purity.

During two of the five analyses, only a single scan was performed for the component molecular mass, and this possibly led to a less accurate determination of the molecular masses of the components.

3.3.8 Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis of purified proteins

The MALDI-TOF analysis was performed by A. Fiedler (Department of Molecular and Cell Biology, University of Cape Town) to assess the purity of the isolated peptide from Fraction 50-B, which was submitted for Edman amino acid sequence determination. Equal volumes of purified protein (10 pmol) were mixed with matrix (sinapinic acid). Samples were analyzed with a DE-PRO MALDI-TOF mass spectrometer (PerSeptive Biosystems, Farmingham, MA, USA) with an accelerating voltage (20 000), grid-voltage (91 – 92%), and guide wire voltage (0.1%) using a linear flight path. Between 85 and 1000 scans were averaged.

3.3.9 N-terminal amino-acid sequence determination

Prof. W. F. B. Brandt (Department of Molecular and Cell Biology, University of Cape Town) performed N-terminal amino-acid sequence determination of the isolated *O. savignyi* defensin B in a gas phase amino acid sequencer constructed as outlined in Hewick *et al.* (1981) and modified as described by Brandt *et al.*. (1984).

3.4 Results

3.4.1 Initial strategies for the isolation of antibacterial proteins from *O. savignyi*.

The first step in the initial procedure to isolate the antibacterial proteins from the hemolymph of *E. coli* pre-treated ticks entailed the use of SE-HPLC. The samples were injected onto a size exclusion column with an exclusion size of 100 kDa. The

components displaying antibacterial activity eluted from the column over a 4 min time period, but co-eluted with a majority of the hemolymph components (Fig 3.1A). This isolation step was therefore not effective at removing a large fraction of contaminating proteins.

The collected samples displaying antibacterial activity from the SE-HPLC were collected during subsequent SE-HPLC runs and were subjected to C_{18} RP-HPLC chromatography (Fig 3.1B). Fractions (1 ml) were collected, dried under vacuum and assayed for antibacterial activity using a radial diffusion assay with *B. subtilis* as indicator organism. Two discrete antibacterial fractions were found to elute from the RP-HPLC column. The fractions were separate from each other, although the one fraction did not elute in an area which showed a significant absorbing fraction on the chromatogram. The chromatogram showed that the antibacterial proteins were well resolved from the majority of contaminating proteins.

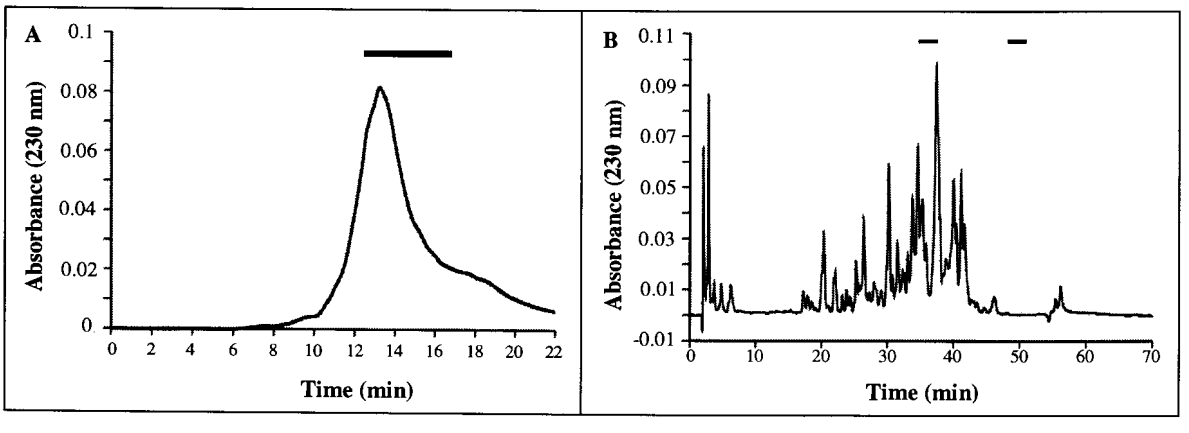

Figure 3.1 A) SE-HPLC chromatogram of *E. coli* induced tick hemolymph. B) The C_{18} RP-HPLC chromatography of the SE-HPLC fractions displaying antibacterial activity. A SE-HPLC isocratic run was performed with a 20 mM acetate elution buffer, pH 4.7 at a flow speed of 1 ml/min. A RP-HPLC gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 60% acetonitrile, 0.1% TFA over 60 min. Fractions (1 ml) were collected, dried *in vacuo* and assayed for antibacterial activity using a radial diffusion assay with *B. subtilis*. Fractions displaying antibacterial activity against *B. subtilis* with the radial diffusion assay are indicated by (▬).

It was subsequently found that there were high molecular mass proteins present in the hemolymph that were adsorbing strongly to the stationary phase of the SE-HPLC column, and this was negatively affecting the separation efficiency of the column. The majority of these proteins were suspected to be vitellogenins (Sonenshine, 1991), a prominent tick egg protein. These proteins occur in high concentrations in the hemolymph of female ticks and have a molecular mass of approximately 94 kDa. The result of this observation was that a new isolation method had to be developed to eliminate the vitellogenins from the hemolymph so as to improve the purification efficiency.

3.4.2 Isolation of antibacterial factors using ammonium sulfate precipitation and RP-HPLC.

The concentration of ammonium sulfate that mediates the precipitation of the vitellogenins, but not the antibacterial proteins, hemolymph from the *E. coli* induced ticks had to be determined. The pellets of the ammonium sulfate precipitated samples were analyzed using tricine SDS-PAGE (Fig 3.2). It can be seen from the gel (lane S) that a significant amount of high molecular mass proteins are present in the crude hemolymph. The 60% (w/v) ammonium sulfate concentration caused the precipitation of a significant amount of high molecular weight proteins from the crude hemolymph. The supernatant of the 60% ammonium sulfate precipitation step was desalted on the C_{18} HPLC column and the antibacterial activity determined to confirm that that the antibacterial factors did not co-precipitate (data not shown).

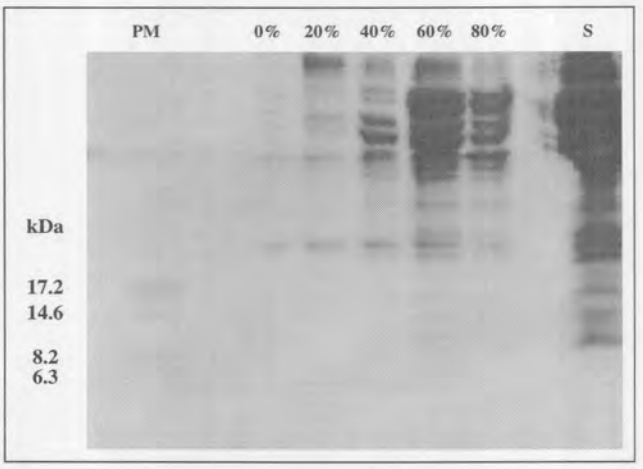

Figure 3.2 Tricine SDS-PAGE gel of ultracentrifugation pellets of hemolymph fractions subjected to ammonium sulfate precipitation. The indicated percentage of a saturated ammonium sulfate solution was added to the hemolymph, followed by ultracentrifugation at 50 000 g for 30 min. The pellet was dissolved in 30 µl reducing Laemmli sample buffer and incubated at 94°C for 4 min. The gel was stained using Coomassie Brilliant blue overnight. Lanes are marked as follows: PM = peptide mass markers, 0% to 80% indicates the ammonium sulfate concentration used to obtain the pellets, S = supernatant of 0% ammonium sulfate precipitation. The sizes of the peptide mass markers are indicated on the side of the gel in kDa.

Subsequently, the initial step in the isolation procedure was a 60°C heat treatment for 10 min followed by a 60% (w/v) ammonium sulfate precipitation step. The supernatant was applied to a C_{18} RP-HPLC column, and the fractions displaying antibacterial activity in subsequent runs were re-applied to the C_{18} RP-HPLC column (Fig 3.3.B).

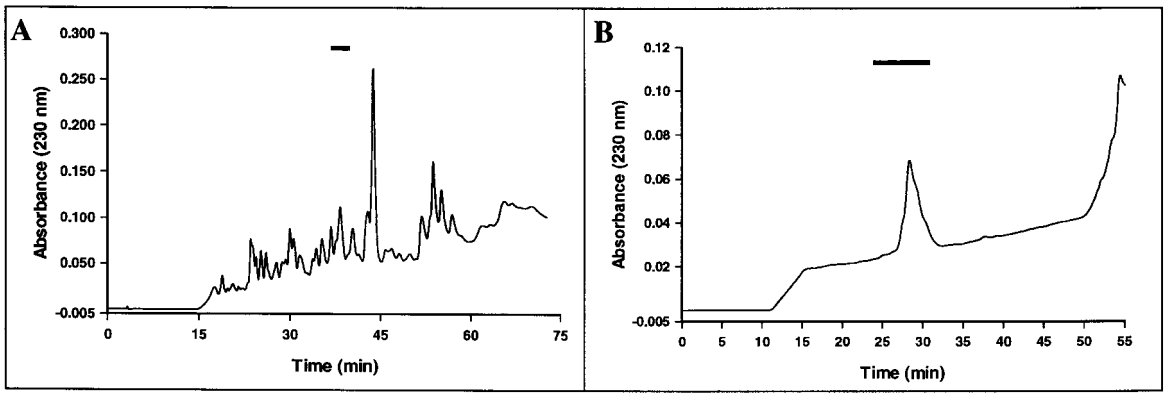

Figure 3.3 (A) C_{18} RP-HPLC chromatograph of 60°C heat-treated, 60% ammonium sulfate precipitated hemolymph. (B) The re-chromatography of the C_{18} RP-HPLC fractions displaying antibacterial activity. The hemolymph of five ticks was subjected to 60°C for 10 min followed by a 60% ammonium sulfate precipitation. The supernatant of an ultracentrifugation step at 50 000 g for 30 min was applied to a C_{18} RP-HPLC column and a gradient run performed from 0.1% acetonitrile, 0.1% TFA to 100% acetonitrile in 60 min. The dried fractions containing antibacterial activity in (**A**) were re-applied to the C_{18} RP-HPLC column (**B**). A step gradient run was performed using 0.1% acetonitrile, 0.1% TFA as buffer A and 100% acetonitrile, 0.1% TFA as buffer B. The gradient was increased from 0% to 25% buffer B for 5 min, followed by a increase from 25% to 52% buffer B in 35 min and from 52% to 80% buffer B in 5 min. Fractions displaying antibacterial activity against *B. subtilis* with the broth dilution assay are indicated by (▬).

The fractions displaying antibacterial activity in Fig 3.3.B were analyzed using ESMS, and the main peak of interest was a protein with a molecular mass of 6568.88 Da (data not shown). The analysis showed that the sample did not contain a pure protein, but the isolation procedure could not be repeated due to problems with the C_{18} RP-HPLC column.

Subsequently, the supernatant from the ultracentrifugation step was applied to the C_5 RP-HPLC column (Fig 3.4.A). The fractions displaying antibacterial activity in subsequent chromatographic analyses were re-applied to the C_5 RP-HPLC column (Fig 3.4.B).

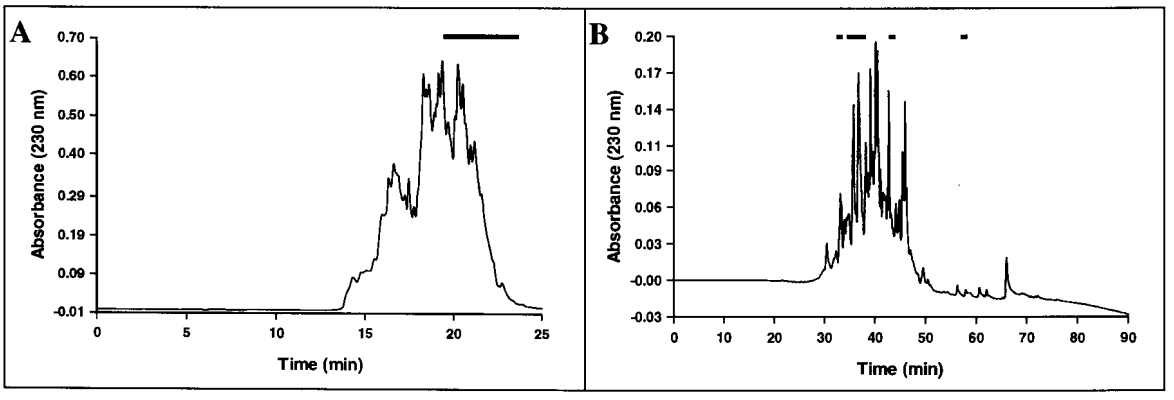

Figure 3.4 (A) C_5 RP-HPLC chromatograph of 60°C heat-treated, 60% ammonium sulfate precipitated hemolymph. (B) The re-chromatography of the C_5 RP-HPLC fractions displaying antibacterial activity. Hemolymph of five ticks was subjected to 60°C for 10 min followed by a 60% ammonium sulfate precipitation. The supernatant of an ultracentrifugation step at 50 000 g for 30 min was applied to a C_5 RP-HPLC column and a gradient run performed from 50 mM Tris, pH 7.6 to 100% acetonitrile in 60 min. The dried fractions containing antibacterial activity in (**A**) were re-applied to the C_5 RP-HPLC column (**B**). A gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 100% acetonitrile, 0.1% TFA in 90 min. Fractions displaying antibacterial activity against *B. subtilis* with the broth dilution assay are indicated by (▬).

The chromatogram in Fig 3.4.B shows four distinct regions displaying antibacterial activity. The subsequent re-chromatography of these samples using elution buffers with lower concentrations of acetonitrile in combination with longer elution gradients were attempted, but no improvements in the purification of the antibacterial factors could be facilitated. The sensitivity of the detector of the Beckman HPLC was insufficient, and the frequent need for repairs to the HPLC pumps seriously affected the reproducibility of the results obtained. Following the acquisition of the Waters HPLC system, a new isolation procedure was attempted (Charlet *et al.*, 1996).

3.4.3 Isolation of the antibacterial factors using acid precipitation and RP-HPLC

The isolation procedure for the defensin peptides from *O. savignyi* is presented in Appendix 1. The hemolymph of 30 ticks was used for acid precipitation, and the supernatant was loaded on a pre-packed bonded silica C_{18} column (500 mg/6 ml, pore diameter 60 – 87 Å, Sigma-Aldrich, Germany). The fractions eluting from 20% to 50% acetonitrile, and from 70% to 90% acetonitrile from the bonded silica C_{18} column displayed antibacterial activity and were collected for subsequent isolation steps.

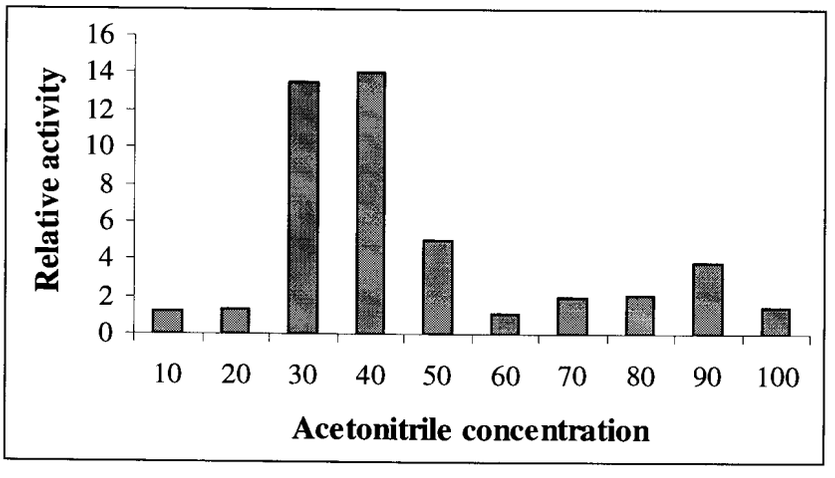

Figure 3.5 Antibacterial properties of *E. coli* treated hemolymph eluting stepwise from the bonded silica C_{18} column. Fractions were eluted from the C_{18} column using the indicated percentage acetonitrile, all buffers contained 0.1% TFA. Fractions were dried under vacuum and the antibacterial activity determined using the broth microdilution assay with *B. subtilis*. The hemolymph of five ticks was used. Relative activity = (Absorbance of negative control – Absorbance of initial negative control) / (Absorbance of sample – Absorbance of initial sample).

To determine the composition of each fraction eluting from the bonded silica C_{18} column, the eluted fractions were analyzed using a 16% tricine SDS-PAGE separating gel under reducing conditions (Fig 3.6). Results shows that the great majority of the high molecular mass proteins are removed during the acid-precipitation step (lane 10, Fig 3.6). A large amount of peptides with molecular masses between 2 kDa and 12 kDa elute between 30%

and 50% acetonitrile, the fraction containing the majority of the antibacterial factors (Fig 3.6). On the other hand, it can be seen that the great majority of peptides have eluted after 70% acetonitrile. This indicates that the fraction eluting between 70% and 90%, containing the minority of the antibacterial factors, have been resolved from the majority of contaminating proteins.

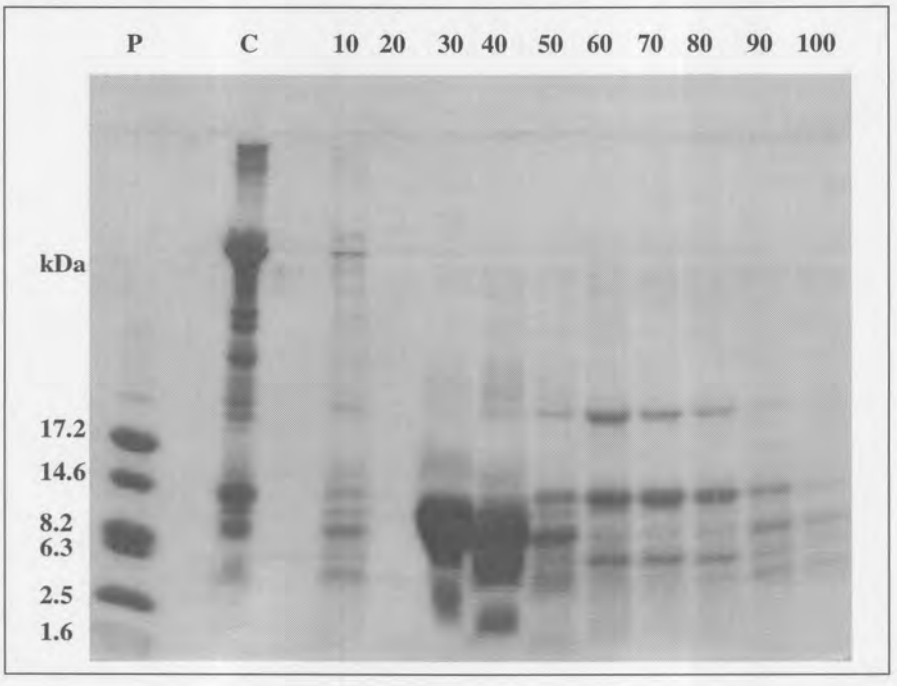

Figure 3.6 Tricine SDS-PAGE of eluted fractions from the bonded silica C_{18} column. Fractions were separated on a 16% separating gel, using mercapto-ethanol as the reducing agent. The vacuum dried fractions were dissolved in 15 µl Lammli sample buffer and incubated at 94°C for 5 min. The gel was stained with Coomassie Brilliant Blue overnight. Lanes: P = peptide mass markers, C = crude hemolymph, 10 – 100 = the percentage acetonitrile used to elute the fraction from the bonded silica C_{18} column. The sizes of the standard peptides in the mass markers (in kDa) are indicated on the gel.

The fractions eluting with 30%, 40% and 50% acetonitrile were pooled and will further be referred to as Fraction 50. The fractions eluting with 80% and 90% acetonitrile were pooled and will further be referred to as Fraction 90.

3.4.3.1 Isolation of antibacterial factors from Fraction 50

The fractions eluting between 20% and 50% acetonitrile from the bonded silica C_{18} column (Fraction 50) were analyzed on a C_5 RP-HPLC-column using the Waters HPLC system (Fig 3.7). Two fractions displaying antibacterial activity were obtained (50-A and 50-B). The fractions were resolved from the majority of the contaminating proteins.

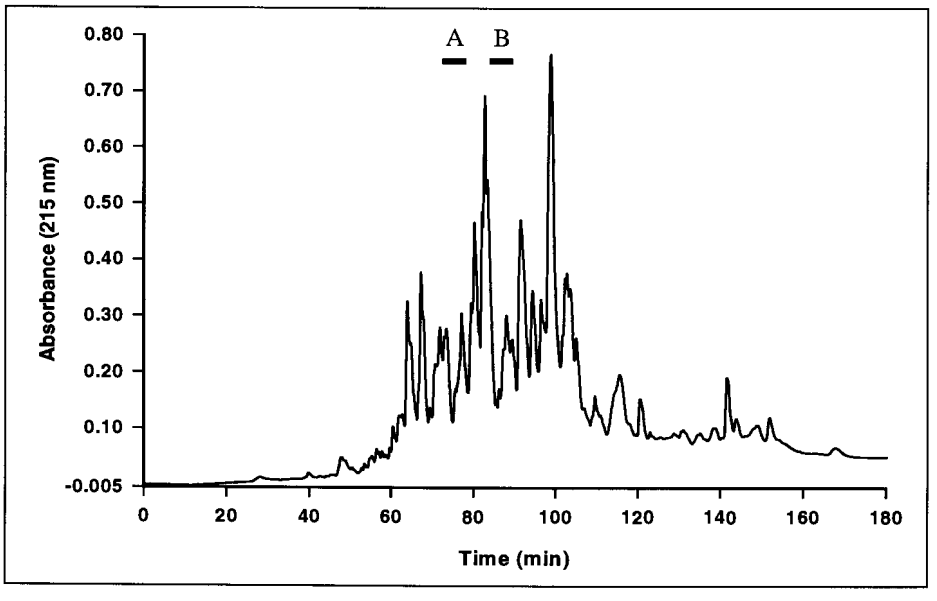

Figure 3.7 RP-HPLC chromatography of Fraction 50. The vacuum dried bonded silica C_{18} fractions were dissolved in 600 µl 0.1% acetonitrile, 0.1% TFA and applied to the column. A gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 60% acetonitrile, 0.1% TFA in 180 min. Fractions displaying antibacterial activity against *B. subtilis* with the broth dilution assay are indicated by (▬).

3.4.3.1.1 Isolation of the antibacterial factor in Fraction 50-A

Fraction 50-A, collected from the RP-HPLC analysis on Fraction 50 (Fig 3.7), was dried *in vacuo*, dissolved in 0.1% acetonitrile, 0.1% TFA and re-applied on the C_5 RP-HPLC column. A gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 70% of 100% methanol in 210 min, peak fractions collected, dried under vacuum and assayed for antibacterial activity with *B. subtilis*. The fraction displaying antibacterial activity (Fig

3.8) did not indicate significant absorption at 215 nm, but the antibacterial response of the constituents of this peak was highly significant (data not shown).

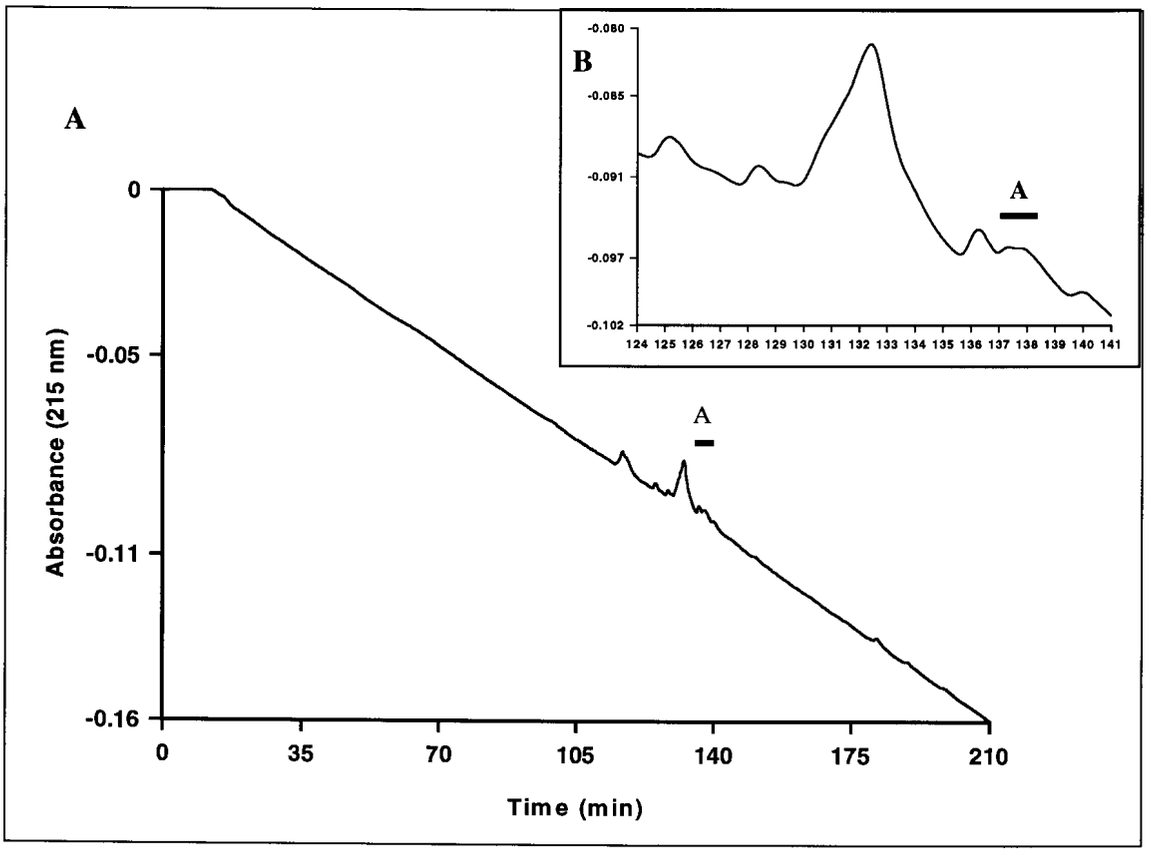

Figure 3.8 (A) C_5 RP-HPLC re-chromatography of Fraction 50-A from Fig 3.7. (B) Magnification of the region on the chromatogram displaying antibacterial activity. Antibacterial fraction A from Fig 3.7 was dried under vacuum, dissolved in 0.1% acetonitrile, 0.1% TFA and a gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 70% of 100% methanol in 210 min. Collected fractions were dried *in vacuo* and analyzed for antibacterial activity against *B. subtilis*. Fractions displaying antibacterial activity are indicated by (▬).

Fraction A of Fig 3.8 was analyzed using ESMS to investigate the composition and molecular mass of the components. The analysis (Fig 3.9) shows that the fraction is relatively pure. The molecular mass of the predominant peak on the spectrum was calculated to be 4166.00 Da. This molecular mass, together with the activity spectrum of

the antibacterial factors potentially classifies the peptide as a member of the defensin class of antibacterial peptides. This peptide is tentatively named *O. savignyi* defensin A, pending future sequence analysis for definite classification.

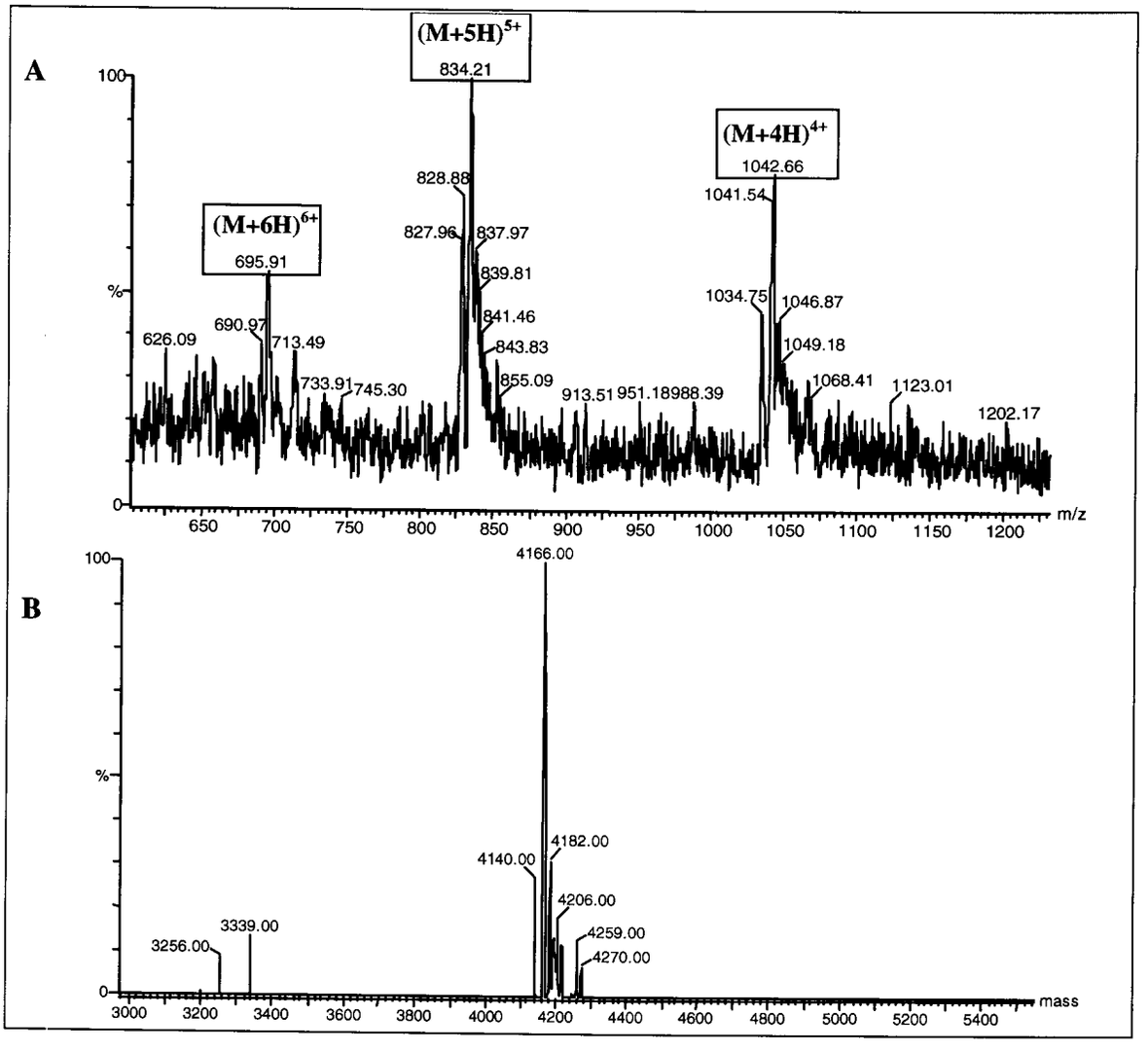

Figure 3.9 (A) Mass spectrum of the isolated peptide from Fraction 50-A following electrospray mass spectrometric analysis. (B) The molecular mass of *O. savignyi* defensin A (4166.00 Da) was calculated by the MaxEnt program. The predominant peaks in **A**, from right to left, represent the three molecular ions $(M+4H)^{4+}$, $(M+5H)^{5+}$ and $(M+6H)^{6+}$ formed during the analysis. The mass/charge (m/z) ratios are indicated above each peak. The calculated molecular masses of the components are indicated above the peaks in **B**.

3.4.3.1.2 Isolation of the antibacterial factor in Fraction 50-B

Fraction 50-B, collected from the RP-HPLC analysis on Fraction 50 (Fig 3.7), was dried under vacuum, dissolved in 0.1% acetonitrile, 0.1% TFA and re-applied on the C_5 RP-HPLC column. A gradient run was performed form 0.1% acetonitrile, 0.1% TFA to 60% of 100% methanol in 180 min (Fig 3.10). Peak fractions were collected, dried under vacuum and assayed for antibacterial activity. The antibacterial fraction did not elute as a single peak, but by collecting the antibacterial fraction in separate regions indicated that the complete fraction displayed antibacterial activity.

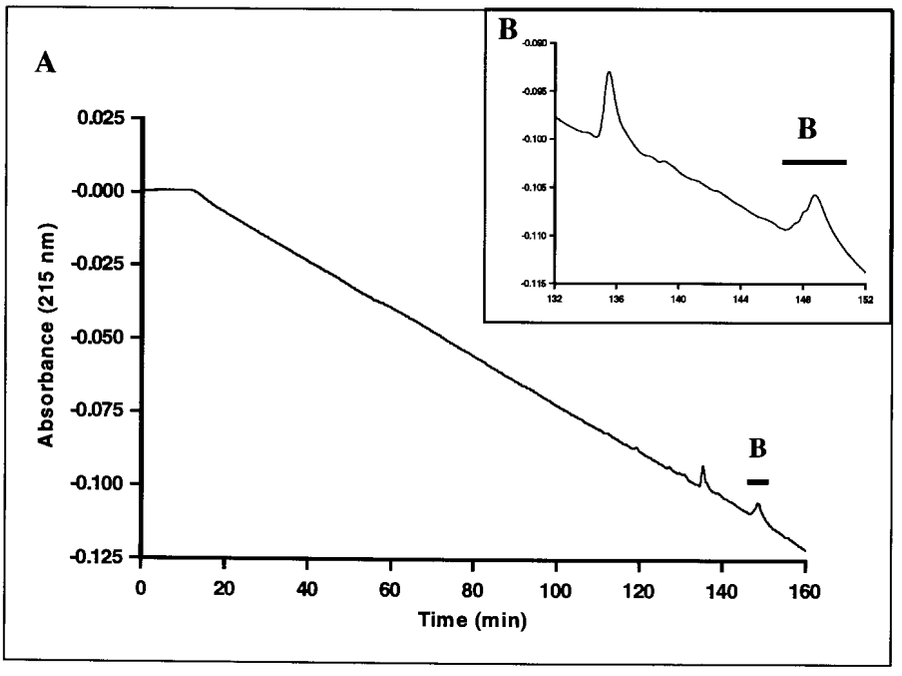

Figure 3.10 (A) C_5 RP-HPLC re-chromatography of Fraction 50-B From Fig 3.7. (B) Magnification of the region on the chromatogram displaying antibacterial activity. Vacuum dried fractions were dissolved in 0.1% acetonitrile, 0.1% TFA and a gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 60% of 100% methanol in 160 min. Fractions displaying antibacterial activity against *B. subtilis* with the broth dilution assay are indicated by (▬).

Fraction B of Fig 3.10 was analyzed using ESMS to investigate the purity and molecular mass of the fraction. The analysis shows that the main component of the fraction is

relatively pure. The molecular mass of the predominant peak on the spectrum was calculated to be 4203.00 Da. This molecular mass, together with the activity spectrum of the antibacterial factors potentially classifies the peptide as a member of the defensin class of antibacterial peptides.

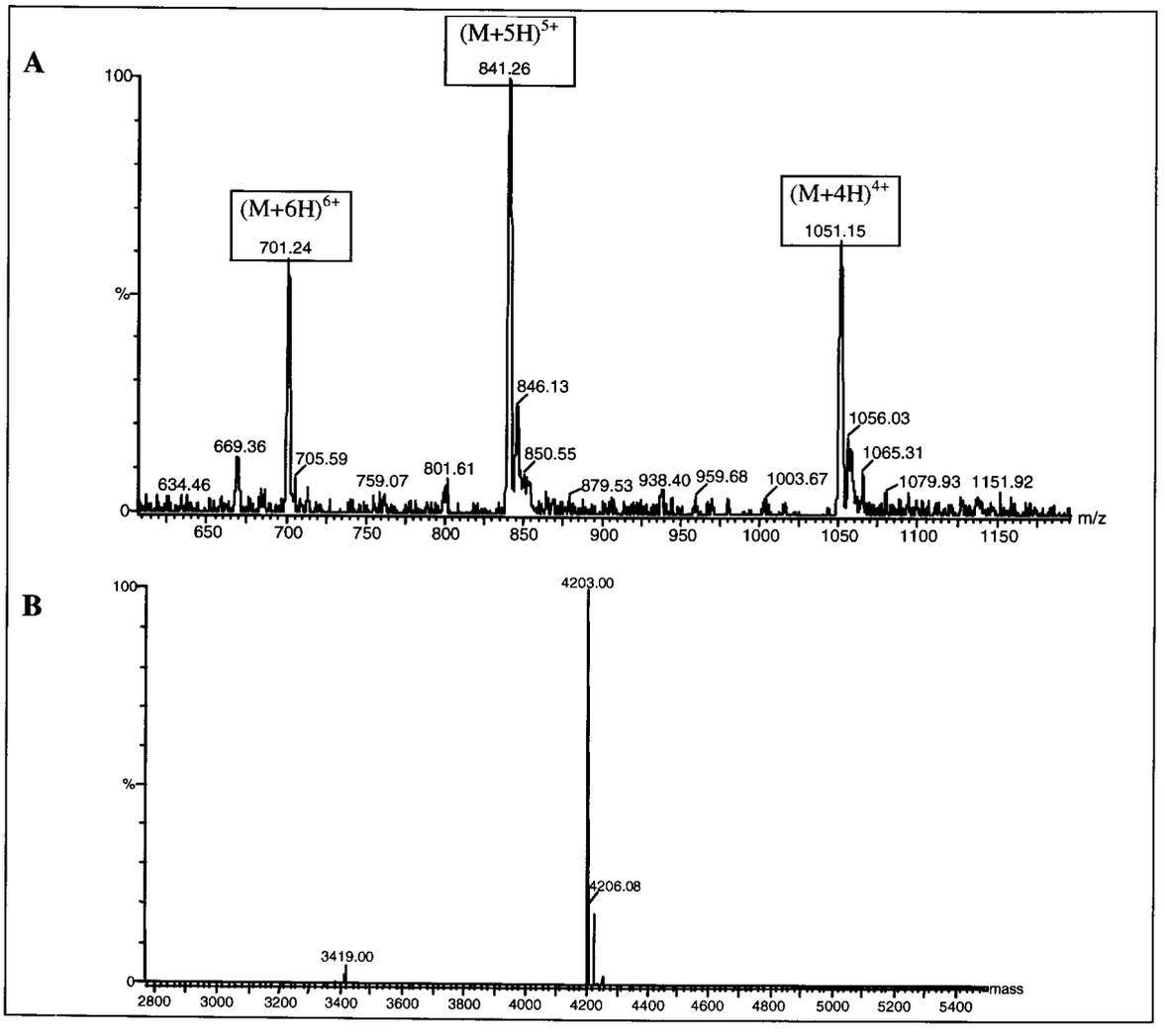

Figure 3.11 (A) Mass spectrum of the isolated peptide from Fraction 50-B following ESMS analysis. (B) The molecular mass of *O. savignyi* defensin B (4203.00 Da) was calculated by the MaxEnt program. The predominant peaks in **A**, from right to left, represent the three molecular ions $(M+4H)^{4+}$, $(M+5H)^{5+}$ and $(M+6H)^{6+}$ formed during the analysis. The mass/charge (m/z) ratios are indicated above each peak. The calculated molecular masses of the components are indicated above the peaks in **B**.

The isolated antibacterial factor from Fraction 50-B was analyzed to determine the N-terminal amino-acid sequence. MALDI-TOF analysis was performed on the fraction prior to sequencing in order to confirm purity (Fig 3.12). The major peak in the mass spectrum has a size of 4205.23 Da which correlates well with the size of 4203.00 obtained for Fraction 50-B using ES-MS. The minor peaks in the spectrum are most probably contaminants, but the concentrations of these contaminants were very low, since the N-terminal sequencing reaction only yielded a single residue per cycle (Table 3.1). Thus, the contaminants did not interfere in the sequencing reaction and their influence and presence can be ignored.

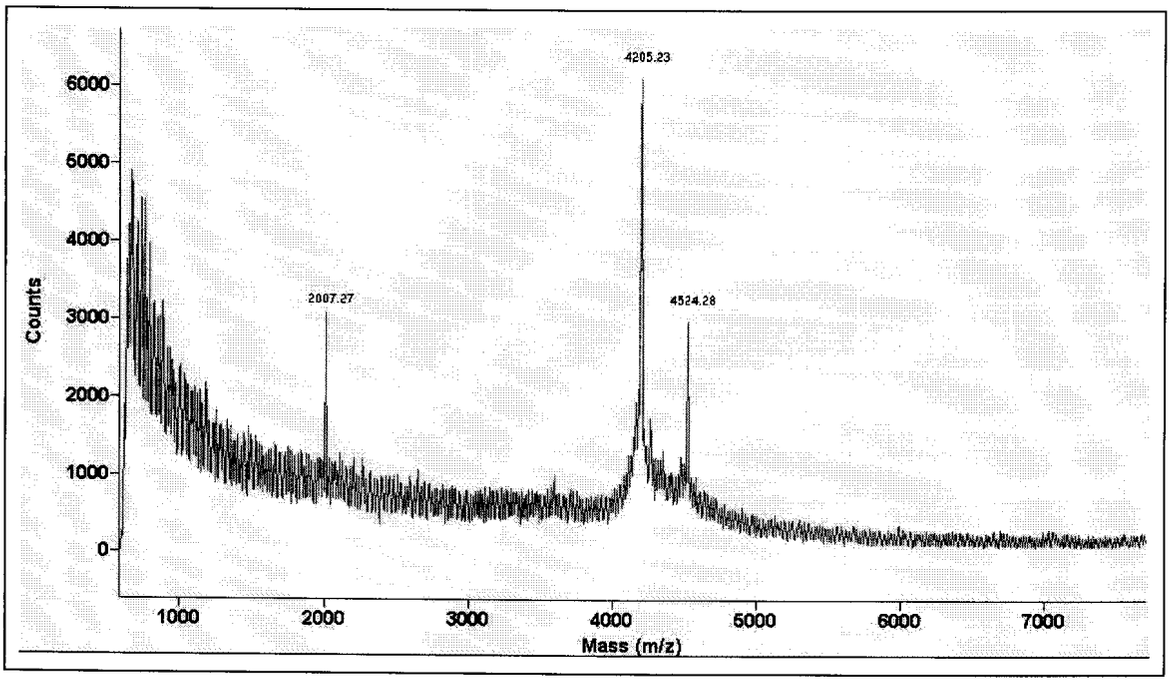

Figure 3.12 MALDI-MS profile of purified antibacterial peptide (50-B) prior to amino acid sequence determination.

The N-terminal amino acid sequence of the purified peptide from Fraction 50-B was determined using Edman-degradation in an automated sequencer (Table 3.1). The cys-residues in the fraction were not alkylated prior to the sequencing reactions, and it was therefore expected that during cycles corresponding to cys-residues no residues would be identified. These residues were labeled as Xxx. Two such unknown residues were found

in the 13-residue N-terminal sequence obtained for the peptide isolated from Fraction 50-B.

Table 3.1 The N-terminal sequence analysis of the isolated peptide from Fraction 50-B.

Cycle	PTH amino acid
1	Gly
2	Gln
3	Gly
4	Xxx
5	Pro
6	Leu
7	Asn
8	Gln
9	Gly
10	Ala
11	Xxx
12	Thr
13	Phe

The N-terminal sequence obtained for Fraction 50-B was aligned with the N-terminal sequences of defensins isolated from other tick species (Table 3.2) and the unknown residues in Fraction 50-B aligned well with the cys-residues of the defensins of the other tick species. This strengthens the hypothesis that these unknown residues in the *O. savignyi* sequence are in fact cys-residues. The significant alignment of the Fraction 50-B sequence with that of the defensins in the other tick species, together with the characterization of the antibacterial activity of the peptides in this fraction, indicates that the peptide of Fraction 50-B is very possibly a member of the insect defensin family.

Table 3.2 N-terminal amino acid sequence alignment of the sequence obtained for the peptide in Fraction 50-B with the defensin peptides isolated from other tick species i.e. *O. moubata* (Nakajima *et al.*, 2001) and *D. variabilis* (Johns *et al.*, 2001). Residues in *O. savignyi* identical with residues in the other species are boxed.

Peptides	Peptide sequence											
O. savignyi Fraction 50-B	G	Q	G	X	P	L	N	Q	G	A	X	T	F
O. moubata defensin A	G	Y	G	C	P	F	N	Q	Y	Q	C	H	S
O. moubata defensin B	G	Y	G	C	P	F	N	Q	Y	Q	C	H	S
D. variabilis defensin	G	F	G	C	P	L	N	Q	G	A	C	H	N

The amount of the isolated peptide in Fraction 50-B was determined as 11.03 µg by using the Micro BCA protein assay kit. Therefore, the average amount of the peptide in a tick following bacterial invasion is at least 0.37 µg.

The dose response curve for the isolated peptide follows a logistic sigmoid pattern. The curve fitting of the data was performed using the FigP v. 2.98 graphical program (FigP Software Corporation, Durham, NC, USA). Serial dilutions of the isolated peptide were performed and the antibacterial activity of the dilutions determined with *B. subtilis*. The positions (Fig 3.13) of the minimal inhibitory concentration (MIC) and the concentration responsible for 50% inhibition of bacterial growth (IC_{50}) are indicated on the graph. The value for MIC was determined to be 0.02 µM and the value for IC_{50} was determined to be 0.90 µM (Du Toit and Rautenbach, 2000).

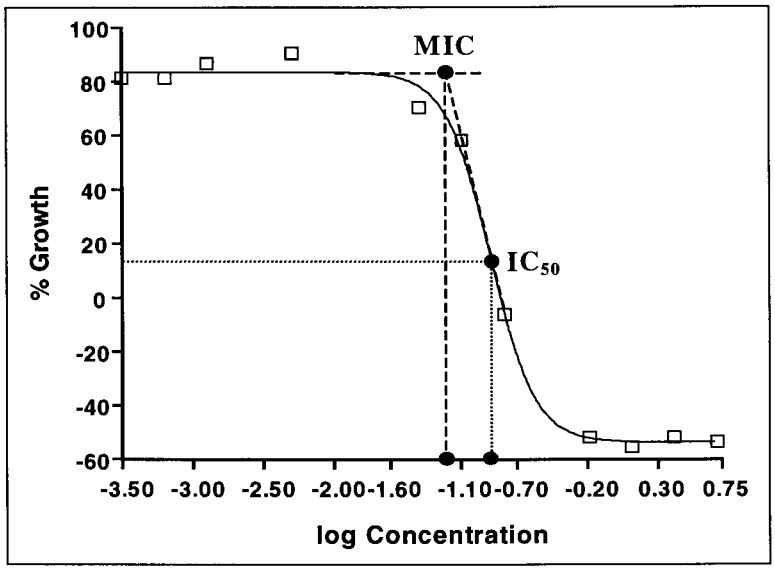

Figure 3.13 The dose response curve for the isolated peptide from Fraction 50-B. Serial dilutions of the peptide was assayed for antibacterial activity with *B. subtilis*. The FigP program was used to fit the data to a logistic sigmoidal model with a curve fitting correlation r^2=0.996. The value of the MIC and IC_{50} can be determined from the graph at the indicated positions (Du Toit and Rautenbach, 2000). The % growth after 3 hours is determined by subtracting the A_{600} value for the peptide dilutions from the A_{600} value of the bacterial growth control. A negative growth indicates bactericidal activity, resulting in the decrease in the initial number of bacteria present.

3.4.3.2 Isolation of antibacterial factors from Fraction 90

The fraction that eluted from 70% to 90% acetonitrile from the bonded silica C_{18} column (Fraction 90) were analyzed on a C_5 RP-HPLC-column using the Waters HPLC system (Fig 3.13). Two fractions displaying antibacterial activity were obtained and the fractions were resolved from the majority of the contaminating proteins.

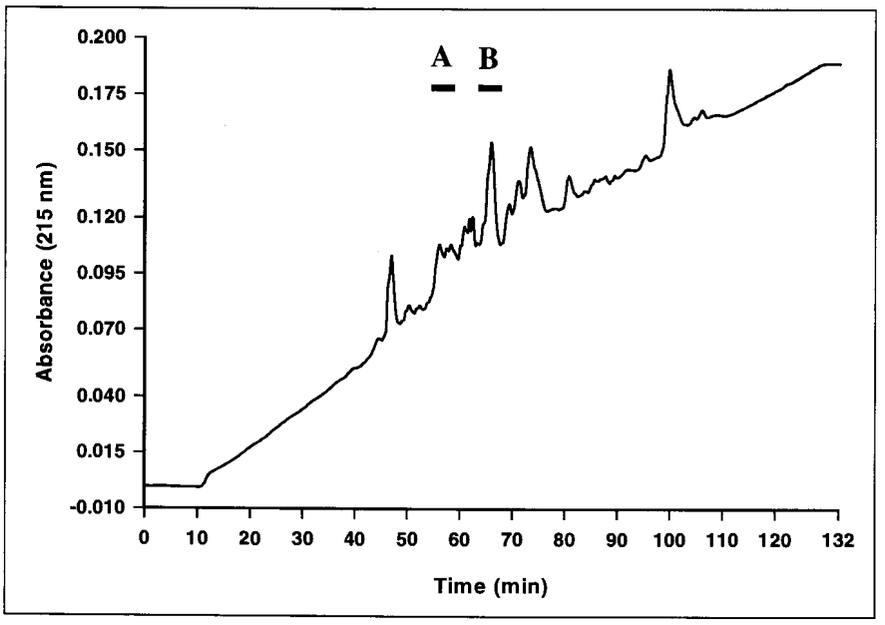

Figure 3.14 RP-HPLC chromatography of Fraction 90. The vacuum dried fractions were dissolved in 600 µl 0.1% acetonitrile, 0.1% TFA and applied to the column. A gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 60% acetonitrile, 0.1% TFA in 120 min. Fractions displaying antibacterial against *B. subtilis* with the broth dilution assay activity are indicated by (▬).

3.4.3.2.1 Isolation of the antibacterial factor from Fraction 90-A

Fraction 90-A, collected from the RP-HPLC analysis on Fraction 90 (Fig 3.14), was dried under vacuum, dissolved in 0.1% acetonitrile, 0.1% TFA and re-applied on the C_5 RP-HPLC column. A gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 30% acetonitrile, 70% methanol in 400 min, peak fractions collected, dried under vacuum and assayed for antibacterial activity with *B. subtilis*. The region on the chromatogram displaying antibacterial activity (Fig 3.15) contains two absorbing fractions, but collection of multiple fractions confirmed the presence of antibacterial activity in the total region.

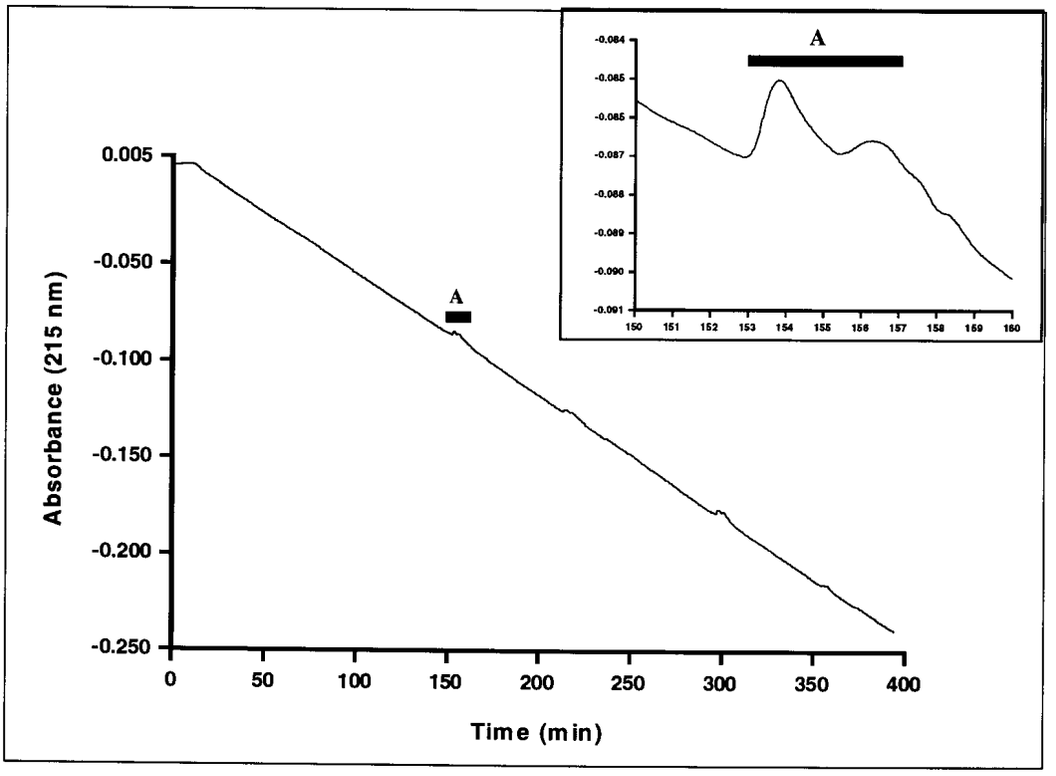

Figure 3.15 (A) C_5 RP-HPLC re-chromatography of Fraction 90-A (Fig 3.14). (B) Magnification of the region on the chromatogram displaying antibacterial activity. Dried fractions were dissolved in 0.1% acetonitrile, 0.1% TFA and a gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 30% acetonitrile, 70% methanol in 400 min. Fractions displaying antibacterial activity against *B. subtilis* with the broth dilution assay are indicated by (▬).

Fraction A of Fig 3.15 was analyzed using ESMS to investigate the composition and molecular mass of the components. The analysis (Fig 3.16) shows that the fraction is not very pure and still contains several contaminating components. It can also be seen that the concentration of the predominant component is low, although the molecular mass of the predominant fraction (4165.13 Da) is similar to the molecular mass of the predominant fraction in Fig 3.9 (4166.00 Da). The difference between the molecular masses is 0.87 Da, and from this negligible difference it is hypothesized that the predominant component in Fig 3.15 is identical to that of the component in Fig 3.8. This

peptide could thus tentatively be named *O. savignyi* defensin A, pending future sequence analysis for definite classification.

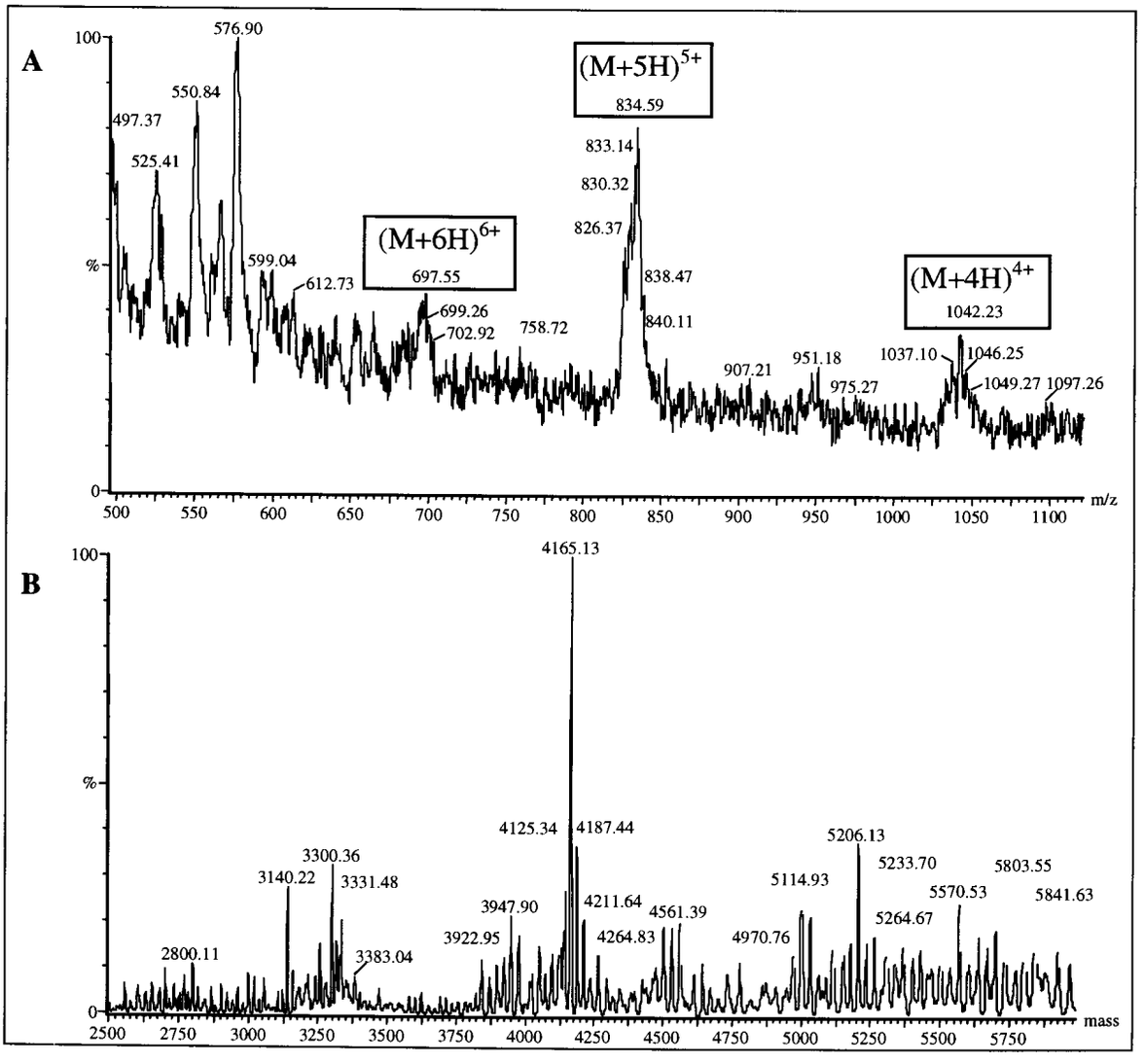

Figure 3.16 (A) Mass spectrum of the isolated peptide from Fraction 90-A following ESMS analysis. (B) The molecular mass of *O. savignyi* defensin A (4165.13 Da) calculated by the MaxEnt program. The predominant peaks in **A**, from right to left, represent the three molecular ions $(M+4H)^{4+}$, $(M+5H)^{5+}$ and $(M+6H)^{6+}$ formed during the analysis. The mass/charge (m/z) ratios are indicated above each peak. The calculated molecular masses of the components are indicated above the peaks in **B**.

3.4.3.2.1 Isolation of the antibacterial factor from Fraction 90-B

Fraction 90-B, collected from the RP-HPLC analysis on Fraction 90 (Fig 3.14), was dried under vacuum, dissolved in 0.1% acetonitrile, 0.1% TFA and re-applied on the C_5 RP-HPLC column. A gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 30% acetonitrile, 70% methanol in 400 min, peak fractions collected, dried under vacuum and assayed for antibacterial activity with *B. subtilis*. The region on the chromatogram displaying antibacterial activity (Fig 3.17) contains two absorbing fractions, but collection of multiple fractions confirmed the presence of activity in the total region.

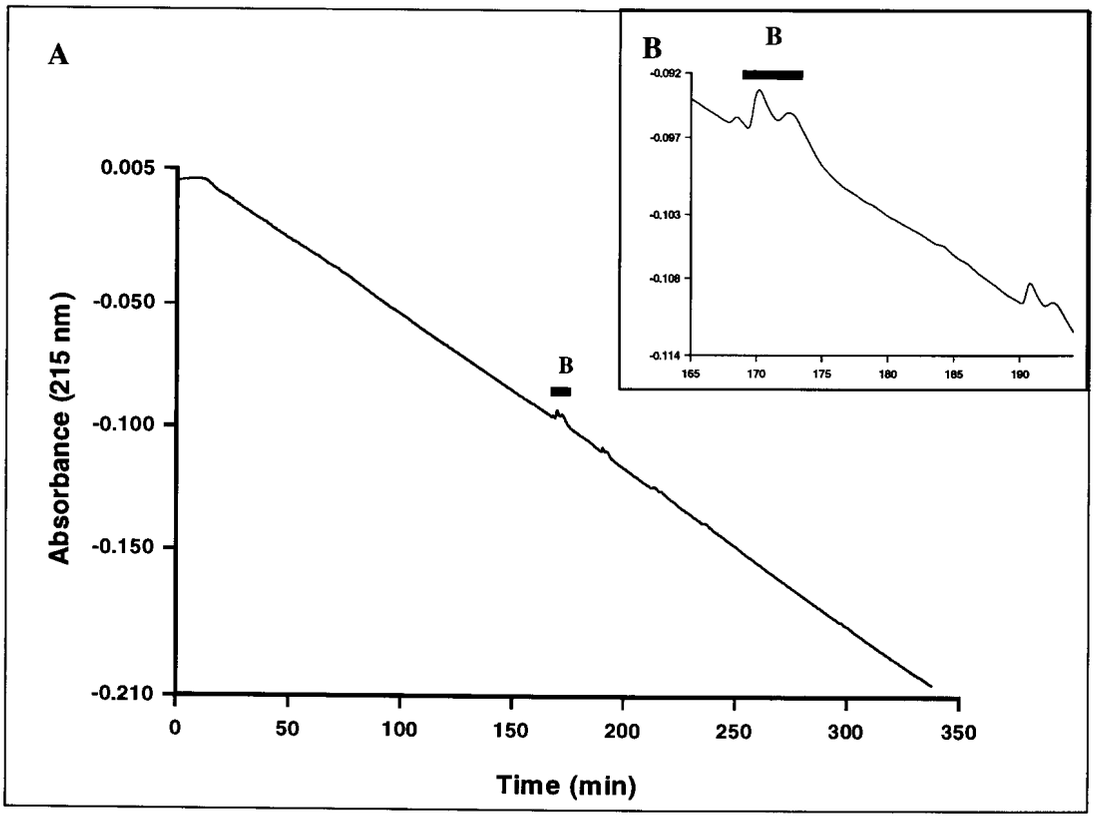

Figure 3.17 (A) C_5 RP-HPLC re-chromatography of Fraction 90-B (Fig 3.13). (B) Magnification of the region on the chromatogram displaying antibacterial activity. Dried fractions were dissolved in 0.1% acetonitrile, 0.1% TFA and a gradient run was performed from 0.1% acetonitrile, 0.1% TFA to 30% acetonitrile, 70% methanol in 400 min. Fractions displaying antibacterial activity against *B. subtilis* with the broth dilution assay are indicated by (▬).

Fraction B of Fig 3.17 was collected in two separate fractions and the fractions were separately analyzed using ESMS to investigate the composition and molecular mass of the components. The analyses of the samples show that the molecular mass of the predominant component in both regions are similar (4197.69 vs 4198.25 Da), thus only the mass spectrum of the second fraction is included (Fig 3.18). The molecular mass of the predominant component (4198.25 Da) is similar to the molecular mass of the predominant component in Fig 3.11 (4203.00 Da). The difference between the molecular masses of the components is 4.75 Da, and the predominant component in this sample is hypothesized to be similar to the component in Fig 3.11. This peptide could thus tentatively be named *O. savignyi* defensin B, pending future sequence analysis for definite classification.

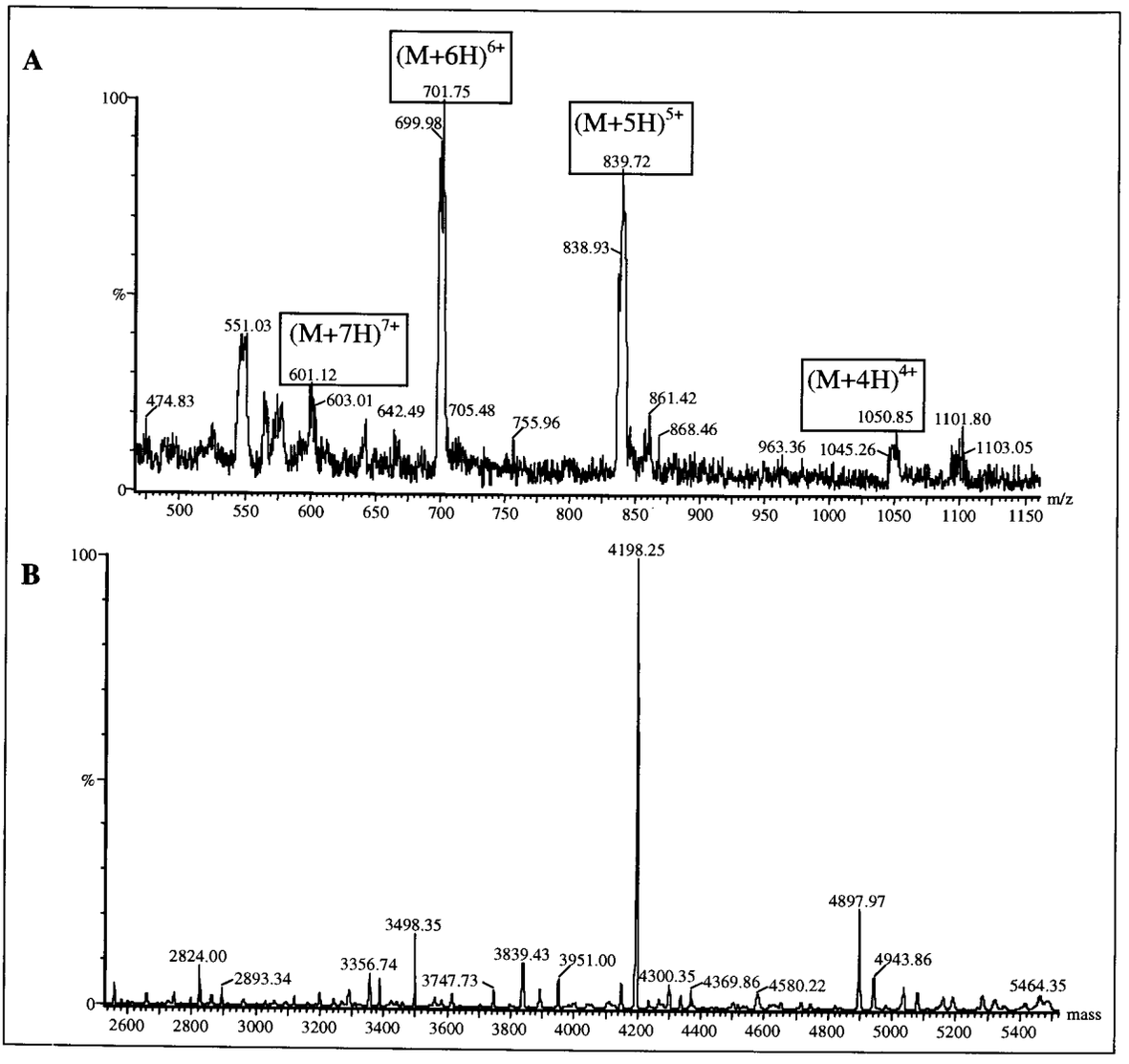

Figure 3.18 (A) Mass spectrum of the isolated peptide from Fraction 90-B following ESMS analysis. (B) The molecular mass of *O. savignyi* defensin B (4198.25 Da) was calculated by the MaxEnt program. The predominant peaks in **A**, from right to left, represent the four molecular ions $(M+4H)^{4+}$, $(M+5H)^{5+}$ and $(M+6H)^{6+}$, and $(M+7H)^{7+}$ formed during the analysis. The mass/charge (m/z) ratios are indicated above each peak. The calculated molecular masses of the components are indicated above the peaks in **B**.

3.4 Discussion

The isolation procedure using SE-HPLC did not result in a significant separation of the antibacterial factors from the contaminating proteins (Fig 3.1.A). The antibacterial factors

did not elute in a narrow range from the SE-HPLC column, and could be an indication of secondary interactions with the matrix of the column, i.e. interaction of positively charged proteins with the unblocked acidic surface silanols of the column matrix. This is supported by the previous finding that elution buffers which have ion concentrations of less than 50 mM cease to allow separation due to size difference alone. The secondary interactions with the column increase with low buffer strengths, and this leads to irreproducible retention times and also to an extended range of elution from the column (Engelhart, 1991). In this study a low ion concentration in the running buffer (20 mM ammonium acetate, pH 4.7) was used due to the volatile nature of the ammonium acetate. Following a freeze-dying step, the low ammonium acetate quantity in the buffer is eliminated without removing or damaging the peptides, and this allows for a more accurate determination of the antibacterial activity in the respective samples. An excess of ammonium acetate interferes with the bacterial growth in the assay and gives rise to false positive results. A higher concentration of the ammonium acetate in the elution buffer would decrease the efficiency of the salt removal during freeze-drying. This would necessitate another freeze-drying step, increasing the risk of damage to the peptides in the sample and giving rise to false negative results during the antibacterial assay.

The subsequent RP-HPLC re-chromatography of the antibacterial fractions from the SE-HPLC (Fig 3.1.B) shows two regions that display antibacterial activity. From the chromatogram it can be seen that the regions are well resolved from the majority of the contaminating proteins. The one region of antibacterial activity is shown to be in a region where no absorbing fractions are apparent. This is a possible indication of the low sensitivity of the Beckman HPLC detector. Unfortunately, a more focussed examination of these antibacterial fractions could not be pursued, since it was found that high molecular mass proteins were adsorbing to the matrix of the SE-HPLC column and this was negatively influencing the separation efficiency of the column. Thus, a gradual decrease in the antibacterial activity of the fractions eluting from the RP-HPLC during the re-chromatography step could be discerned. As discussed previously, a possible reason for this decreased separation efficiency could be the low ion concentration of the

elution buffer that enhances adsorption of proteins to the SE-HPLC matrix. The column could not be reconditioned using the manufacturer's specifications and was discarded.

Studying the tricine SDS-PAGE gel in Fig 3.2 (lane S), it was apparent that the high molecular mass proteins present in large amounts in the hemolymph had to be removed during the first isolation step to decrease the high excess of these proteins in the subsequent isolation procedures. The use of ammonium sulfate to precipitate these proteins was successful (Fig 3.2), as the majority of the high molecular weight proteins precipitated. The desalting of the supernatant obtained from the centrifugation step was necessary, since the presence of the high concentration ammonium sulfate could have a detrimental effect on the antibacterial assay.

The results of the chromatography shown in Fig 3.3 indicates one fraction in the re-chromatography analysis, although it is obvious from the chromatogram in Fig 3.3.B that the fraction is not composed of only one component. The fraction was collected as four fractions, and fractions one and four, the start and end of the absorbing fraction, showed antibacterial activity. It was assumed that the whole absorbing fraction displayed antibacterial activity. The second fraction was analyzed using ESMS to determine the molecular mass of the components and to determine the purity of the components. The two most predominant components in the fraction had molecular masses of 6568.88 and 7585.89 Da respectively (data not shown). These molecular masses were not similar to that of other antibacterial factors isolated from other invertebrates to date. The closest match was that of the big defensin isolated from the horseshoe crab (8 kDa, Section 1.1.1.2.3), although the activity spectrum of the big defensin and the *O. savignyi* antibacterial factors are different. Results (not shown) in the mass spectrometric analysis indicate that the most predominant peaks in the sample were in the size range of 5 - 7 kDa. Thus, the molecular mass of 4 kDa obtained for the defensin peptides are outside the range covered by the analysis. The results of this analysis are therefore inconclusive.

In hindsight the chromatograms in Fig 3.3 are interesting, as only one region of antibacterial activity can be discerned on each chromatogram. Several hypotheses can be

proposed to explain this result. Firstly, the other antibacterial factors present in the crude hemolymph could have been precipitated during the ammonium sulfate precipitation step. Thus, only one factor remained in the supernatant after centrifugation. Secondly, the antibacterial factors formed complexes with each other during the ammonium sulfate precipitation step, causing only one fraction with antibacterial activity. This might lead to questions about the ability of antibacterial factors to retain their activity while involved in complexes. Thirdly, the conditions of the separation were not ideal to result in the resolution of the antibacterial factors. Fourthly, a combination of these hypotheses might be possible. The results obtained in the chromatograms in Fig 3.4 disprove the first two hypotheses. The fact that the conditions are still the same for the initial precipitation step, but that the presence of four distinct areas of antibacterial activity can were observed is an indication that the conditions of the chromatograms in Fig 3.4 are most likely more optimal to resolve the antibacterial factors.

The acid precipitation step used in the isolation of the antibacterial peptides is very effective at precipitating the high molecular mass proteins in the hemolymph. From Figure 3.6 it can be seen that the high molecular proteins in the crude hemolymph (lane C) have precipitated (lane 10). The antibacterial proteins eluted from the C_{18} sep-pak column in two regions, at 30%, 40% and 50% acetonitrile (combined subsequently to form Fraction 50), and at 80% and 90% acetonitrile (combined subsequently to form Fraction 90). The majority of the proteins retained on the column were in the 2 – 14 kDa size range (Fig 3.6). The acid-precipitates were not tested for antibacterial activity, since the relative strength of the antibacterial activity in the supernatant of the precipitation was similar to the strength of the antibacterial activity of the untreated sample.

The chromatography of Fraction 50 (Fig 3.7) shows that there were still many contaminating proteins in the sample, confirmed by the tricine gel of Figure 3.6 (lanes 30, 40 and 50). The antibacterial components in Fraction 50 eluted from the column in two distinct regions, and were resolved from the majority of the contaminating proteins. The re-chromatography of these two regions showed the presence of a few contaminating proteins (Fig 3.8 and 3.10). To change the elution conditions for the re-chromatography it

was decided to use methanol as the hydrophobic eluting solvent. The methanol is more hydrophilic and it was thought that the elution of the components from the column would be more gradual and this would result in a better separation. The presence of TFA in the initial buffer, but absence in the methanol, increased the separation in this experiment since the pH of the eluting buffer increased with an increase in methanol concentration.

The re-chromatography of the antibacterial component in Fraction 50-A (Fig 3.8) displayed a very low absorbance at 215 nm, suggesting a low concentration. Although the easiest and quickest method of verifying the composition and molecular mass of a sample is electrophoresis, the low concentration of the components suggested the use of ESMS, since this technique would yield results with a small concentration of sample. The analysis showed that the antibacterial component is not pure (Fig 3.9), but the molecular mass of the predominant peak in the sample was determined as 4166.00 Da. This molecular mass is in the correct region for the class of insect defensins, a hypothesis strengthened by the antibacterial activity profile of the component, since defensins are active against Gram-positive bacteria. The unambiguous identity of the component could be confirmed by determining the amino acid sequence of the peptide and a subsequent comparison with known sequences in a protein data bank. A shortage of ticks prevented these experiments from being carried out.

The re-chromatography of the antibacterial component in Fraction 50-B (Fig 3.10) also displayed a low absorbance at 215 nm, but the antibacterial activity of this fraction was very potent. Interestingly, antibacterial components eluted in multiple fractions, and this suggested contamination. The fractions were collected separately and assayed for antibacterial activity. Surprisingly, all the fractions showed antibacterial activity. The fractions were analyzed separately using ESMS, and the results show that the molecular mass of the predominant component in all peaks is identical (4203 Da, Fig 3.11). This suggested that the component eluted from the column in different fractions, possibly due to differences in the structure of the peptide. The molecular mass of the isolated peptide is also in the correct range to qualify as a member of the defensin family. Since the defensins have three intra-molecular disulfide bonds, and that disulfide bonds allowed for

the differential unfolding of a peptide, the hypothesis would be acceptable. The amino acid sequence of the peptide was determined using Edman-degradation (Table 3.1), and the resultant sequence aligns well with the known sequences of defensins from other tick species (Table 3.2). The presence of cysteine-residues in the peptide was not determined, as these residues were not permanently reduced prior to the analysis to break any disulfide bonds. It was thus expected that the presence of cysteine-residues would give rise to an undefined amino acid, and the sequence alignment of this sequence in Table 3.2 showed that these undefined residues aligned very well with the cysteine-residues in the other defensin sequences. Searching the Swissprot protein database (http://www.epasy.ch) with the sequence did not indicate a similarity of with any known sequence, since more amino acid residues in the peptide sequence are required for a definite match. The amino acid sequencing reaction could not be repeated due to a shortage of ticks and to the lack of available local sequencing facilities.

The chromatogram with Fraction 90 (Fig 3.13) shows that many of the contaminating proteins were removed, and this was confirmed by the tricine gel of Figure 3.6 (lanes 80 and 90). The antibacterial components in Fraction 90 eluted from the column in two distinct regions, and were resolved from the majority of the contaminating proteins. The re-chromatography of these two regions showed the presence of few contaminating proteins (Fig 3.14 and 3.16). The elution conditions for these components were different from the previous components, since methanol as elution buffer failed to elute the components from the column. To increase the hydrophobicity of the elution solvent, a mixture of 70% methanol, 30% acetonitrile was used. The presence of TFA in the initial solvent, but absence in the methanol/acetonitrile solvent, increased the separation in this experiment since the pH of the eluting buffer increased with an increase in methanol/acetonitrile concentration. To further increase separation efficiency, an extended gradient was used for both components.

The components in Fraction 90 were both isolated using the indicated elution conditions, and due the low absorbance at 215 nm of the components, it was again decided that ESMS would lead to the most efficient molecular mass determination. However, the

ESMS analyses of the antibacterial fractions displayed in Figures 3.14 and 3.16 were not performed to the optimum level of accuracy (Fig 3.15 and Fig 3.17). During vacuum drying steps two contaminants possibly leeched from the plastic of the tubes used (Dr. M. J. van der Merwe, personal communication). The concentrations of these contaminants were in great excess compared to the protein components of the sample and only one scan of the protein molecular ions could be completed. The average of multiple scans of the molecular ions would have given a more accurate representation of the actual molecular mass of the components of the sample. The single scan of the components of the two samples would thus lead to a less accurate molecular mass value. It is therefore hypothesized that there are only two, and not four, defensin isomers in the hemolymph of *O. savignyi*. The rationale behind the hypothesis is the possible inaccurate molecular mass determined by the single scan of the mass spectrometric analysis. Thus, the difference in molecular mass between the peptides in Fractions 50-A and 90-A of 0.87 Da could be negligible, and the two peptides could be identical. Similarly, the difference in molecular mass between the peptides in Fractions 50-B and 90-B of 4.75 Da could also be negligible. The differential local unfolding of the peptides could lead to the difference in chromatographic behavior.

Chapter 4

Molecular biological investigation of lysozyme in the soft tick *Ornithodoros savignyi*

4.1 Introduction

As lysozyme has been isolated from several tick species (Kopácek *et al.*, 1999; Ignatovich *et al.*, 1979; Podboronov *et al.*, 1978) it was hypothesized that it is also present in *O. savignyi*. Since lysozyme was not identified during the isolation of the antibacterial peptides from the hemolymph of *O. savignyi* (Chapter 3), it was decided to use molecular biological methods to screen for the presence of lysozyme and lysozyme mRNA-transcripts. This study was performed in parallel to the isolation of the defensin peptides.

The effectivity of mRNA characterization has increased following the development of methods aimed at the study of mRNA. These include the isolation of RNAs, cloning and bacterial amplification of specific mRNAs, Northern blot studies, *in situ* hybridization and nuclease protection assays. The development of the polymerase chain reaction (PCR) in conjunction with reverse transcription (RT-PCR) allows the study of mRNA almost at the level of single cells (Edwards, Ravassard, 1995). The salient features of this technique are described below.

4.1.1 The polymerase chain reaction

The polymerase chain reaction was first developed by Kary Mullis (Saiki *et al.*, 1985), leading to a Nobel Prize in Chemistry in1993. This technique has developed into a powerful technique in molecular biology, offering an easy way to copy large quantities of a specific DNA sequence (Cohen, 1994). The reaction involves the denaturing of a double stranded DNA template followed by the annealing of two specific single stranded oligonucleotides (primer) to the sequence of interest. A thermostable DNA polymerase is used to synthesize the complementary stranded bound by the primer, and the process is repeated for up to 35 cycles to amplify the target sequence. The result of the PCR is that after n cycles, the reaction contains 2^n copies of the target DNA flanked by the primers.

Several applications for the technique include mutation analysis, monitoring cancer development and cancer therapy, the detection of microbial infections, the gender determination of prenatal cells and the study of molecular evolution (Watson, 1992).

4.1.1.1 Components of a PCR

The general components of the polymerase chain reaction are constant, with only changes in the quantities and concentrations of the components. The components of a PCR are a double stranded DNA template, two single stranded oligonucleotide primers specific for the target sequence of the template, $MgCl_2$, a reaction buffer and a thermostable DNA polymerase enzyme.

4.1.1.1.1 Template

The template is the double stranded DNA that contains the target sequence of interest. Either genomic DNA or cDNA could be utilized, depending on the aim of the experiment and on the availability of the sequence of the desired product. Thus, to determine the transcript sequence of the product, cDNA is used, while genomic DNA is used to obtain the full-length sequence of the gene of interest.

4.1.1.1.2 Primers

The primers are the single stranded oligonucleotides that are used to direct the amplification of a specific sequence on the template DNA. The primers have specific sequences to allow annealing to the specific sites on the target region, and this double stranded region would provide the 3'-OH which the DNA polymerase enzyme requires to extend the strand. Since the desired target of the primers is usually in a great minority, during reactions the primer/template molar ratio is adjusted to ~10^7 to increase the efficiency of the primer binding to the template (Kidd and Ruano, 1995). The design of the primers is discussed in Section 4.1.1.2.

4.1.1.1.3 $MgCl_2$

The $MgCl_2$ concentration is a key variable of the reaction. It stabilizes the double stranded DNA, as well as the annealing of primers to the template. Nucleotides chelate the $MgCl_2$, forming the substrate for the polymerase enzyme, while the polymerase requires $MgCl_2$ as cofactor. High concentrations of $MgCl_2$ stabilize the double stranded DNA to such an extent that the strands do not separate during the

denaturation step. The stabilization of primer annealing to non-specific targets would also be effected. Thus, the optimization of the $MgCl_2$ concentration is of great importance for a successful reaction (Kidd and Ruano, 1995).

4.1.1.1.4 Deoxynucleotides (dNTP)

The four dNTPs must be present in sufficient excess relative to the template DNA (10^{10} to 10^{11} excess) to ensure that their concentration is essentially constant. This would ensure that the reaction does not decrease in efficiency due to the inaccessibility of the reactants. The equal concentration of all four the nucleotides is also essential, since an imbalance in the ratio could promote misincorporation of bases, leading to the amplification of non-specific products (Kidd and Ruano, 1995).

4.1.1.1.5 Reaction buffer

The ionic environment of the reaction is critical for a successful reaction. This is provided by the reaction buffer, usually containing KCl and Tris-HCl, although NaCl-based and ammonium sulfate buffers are also used for specific applications. The pH of the buffer is usually maintained at 8.3, but an increase in temperature leads to a decrease in pH, necessitating the optimization of the buffer to ensure an optimal ionic environment.

4.1.1.1.6 DNA polymerase enzyme

The DNA polymerase from *Thermus aquaticus* (*Taq*) is stable at the high temperature necessary for the denaturation of the double stranded template, negating the need to add fresh enzyme for each amplification cycle. At the optimal temperature of 72°C, the enzyme can add 60 bases per second, and this should be taken into account during primer extension to allow the enzyme to complete the polymerization reaction. When performing a hot-start PCR, the enzyme is usually the last reagent to be added. A hot-start PCR involves the addition of the final reagent at a high temperature (80°C) to prevent the priming and extension of the wrong sequences during the low stringency conditions prior to the first denaturation step (Kidd and Ruano, 1995). The *Taq* enzyme is known to misincorporate non-complementary bases during the polymerization reaction, prompting the discovery of higher fidelity related thermostable DNA polymerases from other organisms (Blair and Zajdel, 1992).

4.1.1.2 Primer design

The design of primers specific for the desired product is a crucial factor for a successful amplification reaction. When the sequence of the product is known and the object of the study is to sequence or amplify the gene of interest, the design of the primers is less complicated. The sequences at the 5' and 3' ends of the product can be used to design a very specific primer-pair that would selectively and exclusively anneal to the desired product. When the sequence of the product is unknown, and the object of the study is primarily to determine the presence of the product and the sequencing of the transcript in question, the design of the primer-pair is more complex. A multiple alignment of a large number of amino acid sequences of related products in other organisms is essential, since this could highlight areas of significant homology. The sequences of these areas can then be used to outline a basic sequence to be used as the forward primer. When the template for the reaction is cDNA, this primer is normally the only one in the primer pair to be specific for the product in question. The reverse primer is usually complementary to the anchor primer sequence used in the synthesis of cDNA from mRNA.

The most important parameter to be considered during primer design is that the primer must form a stable duplex with the specific site on the target DNA without forming duplexes with another primer molecule or hybridization at another target site. Other factors affecting the success of primers includes the dimer formation of primer-pairs, self-complementarity, melting temperature stability, internal stability, and degeneracy of primers (Rychlik, 1993).

4.1.1.2.1 Dimer formation of primer-pairs

Primer-pairs should be chosen without significant complementarity at their 3' ends, as this promotes the formation of primer-dimer artifacts. The formation of these artifacts reduces product yield, but can also lead to more serious problems in the reaction, since it could lead to an unbalanced primer ratio. This could ultimately lead to nonspecific DNA synthesis. The higher the stability of a 3' duplex, the lower the yield of the amplification reaction becomes. Even a high 3' stability is not a pre-requisite, since the *Taq* primer can recognize a transient 3' duplex in very little time, and will thus quickly amplify the primer-duplex (Rychlik, 1993).

4.1.1.2.2 Self-complementarity

Primers forming intramolecular duplexes with negative ΔG values are not very efficient during the amplification reaction. The primers can tolerate some intramolecular association, but the ΔG values must be below a threshold limit, and the position of the complementarity is also very important. A hairpin loop near the 5' end can easily be accommodated, but a 3' hairpin loop will lead to internal primer extension and the elimination of the primer from the reaction (Rychlik, 1993).

4.1.1.2.3 Melting temperature stability

The designed primers do not need to have ~50% GC/AT ratios as usually assumed. The primers do, however, need to contain GC/AT ratios similar to or higher than the target DNA (Rychlik, 1993). The efficiency of the amplification reaction will be further increased if the T_m values of the two primers are roughly equal (Kidd and Ruano, 1995).

4.1.1.2.4 Internal stability

The most effective primers contain a very stable (higher ΔG) 5' terminus and an unstable (lower ΔG) 3' terminus. The unstable 3' end would result in the inability of the 3' end to bind with significant stability to non-target sites on the template. This would prevent the elongation of products from these false primed positions, and only the stable binding of the rest of the primer to the specific target site would initiate the elongation of the target sequence (Rychlik, 1993). Primers with thymidine as the 3' nucleotide are not very effective, since this nucleotide can nonspecifically prime with any other nucleotide. The positioning of an adenosine or thymidine in the 3' triplet is also advantageous, since this will prevent the mismatch tolerance of primers with consecutive guanines or cytosines (Kidd and Ruano, 1995). G or C is preferred on the 3' end due to the stronger H-bond association, compared to the association between A and T (Preston, 1993).

4.1.1.2.5 Degeneracy of primers

Since the genetic code is degenerate, a primer that is designed to be specific for a certain amino acid sequence needs to be degenerate in order to recognize all the possible permutations of the target nucleotide sequence. Thus, a primer with a

degeneracy of 32 can potentially recognize 32 different nucleotide sequences. This means that only 1/32 of the degenerate primer will recognize the desired target sequence, and the low abundance of this sequence would facilitate the exponential amplification of the sequence. The disadvantage is that there is a possibility of the primer recognizing one of the other 31 sequences, leading to the amplification of an undesired product. One way of reducing the complexity of a primer is to substitute an inosine in positions where any nucleotide will be incorporated. The inosine would base-pair equally well with all the nucleotides, although the disadvantage of the inosine is to lower the annealing temperature of the primer. Another possibility is the substitution of a thymidine for those regions where any nucleotide will be incorporated. The thymidine nonspecifically primes on any nucleotide without lowering the annealing temperature of the primer. Thus, the lower the degeneracy of the primer, the higher the probability of amplification of the target DNA sequence (Preston, 1993).

4.1.1.3 Thermal cycling parameters

The thermal cycling profile for most reactions will follow the same pattern. The first step is incubation at 94°C to facilitate the denaturation of the DNA double strand. The length of this incubation needs to be sufficient to ensure the complete separation of the DNA strands. If the time of incubation is too short, the strands would re-anneal rapidly, preventing efficient primer annealing and extension (Boehringer Mannheim). The second step is a decrease in temperature to the desired primer annealing temperature. An optimum annealing temperature and annealing time would ensure that the primers would anneal to the correct target sequences. If the annealing temperature is too low, non-specific annealing might be initiated, while a too high temperature would negate the binding of the primers to the template (Boehringer Mannheim). The annealing time could also be critical during the initial steps, since the primers need to bind to the correct sites on genomic DNA or in a cDNA library, and those sites are likely to be in minority. During the later stages of the reaction, the desired template would be in excess and the time needed for the primers to anneal to the correct sequence would be minimal (Kidd and Ruano, 1995). A "touchdown" protocol is often employed to increase the amplification of the desired template. This protocol involves a high primer annealing temperature for the first few cycles, leading to the higher stringency in amplification of the desired product. The temperature is

sequentially lowered during subsequent cycles, since the desired template is in excess, and the high stringency is not required (Kidd and Ruano, 1995).

A third step involves the increase of the temperature to ~72°C to allow the *Taq* polymerase to extend the primed templates. The enzyme can add 60 bases per second at this temperature, and the time for the primer extension must be sufficient to allow the completion of chain elongation (Boehringer Mannheim).

Fourth, the temperature is increased to 94°C to denature the double stranded products. The annealing, elongation and denaturation steps are repeated to facilitate the amplification of the products. The number of these cycles usually employed varies between 25 and 35. Since the desired product extension could enter the plateau phase, an increase in cycle number would lead to an increase in nonspecific products (Boehringer Mannheim).

The final step is 72°C incubation for 5-15 min to complete the extension of all partially extended products. The annealing of single stranded complementary products will also occur during this incubation period (Boehringer Mannheim).

4.1.2 Aim of this study

The aim of this study is the molecular biological characterization of lysozyme in the soft tick, *Ornithodoros savignyi*. Objectives to reach this aim were:

- The design of a lysozyme-specific degenerate primer from the multiple sequence alignment obtained from the Genbank database
- The optimization of the polymerase chain reaction
- The cloning of the PCR products into a cloning vector
- The sequencing of the cloned PCR products
- The searching of internet databases for sequences homologous to the obtained sequences

4.2 Materials

Formamide was obtained from GIBCO-BRL, USA, while diethyl pyrocarbonate (DEPC), formaldehyde and morpholinopropanesulfonic acid (MOPS) was obtained from Sigma Chemical Co, Germany. FORMAzol™(Molecular Research Centre Inc., Cincinnati, USA) is purified and stabilized formamide that protects RNA from degradation by RNases. For the PCR experiments TaKaRa *Taq*™ polymerase, $MgCl_2$-

free PCR buffer, dNTP mixture and $MgCl_2$ were obtained from Gibco BRL, Gaithersburg, USA. Tri-reagent was obtained from Molecular Research Centre Inc., Cincinnati, USA, while DEPC was from Sigma Aldrich, Germany. The DD-poly-T and the 3'-anchor primers were from Genosys, Cambridge, England, while the degenerate LYZ1 primer was from Roche Molecular Biochemicals. Double distilled, deionized (millipore) water was used for all buffers.

4.3 Methods

4.3.1 Total RNA isolation

TRI-reagent (Molecular Research Centre Inc., Cincinnati, OH) composed of a mixture of phenol and guanidinium thiocyanate in a mono-phase solution, was used for the single step isolation of total RNA. All solutions were prepared using DEPC-treated water (1 ml DEPC per liter millipore water, incubated at 37°C for 3 hours and autoclaved twice).

Ticks were ventrally immobilized in wax, the dorsal cuticle was removed, all the internal organs were removed and placed in eppendorf tubes. The organs were snap frozen in liquid nitrogen and stored at -70°C until use.

Organs were lysed by the addition of 1.5 ml of TRI-reagent, followed by extensive manual homogenization with an eppendorf teflon pestle. The homogenate was left at room temperature for 15 minutes, followed by centrifugation at 10 000 *g* for 15 minutes to remove cell debris. After addition of 0.2 ml chloroform per 1 ml TRI-reagent used, the mixture was vortexed for 15 seconds, left at room temperature for 15 minutes and centrifuged at 10 000 *g* for 15 minutes. RNA remains in the upper aqueous phase, DNA in the interphase and proteins in the organic phase. RNA was further isolated by transferring of the upper phase to a clean eppendorf tube followed by the addition of 0.5 ml 2-propanol per 1 ml TRI-reagent used. The sample was mixed slowly, left at room temperature for 10 minutes and centrifuged at 10 000 *g* for 15 minutes. The pellet was aspirated and placed on ice, washed with 1 ml 75% ethanol per 1 ml TRI-reagent used and centrifuged at 7 500 rpm for 5 minutes. After aspiration, the pellet was air dried and dissolved in 30 µl FORMAzol™. The concentration of the RNA was determined spectrophotometrically (see 4.3.3.1).

4.3.2 Denaturing agarose electrophoresis of RNA

RNA samples were prepared for electrophoresis by the addition of DEPC-water to a 3 µg RNA aliquot, up to a total volume of 7 µl. A 13% (v/v) 10X MOPS (0.4 M morpholinopropanesulfonic acid, 100 mM sodium acetate, 10 mM EDTA, pH 7.0), 20% (v/v) formaldehyde, 67% (v/v) deionized formamide solution was prepared and 15 µl added to the RNA. After the addition of 0.2 µl of a 10 mg/ml stock solution of ethidium bromide, the samples were heated to 55°C for 10 minutes and subsequently snap-cooled on ice. Before loading on the gel, 4 µl tracking dye (15% (w/v) Ficoll and 0.025% (w/v) Bromophenol Blue) was added to each sample. The samples were analyzed on a 1% (w/v) denaturing agarose gel containing 10% (v/v) 10X MOPS, 5.5% (v/v) formaldehyde and DEPC-water. Electrophoresis was performed on a Minicell® EC 370M Electrophoretic Gel System (E-C Apparatus Corporation, St Petersburg, FL, USA) at 79V and RNA bands were visualized on a UV transilluminator (Spectroline® TC-312A, 312 nm).

4.3.3 Quantification of nucleic acids

4.3.3.1 Spectrophotometric quantitation

The concentration of nucleic acids was determined using the Shimadzu UV-160A spectrophotometer. Absorbance values were obtained at 260 nm and 280 nm. The conversion values are as follows:

Double stranded DNA: 1 A_{260} is equivalent to 50 µg/ml.

Single stranded DNA (primers): 1 A_{260} is equivalent to 33 µg/ml.

RNA: 1 A_{260} is equivalent to 40 µg/ml.

The ratios obtained from A_{260}/A_{280} are an indication of the purity of the isolated nucleic acids. The A_{260}/A_{280} ratio of 1.7-1.9 for double stranded DNA and the A_{260}/A_{280} ratio of 1.8-2.0 for RNA indicate pure products with regard to protein contamination.

4.3.3.2 Quantitation of DNA by electrophoresis

A portion of the isolated DNA samples and PCR products was analyzed on an agarose (Promega, Wisconsin, USA)/TAE gel in 1x TAE buffer. In one lane 6 µl of a 100 bp ladder standard was loaded and the gel was visualized by staining with ethidium

bromide. The 500 bp DNA band in the standard is equivalent to 150 ng DNA, and this was used as reference for visual estimation of the concentration of the various samples.

4.3.4 Complementary DNA (cDNA) synthesis from total RNA

For the synthesis of cDNA a DD-poly-T primer with the following sequence was used:

GCT ATC ATT ACC ACA ACA CTC $(T)_{18}$ VN

where V= A/G/C and N= A/T/G/C.

Synthesis of cDNA was performed using the Superscript II RT enzyme (Gibco BRL, Gaithersburg, USA). A 1 µg aliquot of purified total RNA and 5 pmol of the DD-poly-T primer and DEPC-H_2O to a volume of 11 µl were incubated at 70°C for 3 min. After snap cooling on ice, 4 µl of 5X first strand buffer (Gibco BRL, Gaithersburg, USA), 10 mM of dNTPs and 2 µl 0.1 M DTT was added. The mixture was heated in a PTC 200 thermal cycler (MJ Research, Massachusetts, USA) at 42°C for two minutes (hot-start), after which 200 U (Gibco BRL, Gaithersburg, USA) of the Superscript II RT reverse transcriptase was added. The mixture was incubated at 42°C for 60 min, followed by a 10 min incubation at 94°C to inactivate the reverse transcriptase enzyme. The cDNA was stored in silanized tubes at -70°C.

The concentration of the synthesized cDNA can be approximated by the relationship determined by us: 1 µg/µl total RNA = 100 ng/µl cDNA (Birkholtz, 1999).

4.3.5 Degenerate primer design

The National Center for Biotechnology Information (NCBI) Swissprot database was searched for lysozyme amino acid sequences. A total of 39 amino acid sequences (27 vertebrate and 12 invertebrate) were obtained and were aligned using the CLUSTALW Program on the ExPASy (*Ex*pert *P*rotein *A*nalysis *Sy*stem) molecular biology server (http://www.expasy.ch). The conserved areas in the multiple alignment were identified and confirmed using the BLOCKS-maker program (Henikoff and Henikoff, 1994; Henikoff *et al.*, 1995) on the BLOCKS database at the Fred Hutchinson Cancer Research Center (http://www.blocks.fhcrc.com).

The OLIGO version 4.1 program (National Biosciences, Plymouth, USA) was used to evaluate the designed primer in terms of internal stability, self-complementarity, primer-dimer formation, primer-pair complementarity, primer T_m values and degeneracy. An inosine was added at a region of high redundancy, decreasing the degeneracy of the designed primer (LYZ1) to 32.

4.3.6 3'-Random amplification of cDNA ends (3'-RACE)

The 3'-RACE reaction was performed using LYZ1 as the forward primer (see p 108 for sequence) and a reverse (3' anchor) primer with the following sequence:

5' GCT ATC ATT ACC ACA ACA CTC 3'

The DNA polymerase from *Thermus aquaticus* (*Taq*) was used in the amplification reaction (1.25 units per reaction) and was supplied in a storage buffer (20 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% (v/v) Tween 20, 0.5% (v/v) Nonidet P-40, 50% (v/v) glycerol, pH 8.0). The other reactants obtained for use in the PCR reaction were: $MgCl_2$ (25 mM), dNTP mix (2.5 mM for each dNTP), and $MgCl_2$-free PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3). The PCR reaction was optimized for $MgCl_2$ concentration, primer concentration, cDNA equivalent concentration and primer annealing temperature (Cobb and Clarkson, 1994). Amplifications were performed on a Geneamp PCR system 9700 (PE Applied Biosystems, California, USA). A control reaction was included (absence of cDNA) in the optimization reactions to preclude cDNA-independent amplification (data not shown).

4.3.7 Electrophoresis of DNA

PCR products were electrophoresed using 2% (w/v) or 3% (w/v) agarose gels, while plasmids required 2% (w/v) agarose gels (Promega, Wisconsin, USA). Gels were prepared in 1X TAE (40 mM Tris-acetate, 1 mM EDTA) using a Minicell® EC 370M or Maxicell™ EC 360M Electrophoretic Gel Systems (E-C Apparatus Corporation, St Petersburg, Florida, USA). Electrophoresis was performed at 89 V (Minicell®) and 98 V (Maxicell™). Following electrophoresis, gels were stained in 10 µg/ml ethidium bromide, and bands were visualized on a transilluminator (312 nm). Images were captured with a digital camera linked to a computer system.

4.3.8 Silica purification of PCR-products from agarose gels

The PCR products were excised from the agarose gels following ethidium bromide staining, and 2-3 volumes (v/w) of 6M NaI added to each band. The agarose was incubated at 55°C for 5 min, 20 µl of silica (100 mg/ml) in 3M NaI added and slowly mixed for 20 min. The samples were incubated on ice for 15 min (with mixing every 2 min) followed by centrifugation at 13 000 *g* for 20 s. After aspiration, the pellets were washed twice with 500 µl buffer (10 mM Tris, 50 mM NaCl, 2,5 mM EDTA, 50% (v/v) ethanol, pH 7.5). To elute the DNA, 10 µl sterile water was added to the pellets, and after incubation for 5 min at 55°C the solution was centrifuged at 13 000 *g* for 30 sec. The concentration of the purified DNA was approximated using electrophoresis.

4.3.9 Cloning procedures

4.3.9.1 Ligation of PCR products

The DNA was ligated in the pGEM®-T Easy cloning vector (Promega, Wisconsin, USA). The vector system is an AT cloning kit and is supplied linearized with an extra 3'-thymidine overhang in the multiple cloning site (See Appendix II for vector map). The vector requires a 5'-adenine overhang on the PCR-products, a situation that is supplied by the *Taq* polymerase used in the PCR reaction. Ligation with this kit entailed the addition of 5 µl 2X rapid ligation buffer, 1 µl (50 ng) pGEM-T Easy vector, 3 µl purified PCR product and 1 µl T4 DNA ligase (3 Weiss units). The ligation reaction was allowed to continue for 2 days at 4°C.

According to the user manual for the pGEM®-T Easy cloning vector system, the optimal insert:vector ratio for the ligation reaction is between 8:1 and 1:8. A formula was developed to calculate the amount of PCR product needed for ligation if an insert:vector ratio is specified. The formula is:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

Thus, for a chosen insert:vector ratio of 3:1, using 50 ng of a 3 kb vector, the following amount of a 0.7 kb insert is required:

$$\frac{50\,\text{ng vector} \times 0.7\,\text{kb insert}}{3\,\text{kb vector}} \times \frac{3}{1} = 35\,\text{ng insert}$$

4.3.9.2 Preparation of competent cells

Initially, SURE *E. coli* bacteria were grown from glycerol stock in SOC (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, pH 6.8-7.2, autoclaved and glucose added to a final volume of 0.1 mM) on minimal medium M9 agar plates (42.24 mM $Na_2HPO_4.2H_2O$, 22.04 mM KH_2PO_4, 18.7 mM NH_4Cl, 8.56 mM NaCl, 1.5% (w/v) agar, pH 7.4. After autoclaving and cooling of the first components, 2 mM $MgSO_4$, 11 mM glucose, 0.1 mM $CaCl_2$, 1 mM thiamine-HCl were added).

The calcium-manganese-based method was used to prepare competent SURE *E. coli* cells (Hanahan *et al.*, 1991). A colony from the M9 plate was inoculated on a LB-tetracycline agar plate (1% (w/v) agar in LB-broth, 0.1% van 12.5 mg/ml tetracycline) and grown overnight at 30°C to increase competency. Colonies were inoculated in 1 ml SOB-broth (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, pH 6.8 - 7.2, autoclaved). The 1 ml culture was inoculated in 50 ml SOB-broth at 30°C until the $A_{550} \approx 0.3$ ($\pm$ 5 x 10^7 cells) and incubated on ice for 10 min. A 10 µl aliquot was removed, diluted 1:10^6 and 100 µl of the 10, 10^5 and 10^6 dilutions incubated on SOB plates (1% (w/v) agar in SOB) to determine viability of cells. After centrifugation for 15 min at 1000 *g* and aspiration, the pellet was dissolved in 16.7 ml sterile CCMB80 (80 mM $CaCl_2.2H_2O$, 20 mM $MnCl_2.4H_2O$, 10 mM $MnCl_2.6H_2O$, 10 mM KCH_3COO^-, 10% glycerol, pH 6.4). The solution was incubated on ice for 20 min, centrifuged at 1000 *g* for 10 min and the pellet dissolved in 4.2 ml CCMB80 after aspiration. Aliquots of cells ($\pm$200 µl) were placed in pre-cooled tubes, flash-frozen in liquid N_2 and stored at -70°C.

4.3.9.3 Transformation of competent cells

Competent SURE *E. coli* cells were thawed on ice, and 5 µl of ligated plasmid added to 100 µl of the competent cells. The mixture was incubated on ice for 30 min, followed by a 90 sec incubation at 42°C. Following a 2 min incubation on ice, 900 µl of pre-warmed SOC (SOB, 20 mM glucose) was added and the mixture incubated for 1 hour at 30°C to start cell growth. The cells were plated on LB-agar plates containing 100 µg/ml ampicillin and 40 µl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside) and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) spread on the surface. The plates were incubated at 30°C overnight to allow the cells to grow, white colonies were inoculated in 5 ml LB-broth containing 50 µg/µl ampicillin and incubated at 30°C while shaking. Plasmid DNA was isolated according to the miniprep plasmid isolation method (see 4.3.9.4). The isolated plasmid DNA was digested using the *Eco*RI restriction enzyme to confirm the insert size. A mixture of 500 ng plasmid, 10U *Eco*RI enzyme (Promega, Wisconsin, USA) and the appropriate 1x buffer was incubated at 37°C overnight. The digestion mixture was analyzed on a 2% (w/v) agarose (Promega)/TAE gel in 1x TAE at 7.8 V/cm and visualized by staining with EtBr. Cells containing plasmids of interest were stored in 15% glycerol (v/v)/LB at –70°C.

4.3.9.4 Mini-prep plasmid isolation and restriction digestion

The 5 ml overnight culture following transformation was centrifuged at 3000 *g* for 5 - 10 min to pellet the cells. The pellet was suspended in 100 µl Solution I (0.9% (w/v) D-glucose, 25 mM Tris pH 8, 10 mM EDTA) and after the addition of 150 µl of Solution II (1% (w/v) SDS, 0.2 M NaOH) the suspension was incubated for 4 min at room temperature. Thereafter, 250 µl of Solution III (29.45% (w/v) KCH_3COO^-, 11.5% (v/v) CH_3COOH) was added and the suspension incubated on ice for 5 min followed by centrifugation at 10 000 *g* for 15 min. The supernatant was transferred to a clean tube and 1 ml of absolute ethanol was added. The solution was incubated at room temperature and centrifuged for 15 min at 10 000 *g*. The pellet was washed with 500 µl ice-cold 70% (v/v) ethanol and centrifuged at 10 000 *g* for 15 min. Following aspiration, the pellet was dried under vacuum and dissolved in 20 µl TE buffer (10 mM Tris, 1 mM EDTA) containing 0.5 µg/ml RNAse.

Isolated plasmid (5 µl) was digested using 10 units of *Eco*RI (Promega, Wisconsin, USA) in the multicore buffer supplied with the enzymes. Digestion was performed at 37°C for 18 hours, tracking dye was added and the plasmids were analyzed using electrophoresis.

4.3.9.5 "High pure" plasmid isolation

Prior to DNA sequencing, the plasmid DNA from the desired clones was isolated using a High Pure Plasmid Isolation Kit (Promega, Wisconsin, USA). A 10 ml culture in LB-broth containing 50 µg/ml ampicillin was grown overnight at 30°C. The cells were collected by centrifugation at 3000 *g* for 10 min. The manufacturer's instructions were followed henceforth. Briefly, the cell pellet was suspended in suspension buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and RNase. Lysis buffer (0.2 M NaOH, 1% (w/v) SDS) was added and the suspension was mixed by inversion. Chilled binding buffer (4 M guanidine-HCl, 0.5 M potassium acetate, pH 4.2) was added, the sample centrifuged and the supernatant transferred to the upper reservoir of a filter tube. Following centrifugation, wash buffer (20 mM NaCl, 2 mM Tris-HCl, 4 parts ethanol, pH 7.5) was added to the filter tube and again centrifuged. The flow-through was discarded and the tube centrifuged once more to remove residual wash buffer. Elution buffer (10 mM Tris-HCl, pH 8.5) was added to the filter tube, centrifuged and the collected plasmid DNA was stored at -20°C.

4.3.10 Automated DNA sequencing

DNA sequencing of inserts was performed using the ABI PRISM 377 automated nucleotide sequencer (PE Applied Biosystems, California, USA) and using the Big dye terminator kit (Applied Biosystems, Foster City, CA, USA). The T7 and SP6 primers, complementary to the T7 and SP6 promotors of the pGEM-T Easy vector (Appendix II) were used in the sequencing reaction. The primer sequences are:

T7: 5' GTA ATA CGA CTC ACT ATA GGG C 3'

SP6: 5' ATT TAG GTG ACA CTA TAG AAT AC 3'

The cycle-sequencing reaction contained 500 ng double stranded DNA template, 3.2 pmol of the respective primers, 3 µl 5X buffer and 2 µl terminator ready reaction mix in a reaction volume of 20 µl. The sequencing reaction was performed in a Geneamp PCR system 9700 (PE Applied Biosystems, California, USA) with 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min.

The products were transferred to a low adhesion tube and purified by the addition of 16 µl H_2O and 64 µl non-denatured 95% ethanol. The mixture was vortexed and incubated at room temperature for 25 min at room temperature. The tube was then centrifuged at 13 000 *g* for 15 min, the supernatant removed and 100 µl 70% ethanol

added to the pellet. Following vortexing and centrifugation at 13 000 *g* for 10 min the supernatant was removed and the pellet dried under vacuum. The pellet was resuspended in 3 µl loading dye (5:1 ratio of deionized formamide to 25 mM EDTA, pH 8 and blue dextran, 30 mg/ml) and analyzed on a 36 cm gel according to the ABI PRISM 377 Genetic Analyzer User Manual. The raw data was analyzed with the Analysis software (ABI PRISM Sequencing Analysis Version 3.0, PE). Results were confirmed by visual inspection of the electropherograms obtained.

4.3.11 Homology searches of DNA sequences

The 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.*, 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 bachground 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 is considered highly significant.

4.4 Results

4.4.1 Degenerate primer design

The degenerate primer used in the 3'-RACE protocol (LYZ1) was designed based on conserved areas in the multiple amino acid sequence alignment of 39 lysozyme sequences in the NCBI Swissprot Database (Fig 4.1). The boxed areas indicate the conserved residues used in designing the primer.

The conserved residues in the multiple amino acid sequence alignment were confirmed by analysis of the alignment using the BLOCKS program. Blocks are multiply aligned ungapped segments corresponding to the most highly conserved regions of proteins. The graphic representation of the results from the BLOCKS-maker program is given in Fig 4.2.

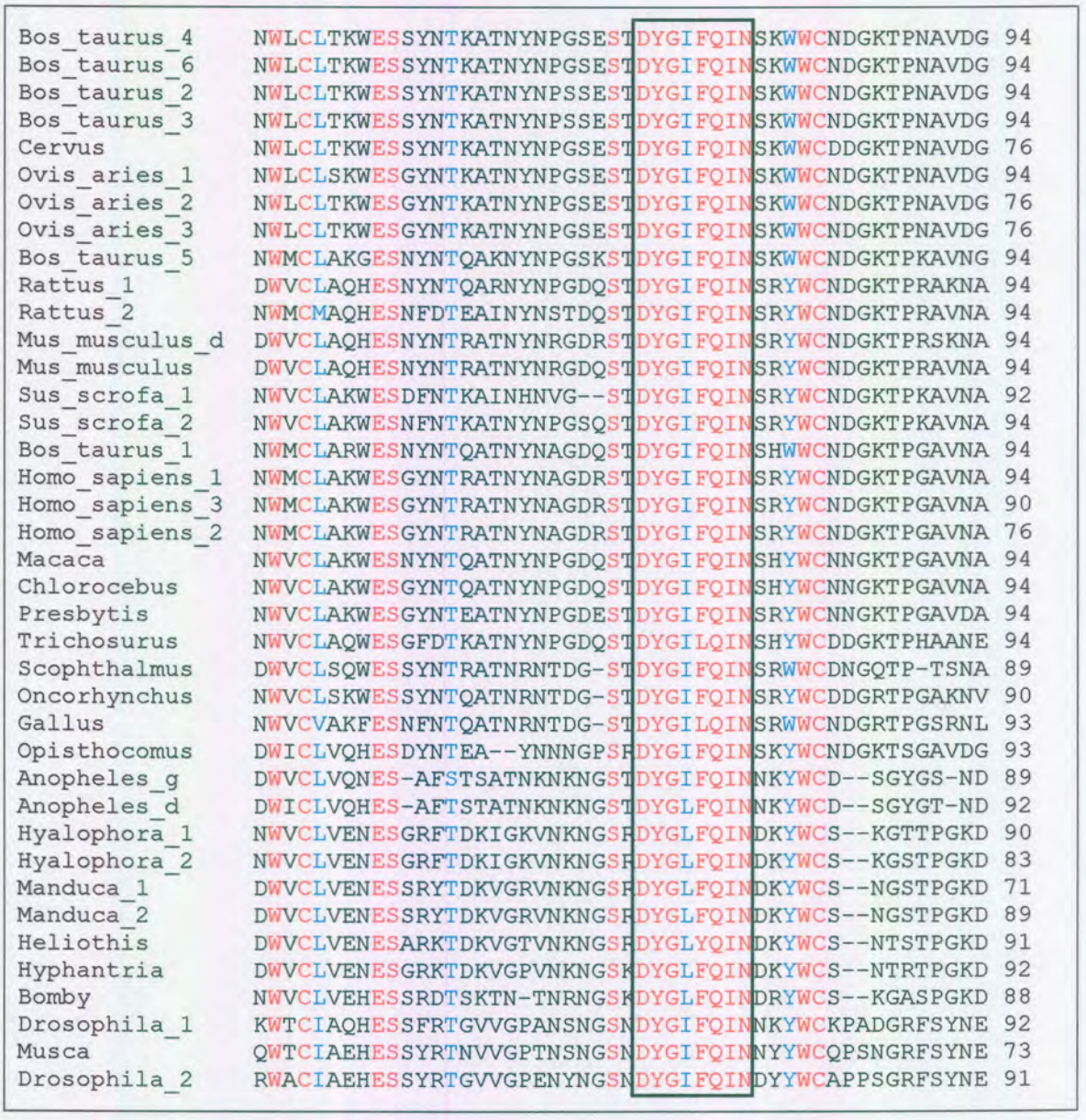

Figure 4.1 Multiple amino acid alignment of 39 lysozyme sequences from vertebrates and invertebrates. Residues in colour indicate residues conserved in all organisms: red residues indicate 100% identity, while blue indicates residues conserved in chemical nature. The boxed residues were used in the primer design. The numbers on the right indicate the position in the corresponding full-length lysozyme sequences. Dashes are included to optimize alignment. The names of the organisms are given in Appendix I.

Figure 4.2 Conserved motif identification in the alignment of the lysozyme sequences. The conserved areas were identified in the multiple amino acid sequence alignment of 39 lysozyme sequences. The color scheme for the amino acids is according to their physiochemical properties. red for acidic amino acids (glu and asp); blue for basic amino acids (lys, arg and his); light grey for polar OH/SH amino acids (ser, thr, and cys); green for amide amino acids (asn and gln); yellow for methionine; black for hydrophobic amino acids (ala, val, leu and ile); orange for aromatic amino acids (tyr, phe and trp); purple for proline; grey for glycine. The Y-axis indicates the relative abundance of the indicated amino acid in the specified position. The X-axis indicates the residue number in the specified area of the alignment. The horizontal bar indicates the residues used in the design of the degenerate primer.

Using the multiple alignment and BLOCKS data, the designed lysozyme specific primer (LYZ1) sequence is:

GA(C/T) TA(C/T) GGT TTI TT(C/T) CA(A/G) ATT AA(C/T) G

The properties of the LYZ1 primer is compared to the properties of the other primers used in the study i.e. the 3'-anchor (reverse) and DD-poly-T (cDNA synthesis) primers in terms of the length, degeneracy, Tm value and stability of the 3'-terminus (Table 4.1).

Table 4.1 Properties of the primers used in the polymerase chain reactions.

Primer	LYZ1	3' Anchor	DD-Poly-T [c]
Primer sequence (5'- 3')	GAY TAY GGT TTI TTY CAR ATT AAYG	GCT ATC ATT ACC ACA ACA CTC	GCT ATC ATT ACC ACA ACA CTC $(T)_{18}$ VN
Length	25	21	41
Degeneracy	32	1	12
T_m Oligo (°C) [a] a) or b)	a) 62.7 b) 60	a) 66.9 b) 60	a) 47.48 b) 40
T_m Equation (°C) [b]	max: 59.7 min: 51.5	56	max: 41 min: 37
-ΔG 3'-end (kcal/mol)	-6.3	-6.4	Not determined

- [a] T_m Oligo: (T_m values at 1 M NaCl)
  a) %G/C method
  b) (2 x [A+T] + 4 x [G+C])
- [b] T_m Equation: 69.3 + 0.41 (%G/C) – (650 / length). Min and max are the calculations for the minimum %G/C and maximum %G/C respectively
- [c] The T_m was calculated for the $(T)_{18}$ VN stretch on its own, as this is the only area involved in hybridization during cDNA synthesis. The T_m for the 3' anchor primer is calculated separately for use in PCR.
- Y=C or T; R=A or G according to IUBMB degenerate nucleotide nomenclature.

From the position of the primer in the full-length lysozyme sequences, it is expected that the PCR would yield a band of ±210 bp. This does not include the untranslated sequence of the mRNA, but the size of the DNA band is not expected to be above 300 bp.

4.4.2 RNA isolation

The internal organs of fifty *O. savignyi* ticks, pre-treated with *E. coli*, were used for total RNA isolation (purity 260/280 = 1.73, concentration 1.51 µg/µl). The isolated RNA was analyzed using denaturing agarose electrophoresis. The electropherogram (Fig 4.3) indicates that the only rRNA species that can be discerned using this technique is the 18S rRNA

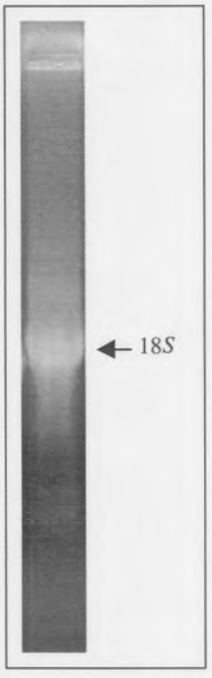

Figure 4.3 Denaturing agarose electrophoresis of RNA isolated from *O. savignyi*. The arrow indicates the position of the 18*S* rRNA band.

The isolated RNA was reverse-transcribed to produce single-stranded cDNA. The cDNA was used as reagent in a 3'-RACE protocol using the degenerate primer specific for lysozyme to drive the forward synthesis and the anchor primer to drive the reverse synthesis. Products of the amplification reactions were analyzed using agarose gel electrophoresis (Fig 4.4).

The initial results indicated that the PCR conditions used for the reaction were not optimal.

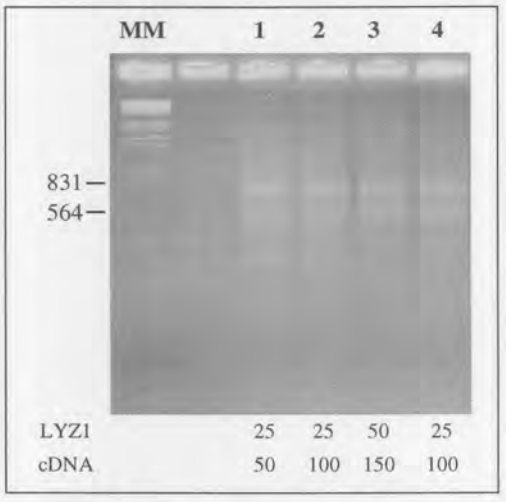

Figure 4.4 Amplifications products of the initial PCR reactions with the LYZ1 primer. Amplification products of the first four reactions (lanes 1-4). The concentration of the reactants of each reaction was: $MgCl_2$ 2 mM, each dNTP 200 nM, anchor primer 5 pmol. The amounts of LYZ1 primer (pmol) and cDNA equivalents (ng) are indicated on the figure. The total volume of the reactions was 25 µl and the *Taq* enzyme was added to the reaction after the first heat-denaturing step (94°C). The thermal cycling parameters for the reactions are: 94°C for 3 min, 80°C for 2 min (addition of enzyme), 30 cycles of 94°C for 30 sec, 56 °C for 30 sec, 72°C for 2 min. The final extension of the products was completed at 72°C for 5 min. The molecular mass markers (MM) are the *Eco*RI/*Hind*III digest of the λ-phage. The sizes of two fragments are included. A 3% (w/v) agarose gel was used for the electrophoretic analysis.

4.4.3 Optimization of the polymerase chain reaction

4.4.3.1 Initial Taguchi optimization of the PCR

Optimization (Fig 4.5) was performed using a Taguchi optimization matrix (Cobb and Clarkson, 1994). The three conditions that were varied were the $MgCl_2$ concentration, LYZ1 primer concentration and amount of cDNA equivalents. To check for the quality of the cDNA used in the amplifications, a positive control was included. In this control the primer for the Factor X inhibitor, isolated from the salivary glands of *O. savignyi*, was used in a single amplification reaction. The products of the amplification reactions were analyzed using agarose gel electrophoresis (Fig 4.5).

The optimization reactions show the presence of three predominant DNA bands, ~700 bp, ~350 bp and ~200 bp in size. The bands are not all evident in all the reactions. The

presence of a DNA band at ~300 bp in the control reaction (lane 10) confirms that the cDNA is still intact.

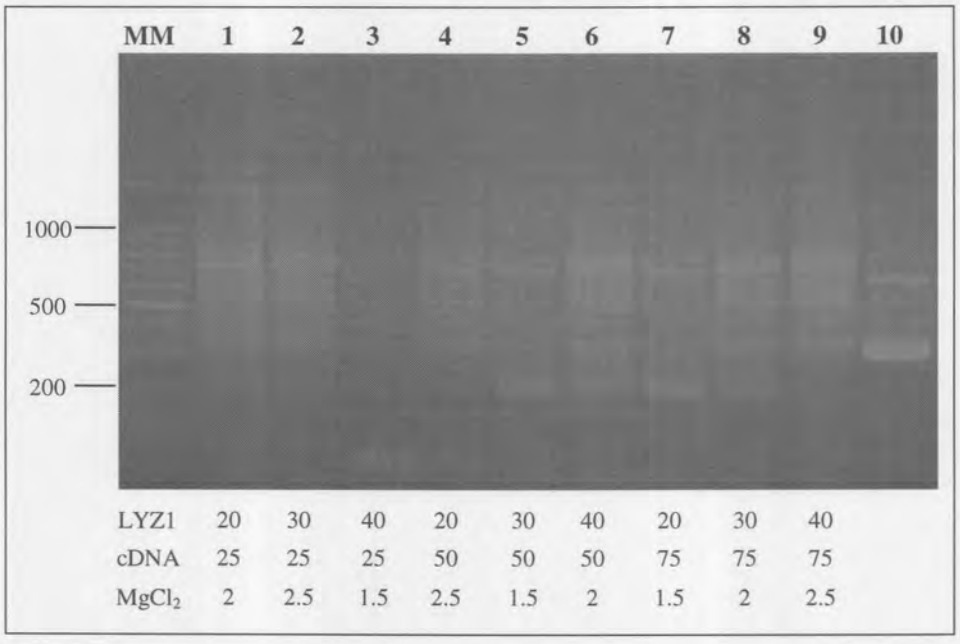

Figure 4.5 Results of the Taguchi optimization of the PCR reactions with the LYZ1 primer. Lanes 1-9 indicate nine different reactions performed. The concentrations of the reactants of each reaction were: each dNTP 200 nM, anchor primer 5 pmol. The amounts of LYZ1 primer (pmol), $MgCl_2$ (µM) and cDNA equivalents (ng) are indicated on the figure. A 100 bp DNA ladder was used as molecular mass marker (MM), with the sizes of the fragments indicated on the figure. Lane 10 is a positive control, using a primer to the Factor X inhibitor from the salivary gland of *O. savignyi*. The total volume of the reactions was 25 µl and the *Taq* enzyme was added to the reaction after the first heat-denaturing step (94°C). The thermal cycling parameters for the reactions are: 94°C for 3 min, 80°C for 2 min (addition of enzyme), 30 cycles of 94°C for 30 sec, 56 °C for 30 sec, 72°C for 2 min. The final extension of the products was completed at 72°C for 5 min. The sizes of two fragments are included. A 2% (w/v) agarose gel was used for the electrophoretic analysis.

Since the size of the expected band is ±200 bp, it was decided to further optimize the PCR using the conditions of Fig 4.5, lane 5.

4.4.3.2 Optimization of the primer annealing temperature

The optimization of the primer annealing temperature was performed using the conditions of the reactions in lanes 5 and 7 of Fig 4.5. The annealing temperatures used were 58°C, 60°C and 62°C. These temperatures were chosen arbitrarily and were all higher than the calculated optimal annealing temperature of the LYZ1 primer (see Table 4.1, p 106). The products of the amplification reactions were analyzed using agarose gel electrophoresis (Fig 4.6). The results of the reactions show that a primer annealing temperature of 60°C is more effective in the amplification of the products, and this temperature was used during subsequent reactions (Lanes 5 and 6, Fig 4.6).

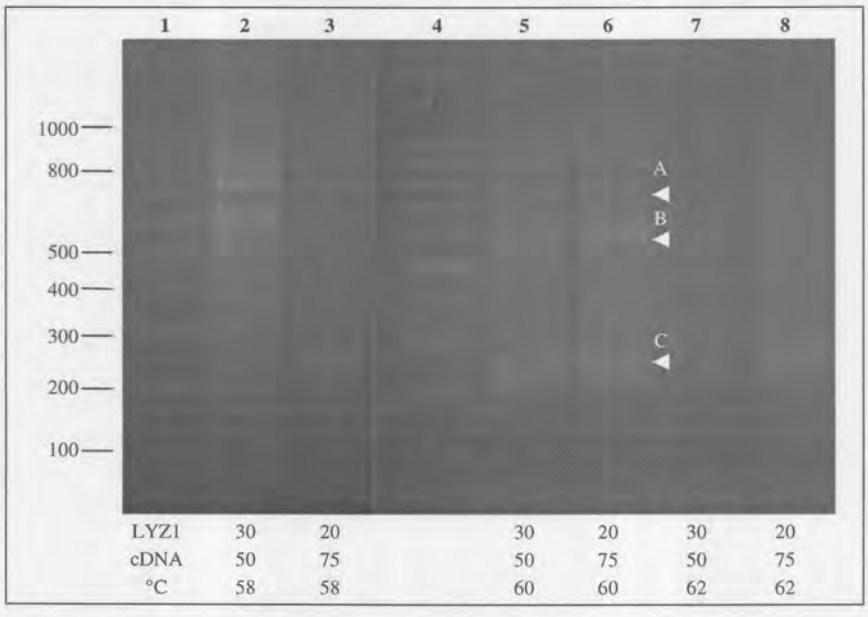

Figure 4.6 Results from optimization of the LYZ1 primer annealing temperature. Lanes 2, 3, 5, 6, 7 and 8 indicate six different reactions performed. The concentration of the reactants of each reaction was: each dNTP 200 nM, $MgCl_2$ 1.5 mM, anchor primer 5 pmol. The amounts of LYZ1 primer (pmol), cDNA equivalents (ng), and primer annealing temperature (°C) are indicated on the figure. Lanes 1 and 4 are standard DNA 100 bp ladders with the sizes of the standard DNA fragments (in bp) included in the figure. The total volume of the reactions was 25 µl and the *Taq* enzyme was added to the reaction after the first heat denaturing step (94°C). The thermal cycling parameters for the reactions are: 94°C for 3 min, 80°C for 2 min (addition of enzyme), 30 cycles of 94°C for 30 sec, the specified annealing temperatures for 30 sec, 72°C for 2 min. The sizes of two fragments are included. A 2% (w/v) agarose gel was used for the electrophoretic analysis. The final extension of the products was completed at 72°C for 5 min. The arrows indicate the bands A, B and C excised for the single primer extension analyses (Fig 4.7).

4.4.3.3 Analyses of single primer extension products

The bands indicated in lane 6, Fig 4.6 (A, B and C) were excised and the DNA extracted by the addition of 100 µl of 10 mM Tris-HCl, pH 8.6. The bands were incubated at 80°C for 10 min and 5 µl of the extracted DNA used as template for the single primer amplification reactions. The products of the amplification reactions were analyzed using agarose gel electrophoresis (Fig 4.7).

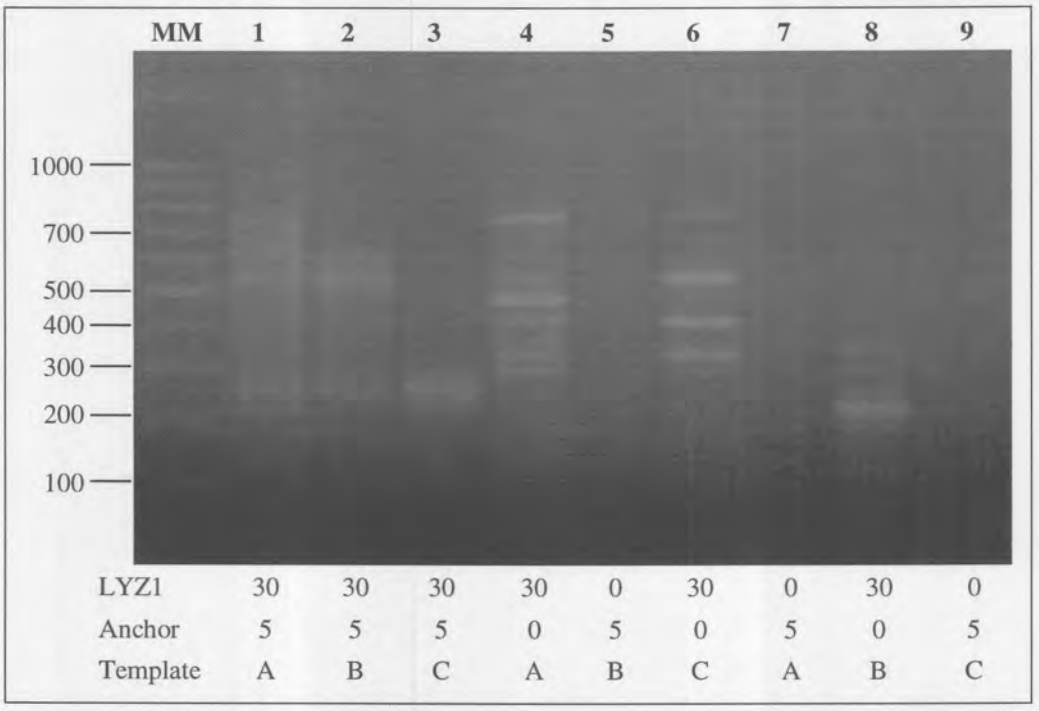

Figure 4.7 Results of single primer amplification analyses of the LYZ1 and 3' anchor primers. Lanes 1-9 indicate nine different reactions performed. The concentration of the reactants of each reaction was: each dNTP 200 nM and $MgCl_2$ 1.5 mM. The amounts of LYZ1 primer (pmol), cDNA template (band A, B or C of lane 6, Fig 4.6), and anchor primer (pmol) are indicated on the figure. A 100 bp DNA ladder was used as molecular mass marker (MM), with the sizes of the fragments indicated on the figure. The total volume of the reactions was 25 µl and the *Taq* enzyme was added to the reaction after the first heat denaturing step (94°C). The thermal cycling parameters for the reactions are: 94°C for 3 min, 80°C for 2 min (addition of enzyme), 30 cycles of 94°C for 30 sec, the specified annealing temperatures for 30 sec, 72°C for 2 min. The final extension of the products was completed at 72°C for 5 min. The sizes of two fragments are included. A 2% (w/v) agarose gel was used for the electrophoretic analysis.

The results of the single primer amplification analyses indicate that the amplification reactions might be driven by the degenerate LYZ1 primer. The products obtained in the absence of the anchor primer were, however, not the same size as the original products obtained with both primers. Subsequent optimization of the PCR reaction was thus continued, since the results of the single primer amplification did not conclusively prove the ineffectivity of the degenerate primer. The anchor primer did not amplify the template DNA in the absence of the degenerate primer.

4.4.3.4 Optimization of the primer-template ratio

A correct primer-template ratio is very important for the sensitivity and selectivity of the PCR. To obtain a more optimal ratio, the LYZ1 primer concentration was increased relative to the previous reactions, and the $MgCl_2$ concentration was increased simultaneously since this parameter is different for each primer-template ratio. The products of the amplification reactions were analyzed using agarose gel electrophoresis (Fig 4.8). The results indicate that the most optimal conditions (Lane 4, Fig 4.8) lead to two predominant products, i.e. a band at ~250 bp and a double band at ~700 bp.

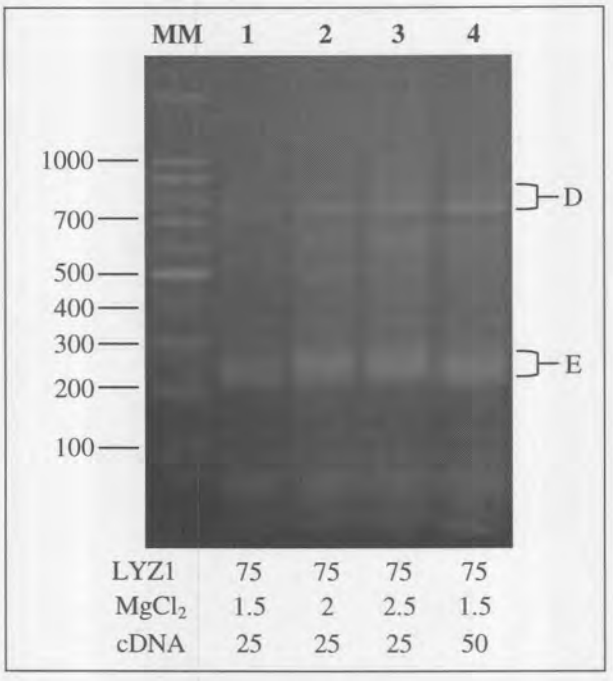

Figure 4.8 Results from optimization of the LYZ1 primer-template ratio. Lanes 1-4 indicate four different reactions performed. The concentrations of the reactants of each reaction were: each dNTP 200 nM, anchor primer 5 pmol. The amounts of LYZ1 primer (pmol), cDNA equivalents (ng), and $MgCl_2$ (mM) are indicated on the figure. A 100 bp DNA ladder was used as molecular mass marker (MM), with the sizes of the fragments indicated on the figure. The total volume of the reactions was 25 µl and the *Taq* enzyme was added to the reaction after the first heat denaturing step (94°C). The thermal cycling parameters for the reactions are: 94°C for 3 min, 80°C for 2 min (addition of enzyme), 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min. The final extension of the products was completed at 72°C for 5 min. A 2% (w/v) agarose gel was used for the electrophoretic analysis.

Following the reaction in lane 4 of Fig 4.8, the two indicated regions (D and E) were cloned into the pGEM®-T Easy cloning vector system in order to obtain the nucleotide sequence of the PCR products. The sequencing results would provide information regarding the identity of the PCR products and confirm the LYZ1 primer specificity.

4.4.4 Cloning of PCR products

The conditions of lane 4, Fig 4.8 were used to complete 10 amplification reactions. The products (Fig 4.8, lane 4: bands D and E) were excised and the DNA isolated

from the agarose gel. The isolated DNA for each band was pooled and 5 µl of the DNA used to estimate the concentration of the isolated DNA using electrophoresis.

The ligation products were used to transform *E. coli* cells, and the cells were plated on ampicillin-containing LB-plates (Section 4.3.9.4). To test the efficiency of the transformation reaction, Bluescript vector was also transformed in the *E. coli* cells. The presence of blue colonies on the control agar plate was an indication of efficient transformation of the cells. On the plates containing cells transformed with the isolated DNA products, 10 white colonies were removed from each plate. These colonies were grown overnight and the plasmids of the cells isolated using a mini-prep isolation method (Section 4.3.9.4).

The isolated plasmids were subjected to restriction digestion using the *Eco*RI restriction enzyme. The digestion was performed overnight at 37°C and the products of the enzymatic reactions analyzed using agarose gel electrophoresis (Fig 4.9).

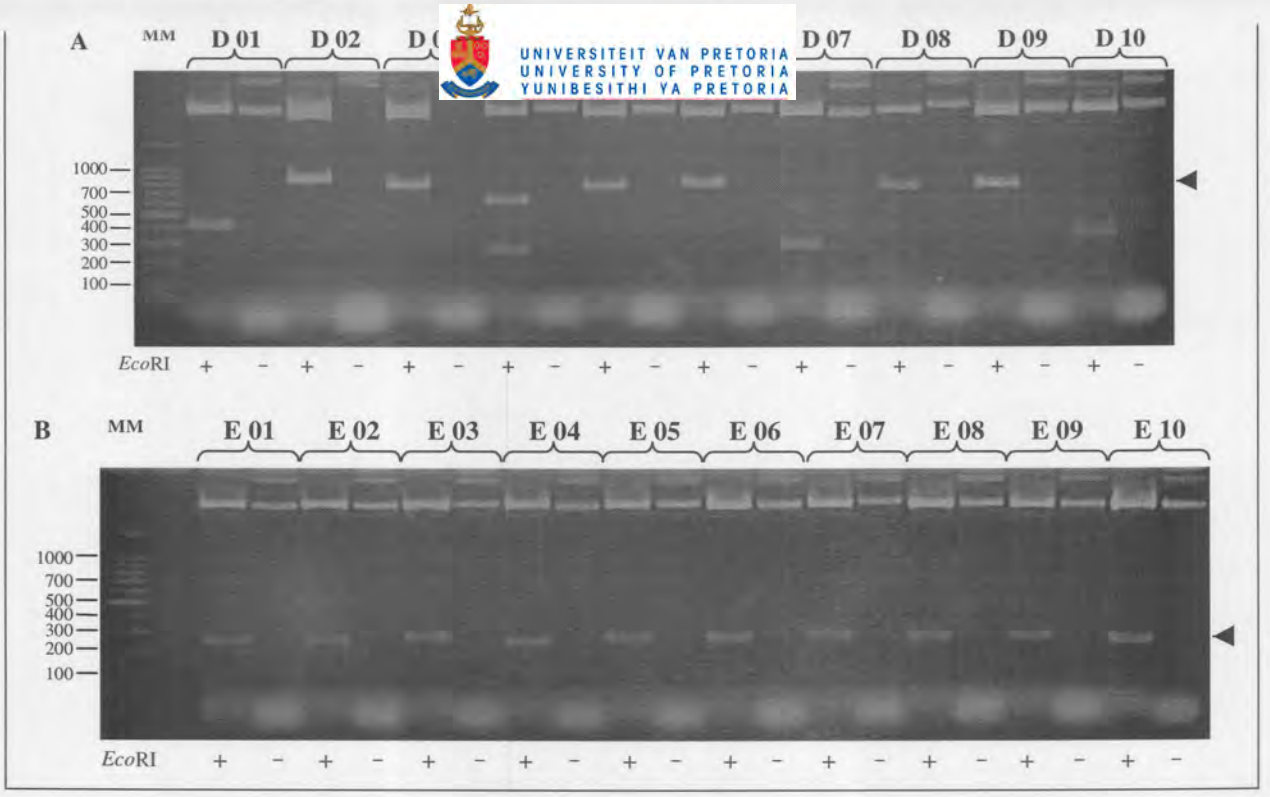

Figure 4.9 Results from restriction digestion of plasmid DNA isolated from transformed *E. coli* cells. **(A)** The digestion of plasmid DNA containing the inserted DNA corresponding to band D in Fig 4.8. The arrow indicates the size of the expected DNA bands (~700 bp). **(B)** The digestion of plasmid DNA containing the inserted DNA corresponding to band E in Fig 4.8. The arrow indicates the size of the expected DNA bands (~250 bp). Digested (+) and undigested (-) plasmid DNA from each colony was loaded together on the gel to indicate the size of the inserted DNA in each plasmid. A 100 bp DNA ladder was used as molecular mass marker (MM), with the sizes of the fragments indicated on the figure. A 2% (w/v) agarose gel was used for the electrophoretic analyses.

The *Eco*RI digestion profiles in Fig 4.9 indicate that all the plasmids contained inserts. The inserts of Fig 4.9 (A) should be between 750 bp and 850 bp in size, judging from the bands in Fig 4.8 (lane D) used for cloning. The actual size of the inserts display a greater variety in size than anticipated. It was decided to obtain the nucleotide sequence of the inserts in lanes D 01 and D 03 of Fig 4.9.

The inserts of Fig 4.9 (B) should be in the region of 250 bp in size, judging from the bands in Fig 4.8 (lane D) used for cloning. This was confirmed, all the inserts falling

in the same size range. As a representative selection, it was decided to obtain the nucleotide sequence of the inserts in lanes E 04 and E 06 (Fig 4.9).

4.4.5 Nucleotide sequencing of the DNA inserts

Cells containing plasmids with inserts from colonies D 01, D 02, E 04 and E 06 were grown for 16 hours in LB-broth containing ampicillin. Plasmid DNA of the cells were isolated using the High Pure Plasmid Isolation Kit (Promega, Wisconsin, USA, Section 4.3.9.5). Isolated plasmid DNA was analyzed using electrophoresis and the concentration of the isolated plasmid DNA estimated from the electropherogram (data not shown).

Using the isolated DNA, the nucleotide sequence of the inserts was determined using the Big Dye terminator kit. Both the SP6 and T7 primers were used, and the sequences of both primers were combined to provide the complete sequence of the insert DNA where possible (D 01 – Table 4.2; D 03 – Table 4.4; E 04 – Table 4.6; E 06 – Table 4.8). The sequences were analyzed using the BioEdit Sequence Alignment Editor (Hall, 1999), and in none of the obtained sequences were the degenerate primer sequence identified.

The BLAST algorithm (Altschul *et al.*, 1997) was used to search for similarities with known protein sequences in the Genbank database (www.ncbi.nlm.nih.gov). The sequence and reverse complement of every DNA sequence were subjected to the database search. The protein translation of each reading frame of each sequence and reverse complement sequence were also used in the similarity search, and only the most significant similarities in all searches were reported (D 01 – Table 4.3; D 03 – Table 4.5; E 04 – Table 4.7; E 06 – Table 4.9). The search results indicate that none of the inserted DNA fragments display significant similarity with any known protein.

Table 4.2 DNA sequence obtained following sequencing of the insert plasmid DNA of clone D 01 (Fig 4.9). Residues in red are part of the plasmid sequence, residues in black are part of the insert sequence, residues in green indicate the anchor primer sequence and residues in blue indicate the *Eco*RI restriction sites. Numbers indicate the position of the residues in the full-length sequence. Gaps are included to separate each set of ten residues in the sequence.

```
GATTGCCTAC TACCTGGAGG CTTCAGGACA AGTNTAAGCG TAGAAGTCAC  50
AGTTGAAGCG AAAAAGCTCG AGAACGCTTT GCTTGAGCAA CTCCAATATC 100
TGCCTGAAAT GATTGAAGAG GAAGTGGCCG AACTTTCCGA CTCCGAAGAC 150
ACCGAGTACG GTTGGCTCAA GAAGAAACTC AAGAAGATGA AGAAAGTCTT 200
CGGCAAAATT TCAGGCCACA TTTCGAAAGG CCCTCACAAA AATAACGATG 250
GCAGGATAGT GGTCCAGCCT TGAGAGCACG GGTGTTCGTT ACTGTGATCA 300
AATATGTTAT GTATATATAC TGCACTACTA ATATATTACA ACCAAAAAAA 350
AAAAAAAAAA AGAGTGTTGT GGTAATGATA GCAATCACTA GTGAATTCGC 400
```

Table 4.3 Results of a BLAST database search with the insert DNA sequence of clone D 01 (Table 4.2). Similarities are shown with known protein sequences in the Genbank database (www.ncbi.nlm.nih.gov).

Significant alignments	Expect value
Mycoplasma synoviae phase-variable hemagglutinin homolog	0.087
Cryptosporidium parvum histone deacetylase (HD1)	0.34
Hepatitis D virus delta antigen (HDAg)	1.4

Table 4.4 DNA sequence obtained following sequencing of the insert plasmid DNA of clone D 03 (Fig 4.9). Residues in red are part of the plasmid sequence, residues in black are part of the insert sequence, residues in green indicate the anchor primer sequence and residues in blue indicate the *Eco*RI restriction sites. Numbers indicate the position of the residues in the full-length sequence. Gaps are included to separate each set of ten residues in the sequence.

```
GATTGCCTAC TACCTGGAGG CTTCGCGACG CCATCGACGT GACCGTATGG  50
GCTAGTGTAA GCAACGAAGA AAAAATTCGG CGAACCGGTG AGGCAGAAGT 100
AGAAGACACG TGAGACACTT TGCGCCAACA TGTAGCCTGG GCCAGTGCAC 150
TTCAGGTTCT TTTCCAAGAC AGACAAGTAA GGNCGGTTTN TGGACCTTCC 200
NTCACGACAG TTTACGTCCC CAATATAAGA CTCCNATCGG CCAGNACTTC 250
CTCAAAAGGA TANCTGCCCA GATCTAANAG GCGTCCCACA ACGTCGTAAG 300
AAGAGGCTTT GCTTTCCTCG GTGAGCGACA GAAGTGGCTA GGATTTACGT 350
CACGTGTCCG CTTTATTCGG AGCTTTTGTT ATGCGCTGAA CATAGTAGTT 400
CGCACGGATC TTCTAGTACA TTTCTTTCTG AGCCTTGACT GAAGAAACGG 450
TGGGAAGCCA CTGGGTTGAT CTGTTTCTTG CACTTCCTAC CTCTGAACAG 500
CGTTATTGTC TACGAACTCG TTTTCTTACA GGTACGAACT CTTGTTCTTT 550
TGCGAATGTA CGAATTGCAA GATAGAGATA AGATAGAACT TAATCTAACT 600
GAAACAGACT CCTTGTACTT GTGTATGAAA TTCAGAAAGT GCTTTTATAG 650
GCTGGCTCTT CAAAAGTTCA ATAAACCTGC TTTAACATCT TCATGCTGCC 700
TGAAAGCACT GTTGTGTACC GAGAAAGTGC CGGAAGGCCC TAGGTGTAAG 750
AAATAGATTG AATGTTGACA AAAAAAAAAA AAAAAAAGAG TGTTGTGGTA 800
ATGATAGCAA TCACTAGTGA ATTCGCGGCC GC                     832
```

Table 4.5 Results of a BLAST database search with the insert DNA sequence of clone D 03 (Table 4.2). Similarities are shown with known protein sequences in the Genbank database (www.ncbi.nlm.nih.gov).

Significant alignments	Expect value
Sus scrofa partial SDHC gene for succinate dehydrogenase complex	9.6
Mus musculus BALB/c IgH I-α gene	9.6
Mus musculus gene for immunoglobulin alpha heavy chain	9.6
Mus musculus Ig germline alpha-chain gene C-switch region	9.6
Mus musculus germline alpha-chain gene constant (C) region	9.6

Table 4.6 DNA sequence obtained following sequencing of the insert plasmid DNA of clone E 04 (Fig 4.9). Residues in red are part of the plasmid sequence, residues in black are part of the insert sequence, residues in green indicate the anchor primer sequence and residues in blue indicate the *Eco*RI restriction sites. Numbers indicate the position of the residues in the full-length sequence. Gaps are included to separate each set of ten residues in the sequence.

```
CCATTAAATC GGAACCNTAA NGGAGCCCCC GACGCCATGG CGGCCGCGGG  50
AATTCGATTG CCTACTACCT GGAGGCTTCC CAGGAGCCGG TGGAGCACCC 100
GGAGCAGGAG CTGCACCTGG TGGAGGCAGT GGAGGACCAA CTATTGAGGA 150
GGTTGATTAA GACTCCTCTT GCTGTGATAA AGTTCAGCTG TGTTTTAGTT 200
CAGTGTTTCT GTGAATAAAA TGTTGAGCTT GTAAAAAAAA AAAAAAAAAA 250
AAAAAAAAAA AAGAGTGTTG TGGTAATGAT AGCAATCACT AGTGAATTCG 300
CGGCCGCCTG CAGGTCGACC ATATGGGAGA GCTCCC               336
```

Table 4.7 Results of a BLAST database search with the insert DNA sequence of clone E 04 (Table 4.2). Similarities are shown with known protein sequences in the Genbank database (www.ncbi.nlm.nih.gov).

Significant alignments	Expect value
Caenorhabditis briggsae cosmid G42P09	0.012
Lycopersicon esculentum heat shock protein (hsc-1)	0.19
Rivulus marmoratus heat shock protein (Hsc71)	0.74
Ring-tailed lemur involucrin gene	0.74
Entamoeba heat shock protein (Hsp70)	0.74
Drosophila melanogaster Heat shock protein (Hsc70-1)	2.9
Xenopus laevis cyclase-associated protein	2.9
Leptinotarsa decemlineata inducible heat shock 70 kDa protein	2.9
Pichia angusta heat shock protein 70 homolog (HSA2)	2.9
Pleurodeles waltl HSP70 protein	2.9
Gallus gallus mRNA for heat shock cognate 70	2.9
Drosophila melanogaster heat shock protein cognate 70 (Hsc1)	2.9

Table 4.8 DNA sequence obtained following sequencing of the insert plasmid DNA of clone E 06 (Fig 4.9). Residues in red are part of the plasmid sequence, residues in black are part of the insert sequence, residues in green indicate the anchor primer sequence and residues in blue indicate the *Eco*RI restriction sites. Numbers indicate the position of the residues in the full-length sequence. Gaps are included to separate each set of ten residues in the sequence.

```
CATATGGTCG ACCTGCAGGC GGCCGCGAAT TCACTAGTGA TTGCCTACTA  50
CCTGGAGGCT TCCCAGGAGC CGGTGGAGCA CCCGGAGCAG GAGCTGCACC 100
TGGTGGAGGC AGTGGAGGAC CAACTATTGA GGAGGTTGAT TAAGACTCCT 150
CTTGCTGTGA TAAAGTTCAG CTGTGTTTTA GTTCAGTGTT TCTGTGAATA 200
AAATGTTGAG CTTGAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA 250
AAAAAAAAAA GGAGTGTTGN GGTAATGATA AATCGAATTC CCGCGGCCGC 300
CATGGCGGCC GGGAGCATGC NACGTCGGGC CCAATTCGCC CTATA       345
```

Table 4.9 Results of a BLAST database search with the insert DNA sequence of clone E 06 (Table 4.2). Similarities are shown with known protein sequences in the Genbank database (www.ncbi.nlm.nih.gov).

Significant alignments	Expect value
Caenorhabditis briggsae cosmid G42P09	0.013
Lycopersicon esculentum heat shock 70 kD protein (hsc-1)	0.21
Rivulus marmoratus Hsc71	0.82
Drosophila yakuba strain BG1013 phosphoglucomutase	0.82
Drosophila simulans strain dpf96_17.4 phosphoglucomutase	0.82
Ring-tailed lemur involucrin	0.82
Entamoeba heat shock protein (Hsp70)	0.82
Drosophila melanogaster heat shock protein cognate 1 (Hsc70-1)	3.3
Xenopus laevis cyclase-associated protein (CAP1)	3.3
Leptinotarsa decemlineata inducible heat shock 70 kDa protein	3.3
Pichia angusta heat shock protein 70 homolog (HSA2)	3.3
Pleurodeles waltl Hsp70	3.3

4.5 Discussion

Since the multiple sequence alignment of the lysozyme sequences (Fig 4.1), in conjunction with the analysis by the BLOCKS program (Fig 4.2) only indicated one

region of homology, this region was the obvious choice for the design of the primer. This also meant that, should the amplification reactions be unspecific, no other region could be used to design a subsequent primer. Alternatively the N-terminal amino acid sequence of the lysozyme isolated from *O. moubata* (Kopácek *et al.*, 1999) could be used to design a primer which could possibly be specific for the lysozyme of *O. savignyi*.

The designed LYZ1 primer conformed to all the prerequisites (Section 4.1.1.2) for a primer destined for use in the PCR. The internal stability profile shows a stable 5' end and a less stable 3' end, with little self-complementarity regions. There is a measure of self-complementarity on the 3' end, but the free energy for the association is within acceptable limits. There is a possible region for complementarity between the upstream (LYZ1) and downstream (3'-anchor) primers, but the association is also within acceptable limits, though the free energy of the association is in the more unfavourable region of the scale (<ΔG). One inosine residue was incorporated to decrease the degeneracy and complexity of the primer mix. There were two other sites on the sequence where inosines could be incorporated, but this would have increased the self-complementarity of the primers to unacceptable limits. The melting temperature would also have been influenced negatively. It was decided to incorporate two thymidine residues at these site, since this residue can associate non-specifically with any other residue (Kidd and Ruano, 1995, Preston, 1993). This approach has been successful in other studies (Joubert, 1998). The difference in the melting temperature between the two primers was 3.1°C, which was also within acceptable limits.

The quantity and quality of the isolated RNA was acceptable, although in *O. savignyi*, the 5*S* and 28*S* rRNA are not observed using this electrophoresis technique (Fig 4.3). Another tick species analyzed, *Argas (Persicargas) walkerae,* display all three major rRNA bands (unpublished results), so this phenomenon does not seem to be a general characteristic of all tick species. The absence of these rRNA species still needs to be fully investigated, but possible explanations include the joining of the 5*S* and 28*S* rRNA species on genomic and/or transcriptional level, although larger bands could

not be distinguished during the electrophoretic analysis (Mans, B. J. personal communication).

The initial PCR analysis showed the presence of multiple DNA products (~700 bp and ~500 bp), all larger than the expected <300 bp size (Fig 4.4). The initial optimization of the reaction (Fig 4.5) resulted in the amplification of three predominant DNA products, ~700 bp, ~350 bp and ~200 bp in size with the smaller products in the correct size range for the expected product. The optimization of the annealing temperature (Fig 4.6) showed that a higher temperature, thus more specific primer annealing conditions, was required for optimal amplification. This temperature is higher than the melting temperature of either of the two primers, but is still effective.

The single primer extension analysis performed on the predominant products of the amplification (Fig 4.7) showed that there is a possibility of the LYZ1 primer being dominant, and this resulted in the amplification of spurious, non-specific products. The absence of this primer, in the presence of the anchor primer, did not result in the amplification of any products. There is also a possibility that, due to the excess of template present, concatamerization of product occurred. This excess of template could lead to the dimerization and higher order polymerization of amplification products, resulting in bands of higher than expected size (Kidd and Ruano, 1995). This was evident in lanes 6 and 8 of Figure 4.7, where larger than expected sizes of the products were observed. From lanes 4 and 6 of Fig 4.7 it can be seen that products were being amplified which were either in very low abundance, or were absent from the template (lanes 1 and 2, Fig 4.7). The results seem to indicate that the LYZ1 primer is not specific enough for the lysozyme transcript. It was subsequently decided to complete the sequencing of the amplification products to evaluate the identity of the amplification products, since no other primer could be designed from the multiple sequence alignment analysis.

Initially, the optimization of the primer/template ratio was performed by serial dilutions of the template in a series of amplification reactions (data not shown). The results showed that this template dilution led to the decrease in product concentration below the detection threshold. Thus, the primer/template ratio was at the lower end of the optimum, and by increasing the template concentration it was found that the

concentration of the products increased (Lane 4 of Fig 4.8). Since the amount of products was sufficient, and the complexity of the product mixture was acceptably decreased relative to prior amplification reaction results, the optimization of the products was considered to be complete. There were two regions of interest, a double band at ~750 bp and a diffuse band at ~250 bp. Since it was technically too challenging to excise the two products of the double band separately, they were excised and pooled. The excision of the diffuse band (lane 4, Fig 4.8) was similarly considered, and a similar conclusion was reached. It was expected that DNA products of different sizes would be obtained following the cloning step. To increase the yield of PCR products for the cloning step, several parallel amplification reactions were completed and the isolated DNA was pooled.

The cloning of the DNA bands in the cloning vector was successful, and the insert analysis of the clones showed that all the selected clones contained inserted DNA of approximately the correct sizes (Fig 4.9 A, B). The insert sizes of the cloned double band D (Fig 4.8) showed some unexpected variation. Six of the clones displayed the correct insert sizes (represented by clones D 02 and D 03), while four of the clones showed aberrant endonuclease digestion profiles (Fig 4.9 A). Evident from clones D 04 and D 07 is that these clones contained inserts that had cleavage sites for the *Eco*RI endonuclease in their sequence. This would explain the double band pattern obtained following the endonuclease digestion of the plasmid DNA. Clones D 01 and D 10 simply contained single inserts of the wrong sizes. Since the DNA for the ligation and cloning reactions was obtained form the excised bands with sizes above 700 bp, the truncation of the inserts were either the result of DNase contamination of the samples, or bacterial-mediated cleavage. Although the insert sizes of these two clones were different from the predominant sizes, they were considered to be significant based on their closer size homology with the expected PCR products (200 – 300 bp). The insert sizes of the diffuse band E (Fig 4.8) showed no significant variation, and the cloning was considered to be very successful (Fig 4.9 B).

Two clones from each set (Fig 4.9 A, B) were chosen for the sequencing reactions. The clones were arbitrarily chosen, and the main criterion was that the selection of clones was to be representative. Thus, all the relevant insert sizes were to be included. The clones of Fig 4.9A displayed three population subsets with insert sizes of ~400

bp, ~700 bp and ~800 bp. Since the 800 bp fragments were considered to be too large when compared with the expected amplification product sizes of 200 – 300 bp, it was decided not to sequence these clones (D 02 and D 06). Representative clones from the 400 bp and 700 bp subsets were chosen (D 01 and D 03) for sequencing. The same process was followed for the clones in Fig 4.9B, and although there was less variation in insert size, two subsets could be discerned with sizes of ~250 and ~275 bp, respectively. One representative clone from each subset was therefore selected for the sequencing reactions (E 04 and E 06).

The results of the sequencing reactions and the subsequent database similarity searches showed that none of the sequenced clones showed any homology with lysozymes from other organisms (Table 4.2 to 4.9). In all the sequences, the 3'-anchor primer sequence could be identified, as well as at least one *Eco*RI consensus sequence, the sites used for the cloning of the inserts. The LYZ1 primer sequences could, however, not be identified in any of the sequences. This is an important result, since it indicates that the amplification reactions were non-specific, and that the LYZ1 primer did not take part in the reactions to a significant degree. This is contrary to the results obtained for the single primer extension analysis (Fig 4.7), which indicated the possibility that the reactions were driven by the LYZ1 primer. The results from Fig 4.7 could possibly be explained by priming by the products themselves and this would be consistent with the aberrant product sizes and with the sequencing results.

The sequences from clones D 01 (Table 4.2) and D 03 (Table 4.4) were not complete for the total length of the inserts (both cloning restriction sites identified). The available sequences did not, however, show significant homology with any proteins, and it was decided not to obtain the total insert sequences. The sequences from clones E 04 (Table 4.6) and E 06 (Table 4.8) did not show significant homology with any proteins, but a common motif between the two sequences is that both sequences show homology with heat shock proteins (Hsp), most notably with Hsp 70. This might suggest that the inserts of these clones, and presumably of all the clones from Fig 4.9B, are sections of Hsp 70 proteins of *O. savignyi*. The LYZ1 primer was therefore not specific enough, and since another primer cannot be designed from the multiple sequence alignment, the best route would be to attempt the isolation of the lysozyme protein and to use the amino acid sequence to design a more specific primer.

Chapter 5

Concluding Discussion

The finding that *O. savignyi* produces antibacterial factors that target only Gram-positive bacteria was unexpected and evocative. This is even more surprising when the induction of activity is taken into account. The pre-treatment of ticks with Gram-negative bacteria should lead to the LPS-mediated induction of an anti-Gram-negative response, while Gram-positive pre-treatment should lead to the peptidoglycan-mediated induction of an anti-Gram-positive response. This is not the case with *O. savignyi*, since pre-treatment with the Gram-negative bacteria gives rise to antibacterial factors that target none of the Gram-negative bacteria tested, but only some Gram-positive bacteria. This suggests several scenarios. Firstly, it could indicate that Gram-negative bacteria are not pathogenic for *O. savignyi*. Any Gram-negative infection could thus lead to the production of factors specifically targeting concurrent opportunistic Gram-positive infections. Secondly, the factors targeting Gram-positive bacteria could be present to control the natural flora of *O. savignyi*, suggesting that the internal flora of this tick species is only composed of Gram-positive bacteria. Thirdly, the fact that *B. subtilis* occurs naturally and ubiquitously in earth could indicate that this bacteria easily infects *O. savignyi* during times when the tick is resting in sand waiting for a host. Thus, the mainly Gram-positive targeting response could rid the tick of this infection transmitted as a result of injury while resting. The widespread presence of bacteria of the *Wolbachia* species in ticks could possibly explain this result. The bacteria are Gram-negative endosymbionts and their necessary presence in ticks would negate the synthesis of anti-Gram-negative peptides by the ticks, to prevent the removal of these symbionts (Noda, 1997).

The rapid induction of the antibacterial response in *O. savignyi* is an interesting phenomenon. Induction of activity in the pupae of the Lepidopteran species, *Hyalophora cecropia*, occurs more slowly, only reaching a maximum 7 to 10 days following bacterial challenge (Hultmark *et al.*, 1980). The behavior and habitat of *O. savignyi* might explain this rapid response. Since the natural habitat of *O. savignyi* is in the semi-arid desert

region of the Northern Province of South Africa, natural resources are scarce. These ticks can survive for an extended time, sometimes years, between blood meals. Since a protracted bacterial response could lead to the waste of precious nutrients, it would be advantageous to mount a very potent antibacterial activity very rapidly. Such an effective, but short-lived response, would ensure that the infection is quickly contained, and would prevent the excessive use of nutrients. The fast deregulation of the antibacterial response also makes it unlikely that the induction of antibacterial activity in the hemolymph is in response to bacteria ingested with the blood meal from the host. Since the ticks last fed several days prior to collection and assaying, this possible feeding-induced response would already be down-regulated at the time of the study. This fast up- and down-regulation of the antibacterial activity also occurs in *D. variabilis* (Johns *et al.*, 2001), while the activity response in *O. moubata* is constitutively expressed (van der Goes van Naters-Yasui *et al.*, 2000). Thus, there is no consensus antibacterial response in ticks, and the differences could be due to environmental factors.

As can be expected, the antibacterial assays were vital components of the study. Since the radial diffusion assay did not have an adequate sensitivity, the broth microdilution assay was predominantly used. Some modifications in the protocol were completed, increasing the handling efficiency without leading to a decrease in sensitivity. The combination of the radial diffusion assay and the broth microdilution assay (Du Toit and Rautenbach, 2000) might lead to a lower threshold of detection, but that level of sensitivity was not required in this study.

The specificity and heat stability of the antibacterial factors suggested that the proteins involved in the immune system might be members of the defensin family. Even though proteolysis of hemolymph proteins did not lead to the complete inactivation of the immune response, the decrease in the activity suggested that the components might be of protein nature. There is also the possibility that the proteins are insensitive to proteolysis, or that there are protease inhibitors present in the hemolymph of the tick. It is unlikely that the proteins might be insensitive to proteolysis, since this is the mechanism through which the concentration of the proteins in other organisms is controlled. The presence of

protease inhibitors in the hemolymph is more feasible, although this has not been conclusively shown to be true. A third possibility is that the hemolymph contains proteases that are responsible for the degradation of the exogenous proteases (trypsin and proteinase K). This aspect has not been studied in detail, but it has been shown that a constitutive level of proteolysis exists in the hemolymph (unpublished results). Thus, the antibacterial factors were hypothesized to be of protein nature.

The initial isolation procedures for the antibacterial factors were not of sufficient effectivity, since the presence of more than one antibacterial fraction were sometimes not detected. This means that the methods were either not sensitive enough, or that the preparation procedures degraded or removed the proteins prior to chromatography steps. The acquisition of a photodiode array detector for the chromatograph also increased the sensitivity of the separation results. The vitellogenins were also a major problem during the size exclusion separation of crude hemolymph. These proteins are one of the main constituents of the protein fraction of the hemolymph, and since mostly female ticks are used during the studies, and the majority of the ticks appeared to be fed at the time of analysis, these proteins were ubiquitous during analyses. The use of the C_{18} sep-pak columns, in conjunction with the acid precipitation step, was very effective in the removal of the vitellogenin proteins, as wells as in retaining the antibacterial factors.

The peptides were not isolated to absolute homogeneity, but the purity of the peptides was sufficient to determine their molecular masses and to obtain the N-terminal amino acid sequence of the one peptide. The purity and yield of the peptides in Fractions 50-A, 90-A and 90-B were not sufficient to allow the determining of the concentrations of these peptides. When comparing the mass analyses of the four peptides, it can be concluded that the defensin B, isolated form Fraction 50-B, is in excess, since the signal to noise ratio of the mass peaks for this peptide is much higher than any of the other peptides.

The database similarity search of the amino acid sequence of defensin B did not result in homology to any other proteins. This was not due to the fact that the peptide is unique, but due to the insufficient sequence length, since a sequence composed of more than 13

amino acids is required to provide significant matches to existing proteins. The alignment of the sequence with that of the known defensin sequences from other tick species (Table 3.2) showed that the isolated peptide displays significant similarity to these sequences. This validates the hypothesis that the *O. savignyi* peptides are members of the defensin family. The absence of concrete sequence data concerning cys-residues is not limiting, since these unknown residues in the *O. savignyi* peptide aligned well with the cys-residues in the other tick defensin sequences. The probability that these unknown residues are cys-residues is therefore very high. The alkylation of the cys-residues prior to amino acid sequencing would confirm the presence of the residues.

The chromatograms for three of the peptides show that the active fractions display multiple absorbing peaks, although the activity range and mass analysis suggest that the peaks are due to the same protein (Fig 3.10, 3.15 and 3.17). As disulfide bonds can be maintained when the protein has been unfolded (Creighton, 1988), this would result in different regions of the protein structure being exposed to the column matrix. This might then lead to the double peaks on the chromatograms, and as both peaks contain the same protein in different conformational states, the activity and mass analysis could be identical. Since this result was obtained by separate collection of the peaks and since the mass analysis on the peaks showed the same profile, it is proposed that this difference in global folding is the reason for the appearance of the double peaks. One way to investigate this is the re-injection of one of the peaks on the chromatograph. There is a possibility that the peptide should again separate in the subsets and display the same peaks in the same ratios. This, however, is not always the case (Mans, B. J. unpublished results), and might mean that the unfolding is according to specific mechanisms for each peptide peak, and that this would stay constant for each conformational state.

The fact that there is more than one defensin peptide in the tick could be through a gene duplication event. Random mutations and/or evolutionary selection could then lead to the polymorphism in the genes. Another possibility is that there is genetic polymorphism in the tick population, and since many individuals were used in the study, the two isomers could be the two main polymorphic subsets. Thus, each tick might have only one

defensin from one of the two subsets and this explains the appearance of more than one defensin peptide. The presence of three different defensins in the tick could also be explained by any of these two mechanisms.

The absence of the lysozyme protein during the isolation of the defensin peptides is interesting. That might mean that the protein does not occur in *O. savignyi*, or that the protein is not present in the hemolymph. Since lysozyme has been isolated from several tick species (Kopácek *et al.*, 1999; Ignatovich *et al.*, 1979; Podboronov *et al.*, 1978), the first hypothesis does not seem valid. The fact that lysozyme was isolated from the midgut of *O. moubata* (Kopácek *et al.*, 1999) seems to indicate that the second hypothesis might be valid. During the isolation studies the midgut was not analyzed for the presence of antibacterial proteins, and that might be the basis of a future investigation of lysozyme in *O. savignyi*. There is also still the possibility that lysozyme was removed during the initial acid precipitation step, and that the lysozymes are present in the hemolymph.

The studies on lysozyme (Kopácek *et al.*, 1999) and defensins (Nakajima *et al.*, 2001) from *O. moubata* showed that both proteins occur in the tick midgut, and that the concentration of both proteins increased in response to tick feeding, and not in response to bacterial infection. The same studies could not be performed on *O. savignyi*, due to the lack of an artificial feeding system for the ticks. This means that the data for the induction of the antibacterial response is incomplete and that a preventative, rather than a responsive role for the defensins is possible. One problem in the search for lysozyme was the absence of an easily available highly specific assay for lysozyme activity. The generally accepted assay for lysozyme is anti-*M. luteus* activity. This is, however, a Gram-positive bacteria and is susceptible to the action of the defensin molecules. This meant that any anti-*M. luteus* activity displayed, indicated a false positive result for the presence of lysozyme in the hemolymph of the tick.

The lysozyme-specific primer that was designed from the multiple sequence alignment analysis of known lysozyme sequences appeared to be acceptable. The stability profile of the designed degenerate primer showed conformity with the requirements, and no

significant self-complementarity could be detected. A possible explanation for the failure of the primer could be due to the two thymidines that are incorporated in the sequence. Although it was successful in other studies, the effectivity has not been confirmed for the lysozyme system, and there is a possibility that the specificity of the primer was compromised. The fact that the primer sequence could not be identified in the sequenced products contradicts this hypothesis, since this suggests that the primer did not contribute significantly to the amplification reactions and that the amplification was totally non-specific. There is also the possibility that the consensus sequence used for the design of the primer is not present in the tick lysozyme sequence. This would negate any primer designed with the help of sequence homology, and the use of the N-terminal amino acid sequence of the isolated lysozyme for primer design is essential.

The results of the sequencing reactions were used in exhaustive similarity searches of the main sequence databases. Not only were the sequences and their complementary and reverse complementary sequences submitted for the database searches, but all the possible translated sequences in all reading frames were also analyzed. This means that even if a gene was cloned in the wrong orientation, the similarity of the sequence with other proteins was tested. The absence of the 5' *Eco*RI in the sequences of clones D 01 (Table 4.2) and D 03 (Table 4.4) is merely an indication that the sequencing of the inserts did not span the entire insert length. The sequencing of the 5' end of clone E 03 was not very efficiently performed, as there are several bases that could not be identified by the sequencing software (indicated by N). These bases were, however, included with the rest of the sequence during the similarity searches since the BLAST algorithm can tolerate a small degree of unspecified (N) bases. The similarity search was also performed on a truncated part of the sequence in which there were no unspecified bases present, and the search still returned no significant similarity with any proteins. The fact that the similarity searches using the sequences of clones E 04 (Table 4.6) and E 05 (Table 4.8) returned similarity with several members of heat shock proteins, especially Hsp 70, can not be considered as significant. The expect value of these similarities were to high ($E>>0.0001$) to suggest any significant matches with known proteins.

There is little possibility of successfully improving on the optimization of the amplification reactions, since the same products were in evidence during all the optimization experiments. There are not many other factors that can be optimized in order to improve the efficiency of the designed LYZ1 primer. This is not a unique situation with *O. savignyi*, since there has been several attempts to use degenerate primers, designed from homologous regions of multiple sequence alignments, in order to amplify and characterize tick proteins. Three exceptions are the cloning of cysteine proteinases (Mulenga *et al.*, 1999; Renard *et al.*, 2000) and a serine proteinase (Mulenga, *et al.*, 2001) from two tick species using primers designed from multiple sequence alignments of the active sites of these proteins. Except for these cases, no other attempts have reported success, and no reasons have been forwarded to explain this lack of success. It is unsure whether the tick has unique structuring of transcripts, or whether they merely have a non-homologous gene sequence to other organisms. The problem is also not due to this particular tick species, since these reports were in evidence during studies on Ixodid as well as Argasid ticks. The solution for these problems might be a fruitful area of investigation, along with the investigation into the absence of the 5*S* and 28*S* ribosomal RNA species of *O. savignyi*.

There are several key research areas on the antibacterial response in *O. savignyi* that could be completed in future. The unequivocal identification of the antibacterial peptides could be completed through the determination of the amino acid sequences of these peptides. The genes encoding peptides of the antibacterial response could be identified and the regulation of these genes studied. The tertiary structures of the peptides could be modeled using the structures of existing peptides as backbone, or the structure of the peptides could be determined using either 3D-NMR or protein crystallography techniques. The specific activity of the peptides could be studied through the use of synthetic membranes (liposomes) to determine the phospholipid requirements for activity. Studies could also focus on the isolation or identification of lysozyme from *O. savignyi*, either by using protein isolation techniques, or through the design of primers specific for lysozyme from other tick species.

SUMMARY

Invertebrates do not possess an adaptive immune system, but rely on several mechanisms similar to the innate immune system of mammals. The synthesis and release of a host of potent antimicrobial proteins is an important component of this immune response.

The antibacterial activity in the hemolymph of *Ornithodoros savignyi* is specific for Gram-positive bacteria, and the synthesis and release of the antibacterial factors need to be induced by challenging the ticks with heat-killed Gram-negative bacterial suspensions. The induction of the factors is very rapid, leading to a maximal response within one hour following bacterial challenge. The factors are stable at high temperatures, and were found to be protein in nature. By using reverse phase high performance liquid chromatography, four fractions exhibiting antibacterial activity were identified in the hemolymph of immune challenged ticks. Four antibacterial peptides were isolated from these fractions, and the mass analyses of the peptides indicate that there are at least two different antibacterial peptides present in the hemolymph. The N-terminal amino acid sequence of one of the peptides was determined, and the analysis showed that the peptide has high homology with defensin peptides isolated from other tick species. This led to the putative classification of the peptides as part of the invertebrate defensin family.

The presence of lysozyme in *O. savignyi* was studied using molecular biological methods. Vertebrate and invertebrate lysozyme sequences were used to design a lysozyme-specific primer, which was used to amplify specific DNA products from whole tick cDNA using the polymerase chain reaction (PCR). The conditions for the amplification reaction were optimized, the products of the optimized reaction were cloned into a cloning vector and the nucleotide sequences of the products were determined. The nucleotide sequences were used for similarity searches of sequence databases to determine homology with sequences of known proteins. It is deduced the degenerate primer was not specific for lysozyme and did not play a significant role in the amplification of the PCR products. This method is thus not feasible for the investigation of the lysozyme of *O. savignyi*.

OPSOMMING

Invertebrate besit nie 'n aanpasbare immuunsisteem nie, maar maak staat op verskeie sisteme wat soortgelyk is aan soogdiere se onaanpasbare immuunsisteem. Die sintese en vrystelling van 'n verskeidenheid antibakteriese proteïne is 'n belangrike komponent van die invertebraat immuunsisteem.

Die antibakteriese aktiwiteit wat voorkom in the hemolimf van *Ornithodoros savignyi* is hoogs spesifiek vir Gram-positiewe bakterieë, en die sintese en vrystelling van die antibakteriese faktore moet geïnduseer word deur die bosluisimmuun sisteem te aktiveer deur die toediening van 'n hitte geïnaktiveerde Gram-negatiewe bakteriese suspensie. Die induksie van die faktore vind baie vinnig plaas, met 'n maksimale respons binne een uur na bakteriese uitdaging. Die faktore is stabiel by hoë temperature en proteïen van aard. Deur gebruik te maak van omgekeerde fase hoë doeltreffendheid vloeistof chromatografie vier fraksies met antibakteriese aktiwiteit in the hemolimf van die bosluise geïdentifiseer. Vier antibacteriese peptiede is vanuit hierdie fraksies geïsoleer. Die massa analises van die peptiede het gewys dat daar minstens twee verskillende peptiede teenwoordig is. Die N-terminale aminosuur-volgorde bepaling van een van die peptiede het gewys dat die peptiede groot homologie toon met defensin peptiede wat vanuit ander bosluis spesies geïsoleer is. Dus word die peptiede voorlopig geklasifiseer as lede van die invertebraat defensin familie.

Die teenwoordigheid van lisosiem in *O. savignyi* lisosiem is ondersoek met behulp van molekulêre biologiese metodes. Verskeie vertebraat- en invertebraat lisosiem volgordes is gebruik om 'n lisosiem-spesifieke voorvoerder te ontwerp wat gebruik is in 'n polimerase ketting reaksie (PKR) om spesifieke DNA produkte te amplifiseer. Die reaksie kondisies is geoptimiseer, die DNA produkte is in 'n kloneringsvektor gekloneer en die nukleotied volgorde van die produkte is bepaal. Die volgordes is gebruik vir databasis soektogte om homoloë proteine op te spoor. Die volgordes het egter nie betekenisvolle homologie getoon met enige bekende volgordes nie. Die voorvoerder was nie spesifiek genoeg vir lisosiem nie en het oneffektief deelgeneem aan die amplifiseringsreaksies. Hierdie metode is dus nie geskik vir die studie van die lisosiem van *O. savignyi* nie.

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APPENDIX 1

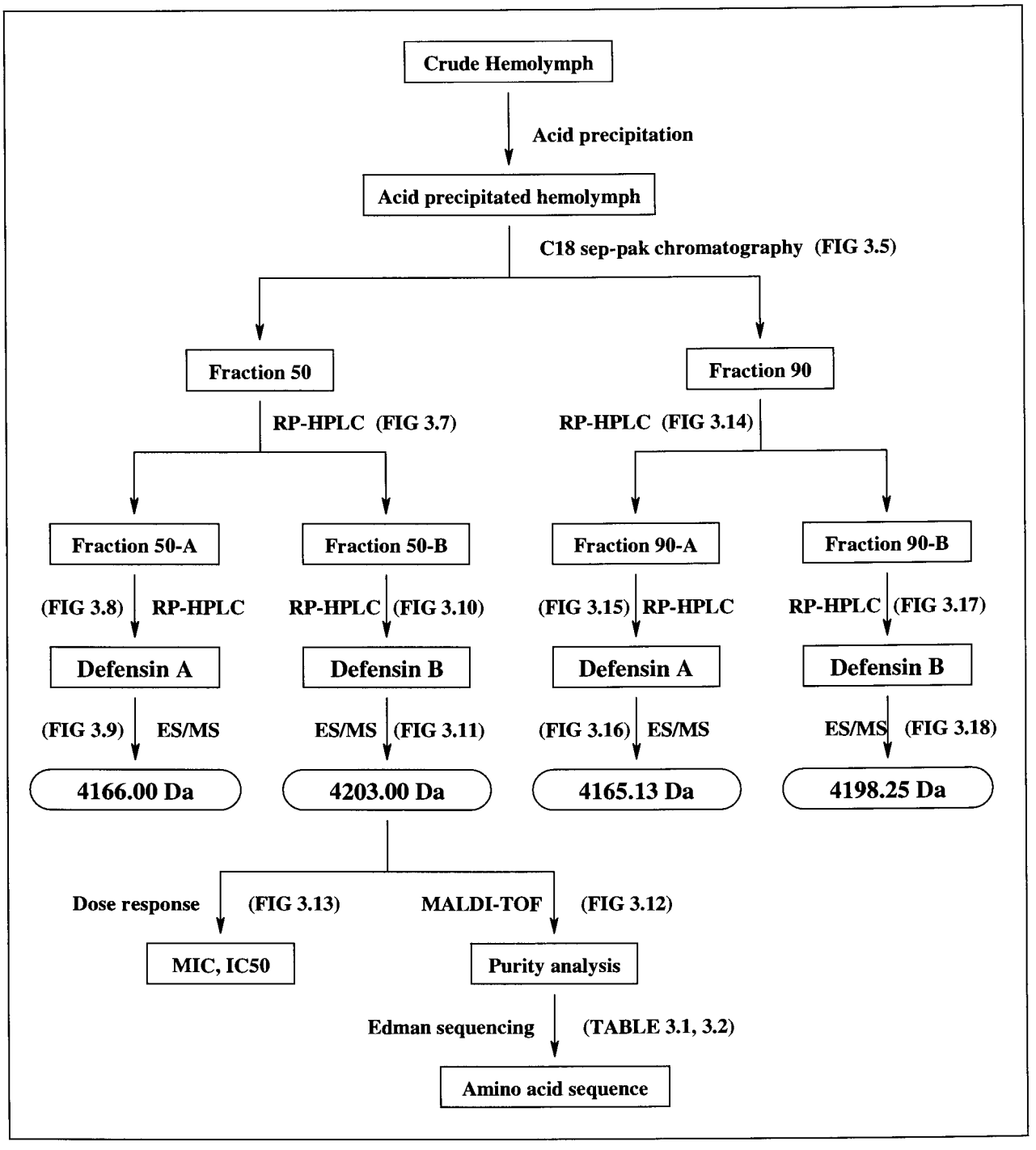

Figure A.1 Isolation procedures of the defensin peptides from the hemolymph of the soft tick *Ornithodoros savignyi*.

APPENDIX 2

Table A.2 Organisms used in the lysozyme multiple sequence alignment (Source: Genbank Database at http://www.ncbi.nlm.nih.gov)

Organism	Common name
Vertebrates	
Bos taurus	Bovine
Cervus axis	Deer
Chlorocebus aethiops	African green monkey
Gallus gallus	Chicken
Homo sapiens	Human
Macaca mulatta	Rhesus monkey
Mus musculus	Mouse
Oncorhynchus mykiss	Rainbow trout
Opisthocomus hoazi	Hoatzin bird
Ovis aries	Sheep
Presbytis entellus	Langur monkey
Rattus norvegicus	Rat
Scopthalmus maximus	Turbot
Sus scrofa	Pig
Trichosurus vulpecula	Brush tailed possum
Invertebrates	
Anopheles	Mosquito
Bombyx mori	Silkworm
Drosophila melanogaster	Fruit fly
Heliothis virescens	Tobacco budworm
Hyalophora cecropia	Giant silk moth
Hyphantria cunea	Fall webworm
Manduca sexta	Tobacco hornworm
Musca domestica	House fly

APPENDIX 3

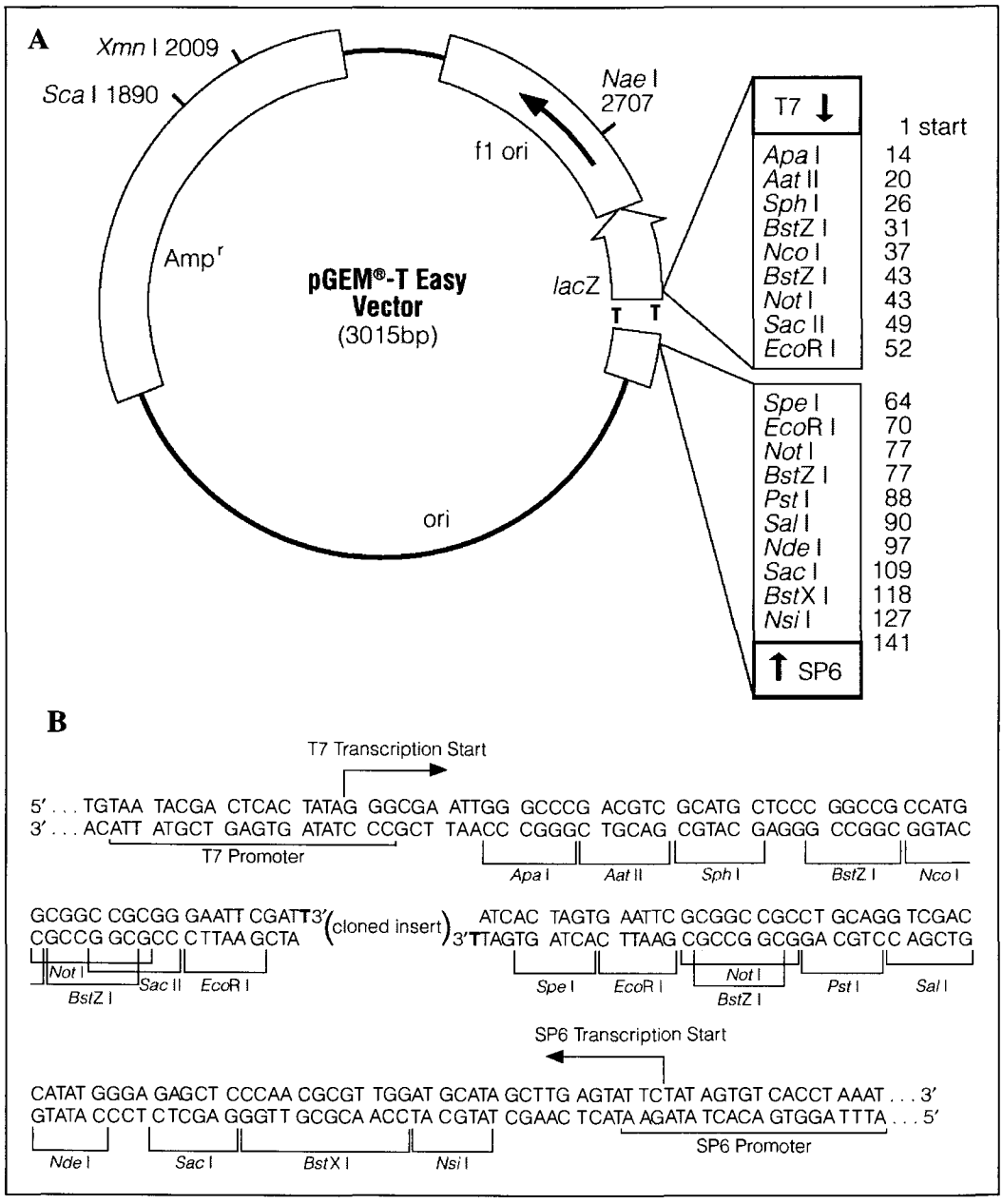

Figure A.3 The (A) Circular map and (B) the multiple cloning site of the pGEM-T Easy vector system used during cloning. The ampicillin resistance (Amp^r) and lacZ genes are indicated on the circular map. The multiple cloning site is flanked by the T7 and SP6 promotors.