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

2.1 Introduction

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

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

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

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

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

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

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

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

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

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

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

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

2.2 Hypothesis

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

2.3 Materials and methods

2.3.1 Ticks

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

2.3.2 Reagents

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

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

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

2.3.3.1 Binding assay

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

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

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

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

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

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

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

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

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

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

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

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

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

2.3.4.1 Challenge of ticks by hemocoelic injection

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

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

2.3.4.2 Challenge of ticks by artificial feeding

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

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

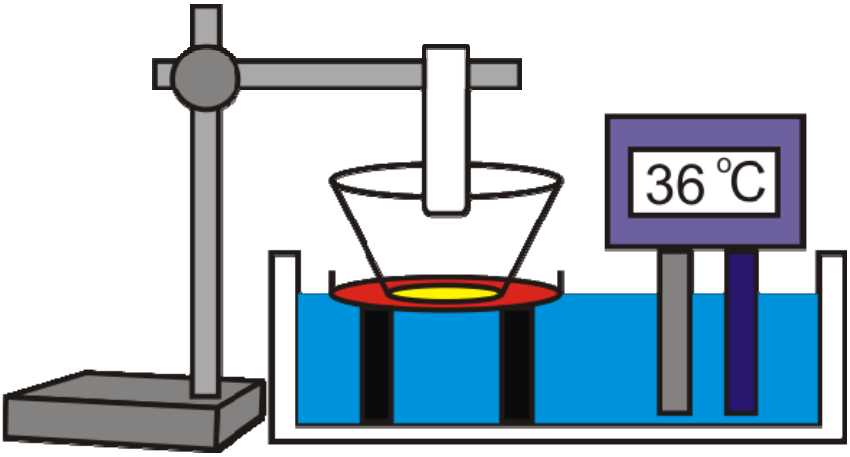

Figure 2.1 An illustration of the feeding apparatus.

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

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

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

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

In-gel tryptic digestion was performed after reduction with dithioerythritol (DTE) and S-carbamidomethylation with iodoacetamide. Gel pieces were washed twice with 50 % (v : v) aqueous ACN containing 25 mM ammonium bicarbonate, then once with ACN and dried in a vacuum concentrator for 20 min. Sequencing-grade, modified porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold by adding 25 mM ammonium bicarbonate to give a final trypsin concentration of 0.01 μg/μl. Gel pieces were rehydrated by adding 10 μl of trypsin solution, and after 30 min enough 25 mM ammonium bicarbonate solution was added to cover the gel pieces. Digests were incubated overnight at 37 ^{o}C.

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

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

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

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

2.3.6 *E. coli* binding proteins in hemocyte extracts

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

2.4 Results and discussion

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

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

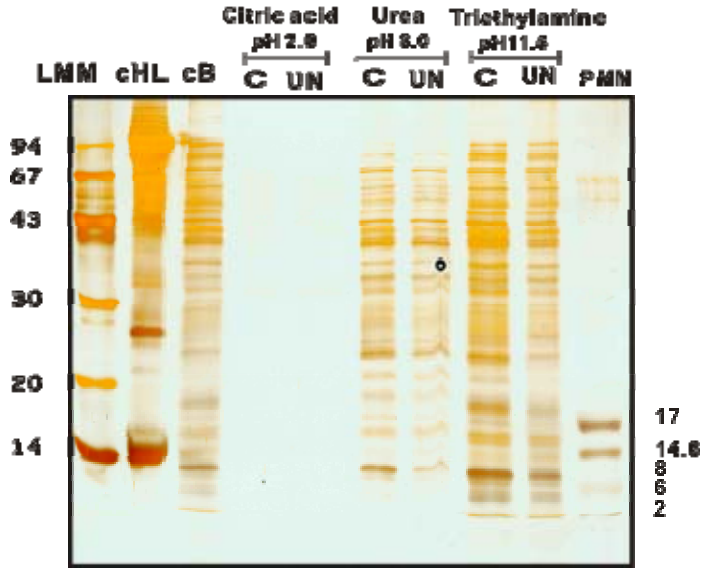

Figure 2.2 SDS-PAGE gel analysis of bacteria binding proteins in plasma of unchallenged ticks. Bacteria cells were incubated with buffer (C) or plasma from unchallenged ticks (UN). Bound proteins were eluted with citric acid (pH 2.0), urea in Tris-HCl (pH 8.0) or triethylamine (pH 11.5) in Tris-HCl.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

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

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

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

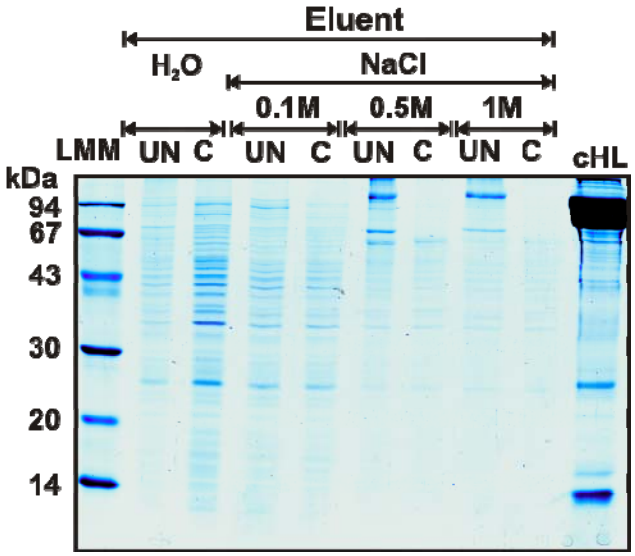

Figure 2.3 SDS-PAGE gel analysis of bacterial binding proteins in plasma of unchallenged ticks. Bacteria cells were incubated with buffer (C) or plasma from unchallenged ticks (UN). Bound proteins were eluted with dddH_2O and NaCl (0.1 M; 0.5 M; 1 M). The eluted proteins were precipitated with acetone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

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

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

2.4.2.1 Hemocoelic challenge

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

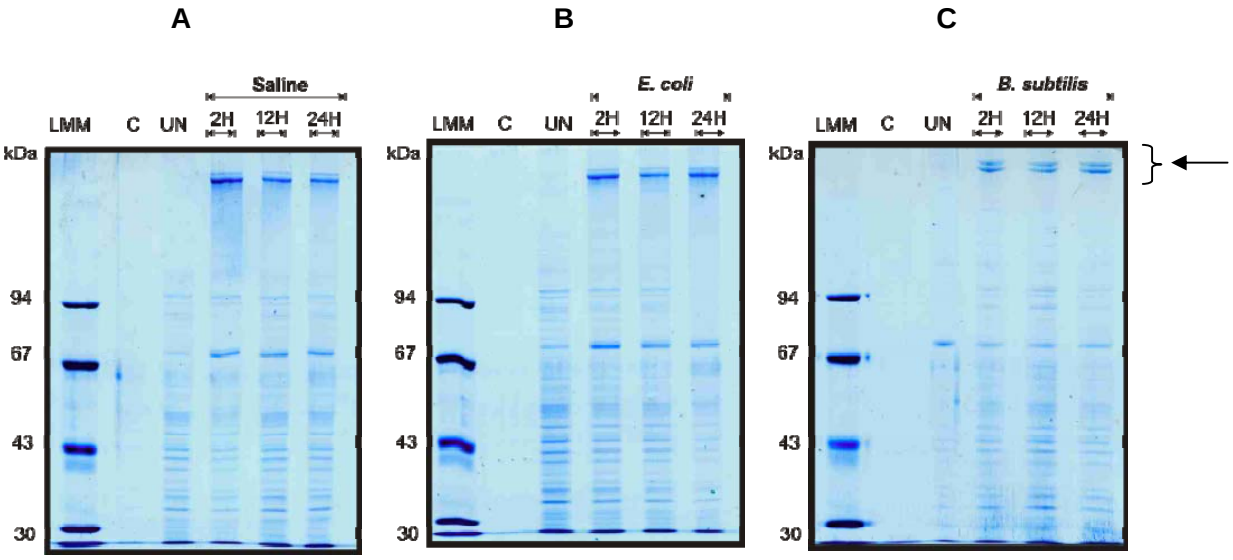

Figure 2.4 SDS-PAGE analysis of bacterial binding proteins, following hemocoelic challenge of ticks. Bacteria cells were incubated with hemolymph plasma obtained from unchallenged ticks (UN) or from ticks injected with either saline (A), heat-killed *E. coli* (B) or *B. subtilis* (C). Hemolymph was obtained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

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

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

2.4.2.2 Challenge by artificial feeding

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

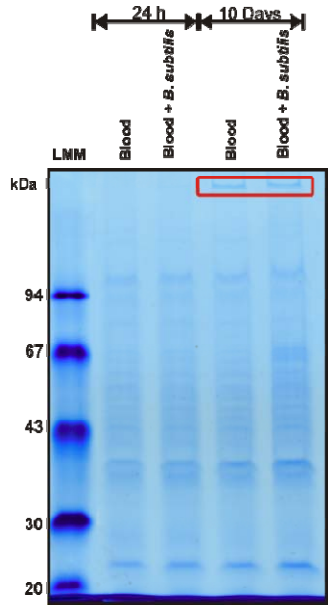

Figure 2.5 SDS-PAGE analysis of bacteria binding proteins following challenge of ticks by artificial feeding. Bacteria cells were incubated with hemolymph plasma obtained from ticks at 24 h and 10 days post-feeding with native blood or blood infected with *B. subtilis*. Bound proteins were eluted with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

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

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

2.4.3 Protein identification

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

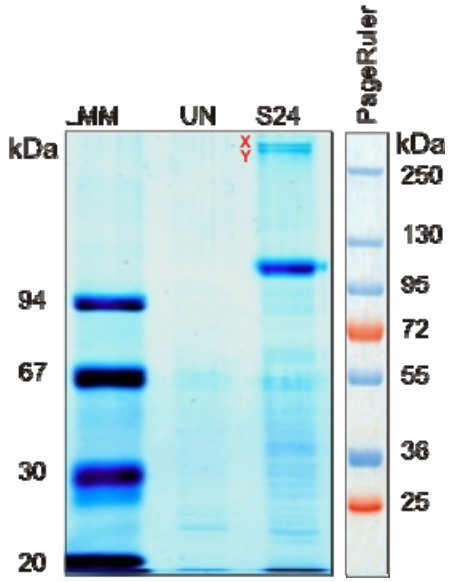

Figure 2.6 SDS-PAGE analysis of bacteria binding proteins following aseptic injury. Bacterial cells were incubated with hemolymph plasma obtained 24 h from ticks injected with sterile saline (S24). The experiment was also performed with plasma obtained from unchallenged ticks (UN). Bound proteins were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

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

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

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

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

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

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

[c] Mowse scores greater than 67 are considered significant

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

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

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

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

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

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

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

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

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

[c] Mowse scores greater than 67 are considered significant

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

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

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

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

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

2.4.4 The source of Protein X and Y

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

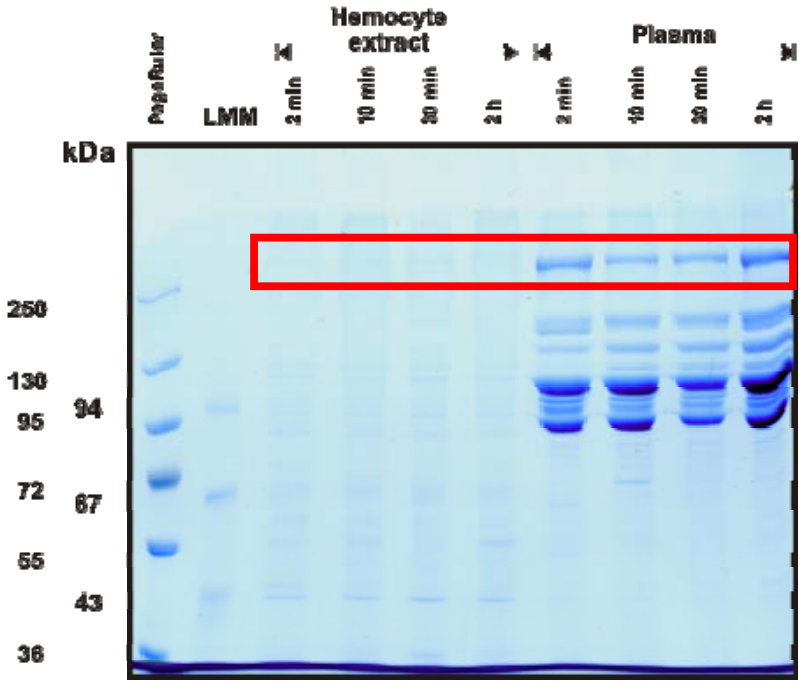

Figure 2.7 SDS-PAGE analysis of hemolymph plasma and hemocyte proteins obtained from ticks at various time intervals (2 min, 10 min, 30 min and 2 h) following aseptic challenge. Samples (10 μg protein per lane) were loaded onto a 7.5 % gel. Bands were visualized by colloidal Coomassie staining. Low molecular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

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

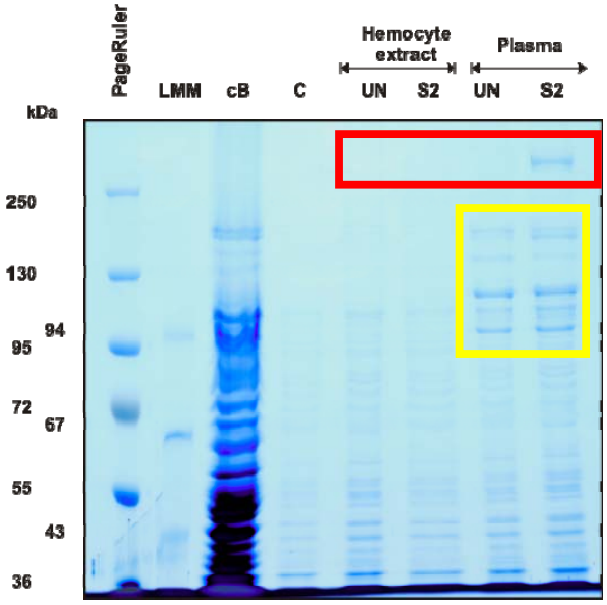

Figure 2.8 SDS-PAGE analysis of hemolymph plasma and hemocyte proteins obtained after 2 h following aseptic challenge. Bacterial cells were incubated either with hemolymph plasma or hemocyte extracts obtained 2 h from ticks injected with sterile saline (S2). The experiment was also performed with plasma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

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