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

4.1 Introduction

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

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

Table 4.1: AMPs from soft ticks

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

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

Table 4.2: AMPs from hard ticks

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

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

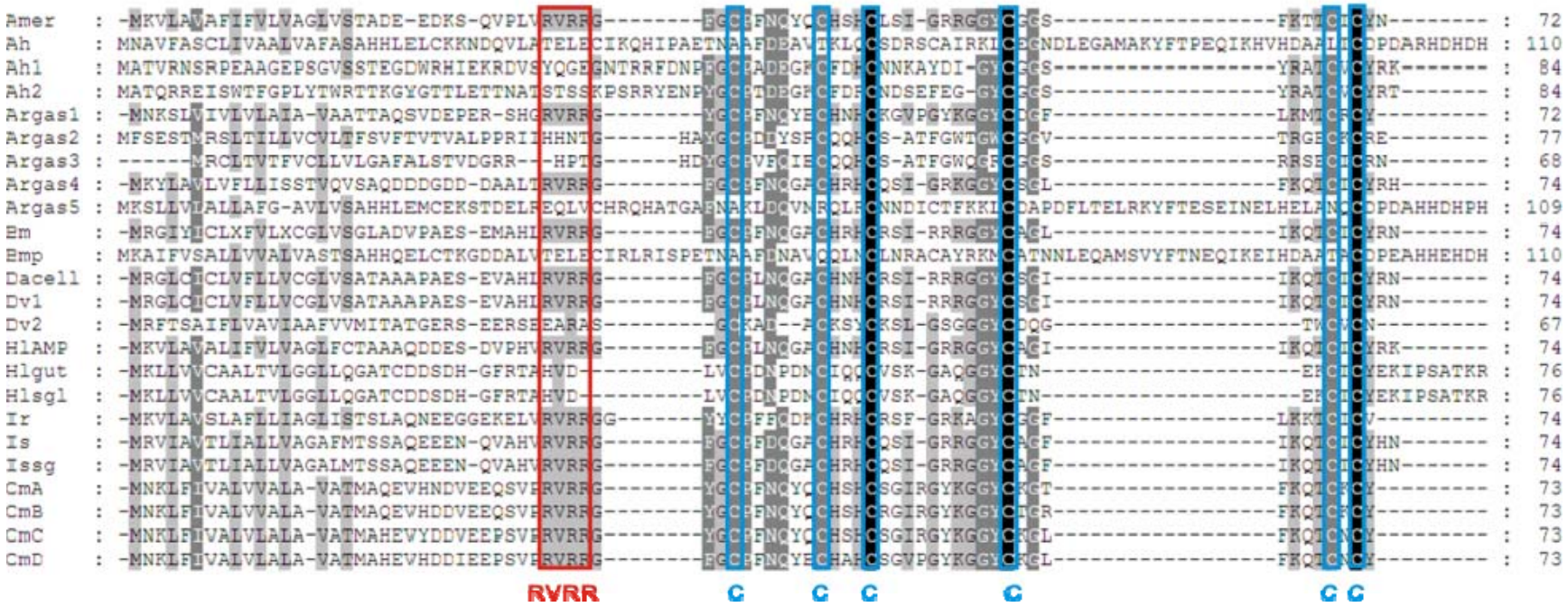

Figure 4.1: Tick defensins and defensin-like sequence alignments. Red indicates the RVRR cleavage site. Blue indicates conserved cysteine residues. Amer (*A. americanum*), Ah (*A. hebraeum*, hebraein), Ah1 and Ah2 (*A. hebraeum*), Argas1, Argas2, Argas3, Argas4, Argas5 (*A. monolakensis*), Bm, Bmp (*R.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

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

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

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

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

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

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

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

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

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

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

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

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

4.2 Hypothesis

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

4.3 Materials and methods

4.3.1 Flow diagram of methods implemented in this chapter

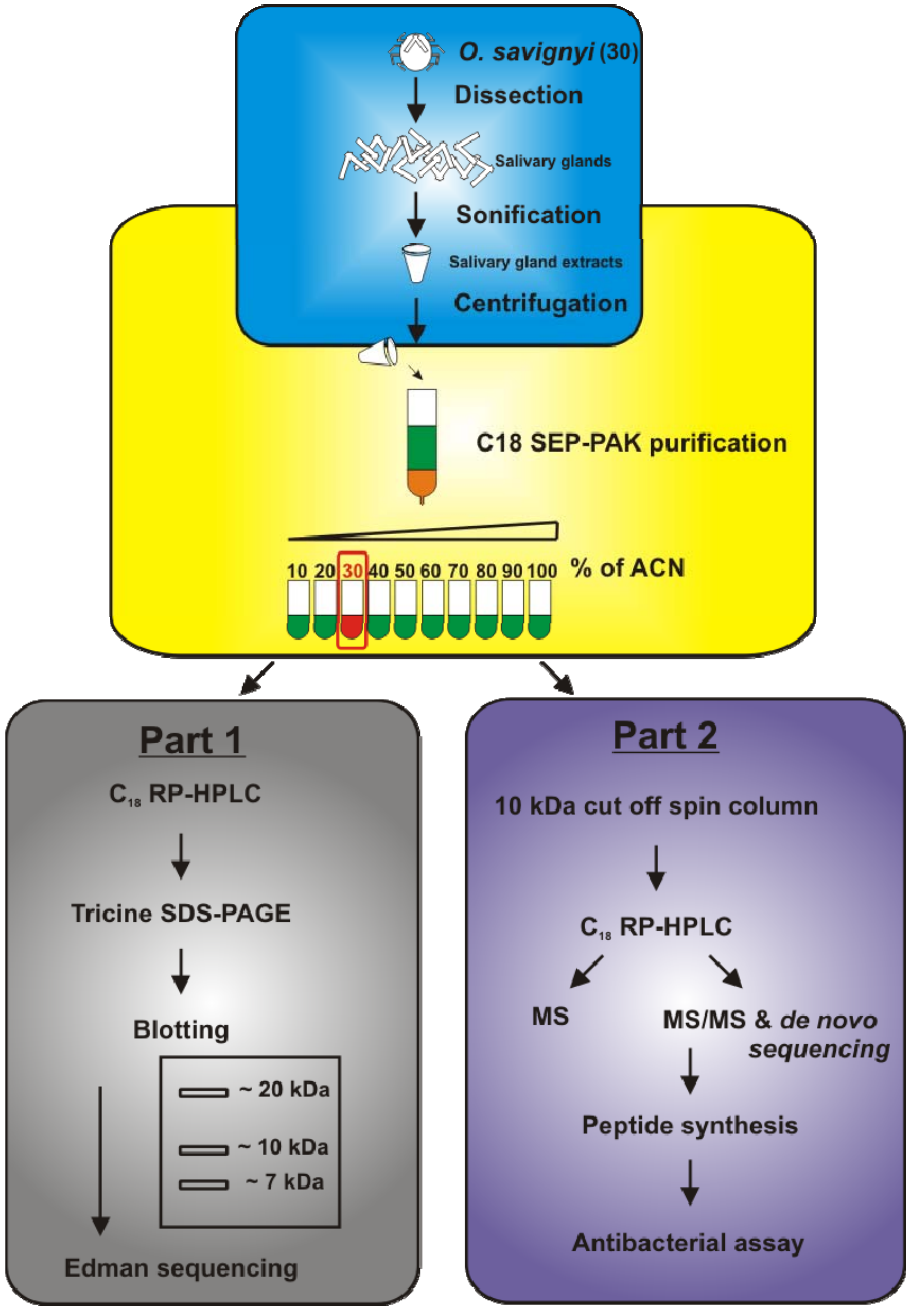

Figure 4.2 Flow diagram of the methods used to identify the Gram-positive antibacterial activity.

4.3.2 Ticks

Ornithodoros savignyi ticks were obtained as described in section 2.3.1

4.3.3 Reagents

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

4.3.4 Sample preparation, collection and extraction

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

4.3.5 Antibacterial assay

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

4.3.6 Purification of the Gram-positive antibacterial activity

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

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

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

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

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

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

4.3.7 Tricine SDS-PAGE analysis

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

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

4.3.8 Blotting of protein bands obtained from tricine SDS-PAGE

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

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

4.3.9 Edman sequencing

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

4.3.10 MS/MS analysis and *de novo* sequencing

MS/MS analysis was performed, at the Technology Facility, Department of Biology, University of York, UK, using the method described by Shevchenko *et al.* (2001). In-solution tryptic digestion was performed after reduction with tris(2-carboxyethyl)phosphine and alkylation with S-methyl methanethiosulfonate. Sequencing-grade, modified porcine trypsin was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold by adding 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.02 μg/μl. Proteins were digested with the addition of 10 μl of trypsin solution, and incubated overnight at 37 ^{o}C.

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

Positive-ion MALDI mass spectra were obtained using a Bruker ultraflex III in reflectron mode, equipped with a Nd:YAG smart beam laser. MS spectra were acquired over a mass range of m/z 800-4000. Final mass spectra were externally calibrated against an adjacent spot containing 6 peptides (des-Arg1-Bradykinin, 904.681; Angiotensin I, 1296.685; Glu1-Fibrinopeptide B, 1750.677; ACTH (1-17 clip), 2093.086; ACTH (18-39 clip), 2465.198; ACTH (7-38 clip), 3657.929.). Monoisotopic masses were obtained using a SNAP algorithm (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583) and a S/N threshold of 2.

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

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

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

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

4.3.11 Anti-bacterial analysis of synthetic actin fragments

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

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

4.4 Results and discussion

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

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

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

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

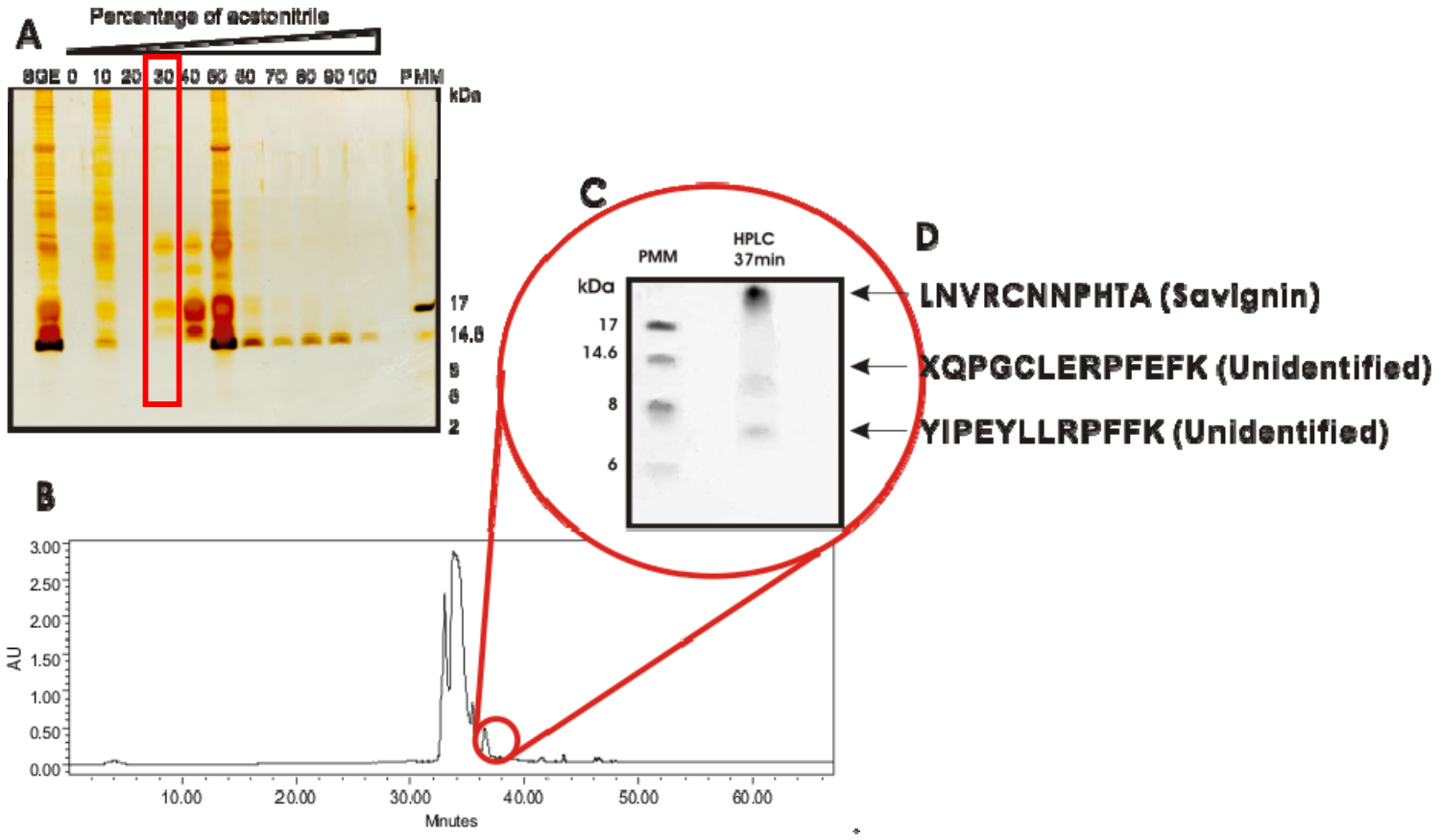

Figure 4.3 Purification of the Gram-positive antibacterial activity from salivary gland extracts and identification using N-terminal sequencing. A. Tricine SDS-PAGE gel of fractions after SEP-PAK C18 purification. B. C_{18} RP-HPLC chromatogram of the SEP-PAK 30 % fraction. Red circle indicates where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

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

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

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

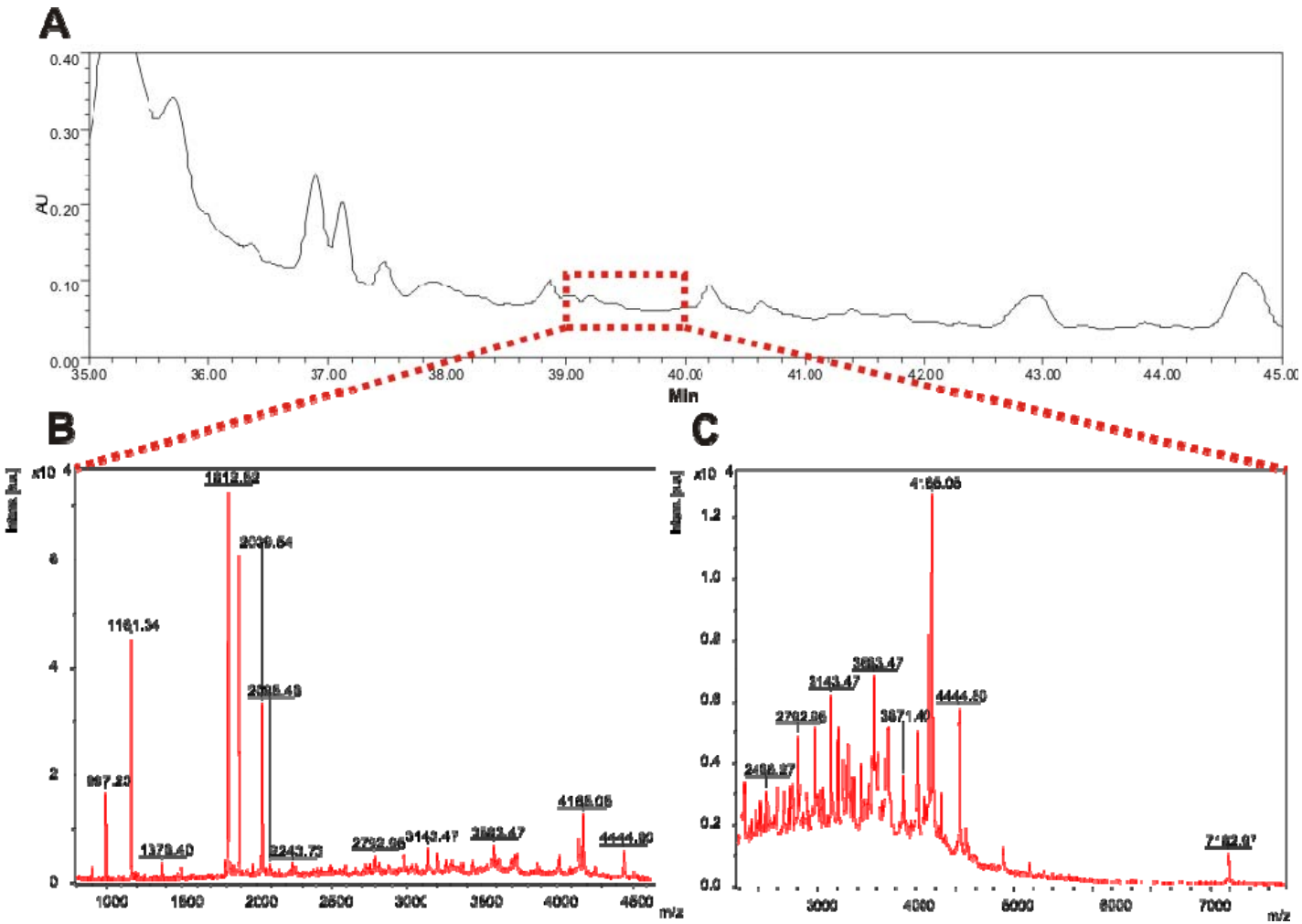

Figure 4.4 Purification of the Gram-positive antibacterial activity from salivary gland extracts and identification using MS analysis. A. C18 RP-HPLC chromatogram of the 10 kDa ultrafiltration permeate. B & C. Mass spectra of the active C18 fraction.

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

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

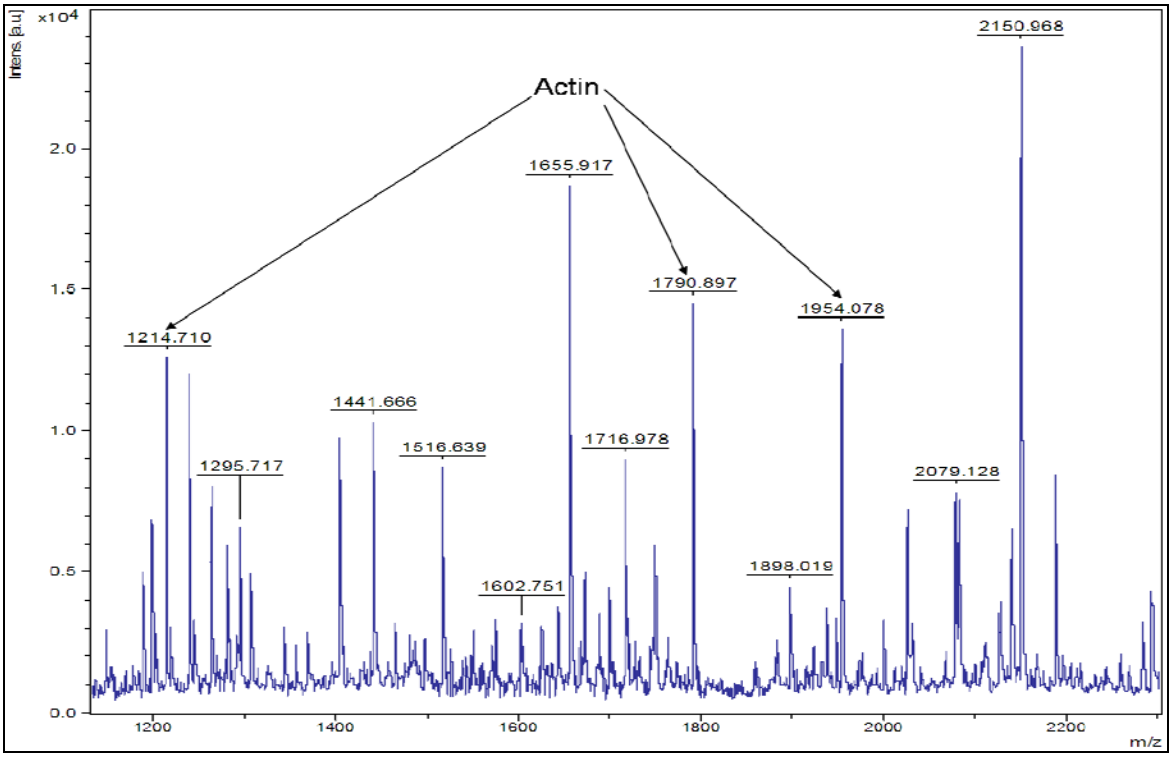

Figure 4.5 MS spectrum showing the three peptides whose MS/MS ion spectra match with actin.

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

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

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

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

[c] Mowse scores greater than 67 are considered significant

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

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

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

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

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

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

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

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

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

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

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

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

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