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

By

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Abbreviations

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

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

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

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Summary

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

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

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

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

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

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