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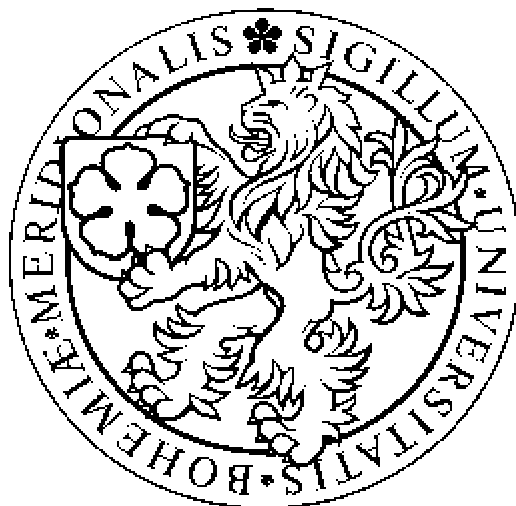

Towards the functional characterization of a “cysteine rich” protein family member from *Ixodes ricinus*

Bachelor Thesis in Biological Chemistry

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Annotation:

The function of a cysteine rich family member from the tick *Ixodes ricinus* was investigated using RNA interference and serine protease inhibition assays of the recombinant polypeptide. Both methodologies suggest that this protein most likely does not influence tick's feeding ability.

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Table of contents

1. Introduction 1
 1.1. Ticks as disease vectors 1
 1.2. Information about the studied gene 2
2. Methods 3
 2.1. Chemicals used 3
 2.2. General methods 7
  2.2.1. Polymerase chain reaction (PCR) 7
  2.2.2. Agarose Gel Electrophoresis 8
  2.2.3. Cleavage of DNA with restriction enzymes 8
  2.2.4. Ligation 8
  2.2.5. Transformation 9
  2.2.6. Polyacrylamide gel electrophoresis (SDS-PAGE) 9
 2.3. Methods used for RNA interference experiment 9
  2.3.1. Purification of linearized plasmid 9
  2.3.2. ssRNA synthesis 10
  2.3.3. Purification of ssRNA 10
  2.3.4. Hybridization 10
  2.3.5. RNA interference 10
 2.4. Methods used for protein expression experiment 11
  2.4.1. Protein expression 11
  2.4.2. Inclusion bodies isolation 11
  2.4.3. Protein refolding 11
  2.4.4. Fast Protein Liquid Chromatography 11
  2.4.5. Serine protease inhibition assays 12
3. Results 13
 3.1. RNA interference 13
 3.2. Protein expression and serine protease inhibition assays 18
4. Conclusion and discussion 23
5. References 24

Goals of the work

1. Prepare dsRNA for knocking-down a cysteine rich protein family member from *Ixodes ricinus.*
2. Prepare the recombinant protein using a prokaryotic expression system.

Table of used shortcuts

PCR – polymerase chain reaction

dNTPs - deoxynucleotides

ssRNA – single strand RNA

dsRNA – double strand RNA

mRNA – messenger RNA

siRNA – small interfering RNA

ssDNA – single strand DNA

dsDNA – double strand DNA

cDNA – complementary DNA

SDS – sodium dodecyl sulphate

PAGE – polyacrylamide gel electrophoresis

DEPC - Diethylpyrocarbonate

rpm – rotations per minute

RT – room temperature

OD – optical density

IPTG - Isopropyl β-D-1-thiogalactopyranoside

LB medium – lysogeny broth medium

DTT – dithiotreitol

FPLC – fast protein liquid chromatography

HPLC – high performance liquid chromatography (high pressure liquid chromatography)

RISC – RNA-induced silencing complex

GFP – green fluorescent protein

1. Introduction

1.1. Ticks as disease vectors

Ticks are blood-sucking ectoparasites that parasitize vertebrates. They are the largest-bodied members of the entire *Acari* order (class *Arachnida,* phylum *Arthropoda*), and some of them grow to 2–3 cm in length during a blood meal. Their chelicerae are smooth and adapted for slicing the host skin, and altogether form the capitulum.

The ixodids, one of two tick families, are also known as hard ticks because of a sclerotized shield covering the entire dorsum. They parasitize reptiles, birds, and mammals. They generally remain attached to their hosts for days or even weeks, feeding on blood [1].

The European tick, *Ixodes ricinus,* is a three-host tick with a 2-6 year life cycle depending on its location. This tick has separate spring and fall feeding populations. After molting, the 6 legs larvae ascend to the grass and twig to assume a good position for host seeking but, here, they tend to lose body water causing them to descend again to a microclimate on or near the ground where the relative humidity is high. The 8 legs nymphs seek a host the next spring or fall, engorge for 3-5 days, then they drop off the host and they molt. The resulting adults (also 8 legs) seek a host the next year. Only adult females feed on hosts; however males can also be found on them trying to copulate. Females feed from 5 to 14 days and they usually lay between 500 and 2000 eggs. Larvae hatch by late spring or late fall, but they usually start feeding during the following spring or fall [2,3].

Figure 1: Life stages of Ixodes ricinus. Marks on the left side and the bottom represent millimetres. A – larva unfed, B – nymph unfed, C – nymph engorged, D – male unfed, E – female unfed, F – female engorged. (http://www.dartmoorcam.co.uk/dartmoortickwatch)

Vertebrate blood is the source of nutrients for ticks. As an adaptation to blood feeding, tick salivary glands secrete a complex mixture of salivary components. These salivary compounds help the tick to overcome their host's defences against blood loss (hemostasis). They also suppress inflammatory reactions at the feeding site that may cause pain and itching or disrupt blood flow. As a result the saliva of ticks contains anti-clotting, antiplatelet, vasodilatory, anti-inflammatory and immunomodulatory components [4].

Ticks transmit important diseases. Disease agents transmitted to animals by ticks include viruses, bacteria, rickettsiae, fungi, and protozoans. Helminths can rarely also been found in ticks. One of most common diseases in the Central Europe is the Lyme disease caused by the bacteria *Borrelia* transmitted by *I. ricinus* [5].

1.2. Information about the studied gene

Contig-763 is a cysteine-rich protein family member. It is a gene putatively expressed in the salivary glands of *I. ricinus.* My co-supervisor Jindřich Chmelař undertook a highthroughput transcriptome analysis targeting *I. ricinus* salivary glands where he described 268 contigs with two or more sequences and 1006 singletons [10]. All genes await their functional characterization and one of them was contig-763. The sequence of the encoded polypeptide is shown in Table 1.

Table 1: Sequence of contig-763

1-20	M	I	R	M	M	I	L	P	M	S	V	V	L	L	A	T	S	D	Y	I
21-40	H	A	T	D	C	S	T	G	L	E	S	Y	M	K	T	K	C	M	G	L
41-60	R	L	R	F	L	E	R	S	D	C	T	F	T	C	E	G	V	N	S	A
61-80	G	Q	N	Q	T	T	K	L	K	L	V	D	G	L	P	C	G	T	C	K
81-100	E	C	C	D	G	T	C	T	L	V	H	F	S	S	L	Y	P	P	T	L
100-105	K	S	C	A	K															

The protein physicochemical parameters were determined using the web-server us.expasy.org and they are listed in Table 2.

Table 2: Parameters of contig-763

molecular weight	11515.5
pI	8.04
Length	105 amino acids
extinction coefficient [M^{-1} cm^{-1} at 280nm]	5095
abs 0.1% (=1 g/l)	0.442

2. Methods

2.1. Chemicals used

Table 3: Used solutions, chemicals, media, vectors, cells and kits

PCR, agarose gel electrophoresis	
2x PCR Mastermix (Fermentas)	0.05 u/µl *Taq* DNA Polymerase, Reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP
10x High Fidelity polymerase (Fermentas)	2 u/µl HiFi polymerase in 20 mM Tris-HCl (pH 7.4 at 25 °C), 0.1 mM EDTA, 1 mM DTT, 100 mM KCl, stabilizers, 200 µg/ml BSA and 50 % glycerol
dNTPs (Fermentas)	100mM aqueous solution, pH7
Nuclease-free water (Fermentas)	
1x TAE buffer	40mM Tris-HCl, 1mM EDTA
Ethidium bromide	10mg/ml aqueous solution
Agarose for DNA electrophoresis (Serva)	
6x DNA loading dye (Fermentas)	10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA
DEPC water	0.1% v/v diethylpyrocarbonate in water
100bp Plus DNA Ladder (Fermentas)	0.5 µg/µl in 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA
Platinum supermix (Invitrogen)	22 u/ml complexed recombinant Taq DNA polymerase with Platinum® Taq Antibody, 22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl2, 220 µM dGTP, 220 µM dATP, 220 µM dTTP, 220 µM dCTP, and stabilizers

Restriction, ligation	
10x Tango buffer (Fermentas)	330 mM Tris-acetate (pH 7.9 at 37°C), 100 mM Mg-acetate, 660 mM K-acetate, 1 mg/ml BSA
Restriction endonucleases	ApaI, XbaI, XhoI, NdeI (Fermentas), 10u/µl
Vectors	PET17 (Merck), PLL10

T4 ligase (Fermentas)	5 u/µl T4 ligase in 10 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM KCl, 0.1 mM EDTA and 50% (v/v) glycerol
10x T4 ligase buffer (Fermentas)	400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8 at 25°C)

Bacterial cultivation	
Competent cells	*E.coli* TOP10 (Invitrogen)
SOC medium (Invitrogen)	2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM glucose
LB medium	1% tryptone, 0.5% yeast extract, 1% NaCl, pH7
LB agar	1.5% agar in LB medium
1000x Ampicillin	100mg/ml Amp. in water

dsRNA synthesis	
Proteinase K	20 mg Proteinase K in 150ml 10mM Tris-HCl (pH8), 2mM $CaCl_2$
10% SDS (Sodium dodecyl sulphate)	
Phenol-Chloroform 5:1 (Sigma)	
Chloroform (Sigma)	
Isopropanol (Sigma)	
80% ethanol (-20°C)	
DEPC water	0.1% v/v diethylpyrocarbonate in water

Protein expression, purification and refolding	
Competent cells	*E.coli* BL21-pLysS (Invitrogen)
SOC medium (Invitrogen)	2% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 20mM glucose
LB medium	1% tryptone, 0.5% yeast extract, 1% NaCl, pH7
LB agar	1.5% agar in LB medium
1000x Ampicillin	100 mg/ml Amp. in water
1000x Chloramphenicol	35 mg/ml Chl. in water
IPTG (Invitrogen)	Isopropyl β-D-1-thiogalactopyranoside in water, 1mM
Lysis buffer	0.4M NaCl, 0.1M KCl, 10% glycerol, 0.5% Triton X-100, 10mM imidazol
Resuspending buffer	20mM Tris-HCl, pH8

Isolation buffer	2M urea, 20mM Tris-HCl, 0.5M NaCl, 10mM imidazol, 1mM 2-mercaptoethanol, 2% Triton X-100, pH8
Solubilizing buffer	6M guanidine hydrochloride
Reducing agent	100x DTT (Dithiotreitol) 1M, (Fermentas)
Refolding buffer	50mM Tris-HCl, 300mM NaCl, pH7.4
Dialysis buffer	50mM sodium acetate
Buffer A for FPLC	50mM sodium acetate, pH6
Buffer B for FPLC	50mM sodium acetate, 1M NaCl, pH6

SDS-PAGE	
Sample buffer	4x NuPAGE LDS Sample buffer (Invitrogen)
Running buffer	1x NuPAGE Bis-Tris Running buffer (Invitrogen)
Reducing agent	10x NuPAGE Sample Reducing agent (Invitrogen)
Molecular weight marker	See Blue Plus 2 Prestained Standard (Invitrogen)
Gel for SDS-PAGE	NuPAGE precast Bis-Tris gel, 12% (Invitrogen)
Staining solution	0.05% Coomassie Brilliant Blue R-250, 50% methanol, 10% acetic acid
Destaining solution	20% MeOH and 5% acetic acid in water

Serine protease inhibition assay	
Enzyme	**Assay buffer**
elastase, proteinase 3, chymase	50 mM Hepes buffer, pH 7.4, 100 mM NaCl, 0.01% Triton X-100
trypsin, a-chymotrypsin, factor XIa, factor XIIa, thrombin	50 mM Tris-HCl, pH 8, 150 mM NaCl, 20 mM $CaCl_2$, 0.01% Triton X-100
β–tryptase	50 mM Tris-HCl, pH 8, 50 mM NaCl, 0.05% Triton X-100
kallikrein, matriptase	20 mM Tris–HCl, pH 8.5, 150 mM NaCl, 0.02% Triton X-100
factor Xa	20 mM Tris-HCl, pH 8, 200 mM NaCl, 5 mM $CaCl_2$, 0.1% BSA
uPA, tPA	20 mM Tris-HCl, pH 8.5, 0.05% Triton X-100
cathepsin G	50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Triton X-100

Enzyme	Substrate
elastase, proteinase 3	Suc-Ala-Ala-Pro-Val-AMC
thrombin	Boc-Asp-Pro-Arg-AMC
trypsin, factor Xia, uPA (Sigma)	Boc-Gln-Ala-Arg-AMC
β–tryptase	Boc-Phe-Ser-Arg-AMC
chymase (Bachem Bioscience, Inc.)	Suc-Leu-Leu-Val-Tyr-AMC
a-chymotrypsin and chymase (EMD Biosciences)	Suc-Ala-Ala-Pro-Val-AMC
factor Xa, factor XIIa, t-PA, matriptase, and kallikrein (American Diagnostica Inc.)	methylsulfonyl-D-cyclohexylalanyl-Gly-Arg-AMC acetate

Enzyme	Concentration, producer
Thrombin	0.01nM; Sigma
α-chymotrypsin	0.025nM; Sigma
chymase	0.9nM; Sigma
b-tryptase	0.01nM; Promega
factor Xa	0.2nM; EMD Biosciences
factor XIIa	0.1nM; haematologic Technologies Inc.
kallikrein	0.04nM; Fitzgerald Industries Int.
elastase	0.18nM; Elastin Products
cathepsin G	4.4nM; Molecular Innovations
Factor XIa	0.06nM; Molecular Innovations
uPA	0.125nM; Molecular Innovations
tPA	0.01nM; Molecular Innovations
matriptase	0.1nM; R&D Systems
proteinase 3	5.8nM; Merck
sequencing-grade trypsin	0.1nM; Roche

Used kits	
Kit:	**Used for:**
JET Quick-Gel Extraction kit (Genomed)	Purification of DNA from an agarose gel
JET Quick PCR product purification spin kit/250 (Genomed)	Purification of product after the cleavage with restriction enzymes
JET Quick-Plasmid Miniprep (Genomed)	Isolation of plasmid from bacteria (miniprep)
JET Star LFU/Plasmid purification MAXI Kit/20 (Genomed)	Plasmid purification, isolation of plasmid form bacteria (maxiprep)
MEGAscript T7 High yield Transcription Kit (Ambion)	ssRNA synthesis

2.2. General Methods

2.2.1. Polymerase Chain Reaction (PCR)

PCR is a technique widely used in molecular biology. It is a method used for amplifying specifically a selected DNA sequence. It is based on a repeating enzyme synthesis of new chains of DNA in the direction 5'→3' using the enzyme DNA polymerase.

First, dsDNA must undergo heat denaturation to single strands. Two short, chemically synthesized oligonucleotides (called primers) bind to the complementary strands of DNA and define specifically the nucleotide sequence fragment to be amplified. An enzyme, called *Taq* DNA polymerase (isolated from *Thermus aquaticus*), can extend the sequence defined by the primers at temperatures up to 72 °C and resists temperatures needed for DNA denaturation. The synthesis of new strands on both matrix chains proceeds in the presence of deoxynucleotides and DNA polymerase. Repeated cycles of DNA denaturation, primer annealing and elongation quickly amplify the sequence of interest. During each cycle, the number of copies of the desired sequence is doubled; therefore, the amount of sequence copies increases exponentially, while all other sequences in the original DNA sample remain unamplified [6, 7].

For PCR amplification of DNA sequences that were used for RNAi experiments, 1 and1 µl of 0.01 mM of each of the two primers, 12.5 µl PCR mastermix, 1 µl cDNA (ca 4ng cDNA) and 9.5 µl H_2O were used. For PCR amplification of DNA sequences to be used for gene overexpression in bacteria, 2 and 2 µl of 0.01mM of each of the two primers, 3 µl ssDNA (from ticks after 6 days of feeding, ca 4ng DNA) and 43 µl Platinum Supermix were used. Settings for the cycler are presented in Table 4. Steps 2-4 were repeated 30times; Steps 1, 5 and 6 were run just once as initial/terminal steps.

Table 4: Settings in the PCR cycler

Step	1	2	3	4	5	6
Time	2 min	30 s	30 s	1 min	8 min	-
Temperature	94°C	94°C	55°C	72°C	72°C	4°C

The sequences of primers were:

RNAi rev: 5'-TTG GGC CCT TAT TTT GCG CAT GAC TTC-3'

RNAi fwd: 5'-AAT CTA GAA CCG ACT GCA GTA CCG GAC-3'

exp. rev: 5'-TTC TCG AGT TAT TTT GCG CAT GAC TTC AG-3'

exp. fwd: 5'-AAC ATA TGA CCG ACT GCA GTA CCG GAC-3'

2.2.2. Agarose Gel Electrophoresis

Gel electrophoresis is a separation method most commonly used for biological macromolecules like DNA, RNA or proteins. Agarose gels are suitable for separation of nucleic acid molecules from 100bp to 50kb long. The speed that the DNA (or RNA) molecules migrate in the gel is called electrophoretical mobility and is directly proportional to the size of the molecule. The DNA charge can be neglected since DNA molecules have constant charge/length ratio. Its size can therefore be estimated by comparison of its electrophoretical mobility with the mobility of standard DNA fragments known also as molecular weight markers. Ethidium bromide is commonly added to agarose gel to bind to the DNA. After excitation by UV light DNA bands (with bound ethidium bromide) emit fluorescence [7].

For gel preparation 0.5g agarose, 0.1 μl ethidium bromide and 50 ml TAE buffer (1x) were dissolved and heated. The sample was mixed with 5 μl DNA loading dye (6x) and loaded into the wells of a gel. 10 μl of 100bp Plus DNA Ladder were used as a molecular weight marker. Electrophoresis was performed at constant voltage of 100V for 30min. Bands of DNA were visible under UV light. DNA was isolated from the gel using the JET Quick-Gel Extraction kit.

2.2.3. Cleavage of DNA with restriction enzymes

Restriction endonucleases are sequence specific enzymes that are able to cleave dsDNA. These enzymes create either blunt ends (both DNA strands cut at the same length) or sticky ends (one DNA strand is longer) [7]. All enzymes used for the experiment form sticky ends and they are therefore more suitable for sticky-end ligation, a procedure with which an exogenous piece of DNA is inserted in a bacterial plasmid (please see below).

The enzymes ApaI and XbaI were used for either DNA or plasmid cleavage. The reaction mixture, containing 6 μl each enzyme (10u/μl), 5 μl Tango buffer (10x), 10 μg DNA sample (or plasmid) and water up to 50 μl, was incubated for 2h at 37°C followed by inactivation of enzymes at 70°C for 10min. The JET Quick PCR product purification spin kit/250 was used for the purification of the cleaved DNA.

2.2.4. Ligation

Ligation is a process of inserting a DNA fragment into a plasmid. Both, plasmid and desired DNA piece have the same sticky ends made in the previous step. Complementary short ssDNA sequences at the ends of DNA molecules are connected using an appropriate enzyme - T4 DNA Ligase.

Mixture of 15 μl digested DNA, 2 μl restricted plasmid, 2 μl T4 DNA Ligase buffer (10x) and 1 μl ligase T4 DNA Ligase (2x) was incubated at 4°C overnight.

2.2.5. Transformation

Transformation is a technique used to introduce a foreign plasmid into bacteria. Bacteria with an artificially disrupted cell wall (also known as competent bacteria) allow us to insert plasmids into prokaryotic organisms. The bacteria amplify the plasmid and make many copies of it. The whole methodology is based on the natural ability of a plasmid to transfer genetic information among bacteria.

E.coli competent bacterial cells were used as acceptors of plasmid; TOP10 cells for RNAi and BL21-pLysS cells for protein expression. 50 μl of bacterial cells were mixed with 10 μl ligation product and incubated on ice for 30 min. Heat shock at 42°C was done afterwards for exactly 1 min. The plasmids enter the cells at this step. Sample was kept on ice again, for 2 min. 125 μl of SOC medium was added and the mixture was shaken in an incubator for 1 hour at 37°C. After that the bacteria were transferred onto an agar plate and incubated overnight at 37°C in the presence of the appropriate antibiotic (ampicillin).

2.2.6. Polyacrylamide gel electrophoresis (SDS - PAGE)

PAGE is an important technique in many areas of molecular biology. In DNA sequencing it provides a fundamental method of nucleic acid separation. It is also an important tool for protein separation, especially with the detergent sodium dodecyl sulphate (SDS). SDS binds to protein samples end it ensures protein denaturation and identical charge per unit mass of protein which results in fractionation by size after applying an electrical current in standard SDS-PAGE protocols [8].

12% polyacrylamide gels were used for protein separation. Protein samples were mixed with NuPAGE sample buffer (4x) and NuPAGE reducing agent (10x). The mixture was then denatured for 10 minutes at 70°C. Electrophoresis was performed at constant voltage of 150V for 35 minutes in an electrophoretic tank filled with SDS-PAGE running buffer. After the run, gels were stained using Coomassie Brilliant Blue and the protein bands were visualized by washing the gel in a Coomassie destaining solution.

2.3. Methods used for RNA interference experiments

2.3.1. Purification of linearized plasmid

25 μl proteinase K and 3.75 μl of 10% SDS were added to 50 μl mixture containing 10μg linearized plasmid and incubated for 30 min. 80 μl phenol-chloroform was added, vortexed

and centrifuged at max speed (15000 rpm) for 5min. The aqueous phase was removed and mixed with 80 µl of chloroform and centrifuged again. 80 µl isopropanol was added to the aqueous phase after centrifugation, mixed and incubated at RT for 15min. After 30 min centrifugation at maximum speed, the resulting pellet was washed with 80% ethanol (-20°C) and centrifuged at max speed for 8 min. The dry pellet was then dissolved in 20 µl DEPC water. Concentration was determined using a Nanodrop® instrument (Thermo Scientific).

2.3.2. ssRNA synthesis

Synthesis of ssRNA was performed using the MEGAscript T7 High yield Transcription Kit (Ambion). For the reaction 2 µl of each dNTP, 2 µl buffer, 1 µg linearized plasmid, 2 µl enzyme mix and water up to 20 ml were used. All used chemicals were part of the kit. The mixture was incubated at 37°C for 8 hour or overnight. This step was done for the plasmid that was previously digested with ApaI and XbaI.

2.3.3. Purification of ssRNA

1 µl DNAse (from the MEGAscript T7 High yield Transcription Kit , 2 u/µl) was added to the reaction mixture and the whole sample was incubated at 37°C for 15min. After that 115 µl DEPC water, 15 µl ammonium acetate (from the same kit) and 150 µl phenol-chloroform was added, mixed by vortexing and centrifuged at maximal rpm for 5 minutes. The aqueous phase was removed and mixed with 150 µl chloroform and centrifuged again. 110 µl isopropanol was added to the aqueous phase after centrifugation, mixed and incubated at room temperature (RT) for 15min. The sample was centrifuged for 30 min and the resulting dry pellet was consequently dissolved in 10 µl DEPC water. The concentration was determined using a Nanodrop® instrument (Thermo Scientific); RNA concentrations higher than 3000µg/ml are the anticipated result for further progress in the next steps of the protocol.

2.3.4. Hybridization

The two complementary strands of ssRNA, synthesized from the previous step, have to be hybridized to form dsRNA. Each ssRNA (one from cleavage with ApaI and the other with XbaI) was diluted to conc. 3µg/µl. Both ssRNA solutions were mixed in 1:1 molar ratio in a microtube that was placed into a cylinder with boiling water isolated by aluminium foil. The tube was then slowly cooled down for at least 8 hours in RT.

2.3.5. RNA interference

RNAi is a mechanism which can be used for gene expression downregulation at the post-transcriptional level. This method uses a dsRNA with the same sequence as the target

transcript. Double stranded RNA is recognized by an enzyme called “Dicer” which cleaves it to small fragments (21-23 nucleotides) called siRNA. In the next step siRNA binds to a nuclease complex called “RISC”. RISC targets siRNA to the complementary mRNA which is consequently cleaved. The disadvantage of RNAi is its lower specificity – it can influence expression of more than one similar in sequence genes [7].

Double stranded RNA (c=3µg/µl) was injected into ticks using a micro injector to an amount that will cause a minor optical enlargement of the tick (ca 0.5µl). Guinea pigs were exposed to these ticks – 25 ticks on one guinea pig. Tick’s feeding duration and their mortality were controlled every day. Two different tick groups were attached to the animals; ticks with suppressed contig-763 and ticks with dsRNA for silencing of GFP as a control. After one month the experiment was repeated using the same guinea pigs to see whether some immune recognition of tick salivary antigens differentially evolved in the two groups.

Success rate of the gene silencing was checked by RNA isolation from tick’s tissues (ovaries, gut and salivary glands) and reverse transcription to cDNA. PCR amplification of the genes and agarose gel electrophoresis reflected the efficiency of gene silencing.

2.4. Methods used for protein expression

2.4.1. Protein expression

Protein expression was performed in 1l LB medium with 1ml ampicilline (100mg/ml) and 1 ml chloramphenicol (35mg/ml). One day before the expression, the bacterial cells were grown in the same medium to increase their population before gene overexpression is induced. 20 ml of cell culture was added to 1L LB medium with both antibiotics and OD was measured; ideal value should be up to 0.1. The OD of the bacterial culture was measured until reached 0.6-0.8 (ca 2.5h). After that, 1 ml IPTG (1mM final concentration in the culture) was added to induce protein expression and the culture was incubated at 37°C for 4 hours. Cells were then collected by centrifugation.

2.4.2. Inclusion bodies isolation

Isolated cells were dissolved in 50 ml Resuspending buffer and disrupted by applying ultrasonic waves (maximum power, 3x30s). After centrifugation, the supernatant (cytosolic fraction) was stored in freezer. The resulting pellet was dissolved in 30 ml Isolation buffer and it was sonicated again. The resulting supernatant (membrane fraction) was stored again as well as the pellet (inclusion bodies). The protein presence in these 3 fractions was determined using SDS-PAGE.

2.4.3. Protein isolation + refolding

The overexpressed protein was present in the inclusion bodies of the bacteria. Inclusion bodies were dissolved in 10ml 6M guanidine hydrochloride 25 mM Tris pH 8. The sample was centrifuged at 10000g for 15 min and the supernatant was mixed with 0.1ml DTT (10mM final concentration). Protein refolding was done in 1l Refolding buffer with constant stirring for 3 hours at RT.

2.4.4. Fast Protein Liquid Chromatography

FPLC is a separation method similar to HPLC optimized to run biological macromolecules on pressure-fragile columns. These columns use inert surfaces necessary for buffers containing high concentrations of halide salts. Most often the FPLC separation techniques are based on size fractionation or strong anion exchange [9].

Polymeric ”MonoS 5/5“ monobeads column was used for chromatographic run, this column is suitable for proteins with pI > 7. Elution of the protein from the column was ensured by increasing the gradient of salt in the mobile phase of the column. First the column was flushed with Buffer A and then NaCl gradient was continuously increased by changing the ratio of Buffer A and B. 2ml fractions were automatically collected; manual fraction collection was also applied to collect fractions of high amount of protein – as judged by the high absorbance at the column chromatogram.

2.4.5. Serine protease inhibition assays

All assays were performed at 30°C in triplicates. For the screen, 0.9 and 1.2 µM of the two different pure fractions of protein was pre-incubated with each enzyme for 10 min before the addition of the corresponding substrate; *ANOVA* analysis was used for statistical analysis of the observed inhibition in the presence of the protein, and statistical significance was considered when $p < 0.05$ when comparing the enzymatic activity in the presence or absence of the inhibitor. Protein samples were dissolved in 20mM Tris, pH8.

All substrates were used in 250 µM final concentration in all assays. Substrate hydrolysis rate was followed in a Tecan Infinite M200 96-well plate fluorescence reader (Tecan group Ltd) using 365 nm excitation and 450 nm emission wavelength with a cutoff at 435 nm.

3. Results

3.1. RNA interference

The goal of RNA interference was to silence a specific gene normally expressed in the salivary glands of a tick and observe whether the gene silencing influences tick's feeding ability or its reproduction.

The sequence of contig-763 was first amplified using PCR. After the run of the PCR products on an agarose gel, the band of the appropriate molecular weight was isolated from the gel using JET Quick-Gel Extraction kit and sequenced *(Figure 2)*.

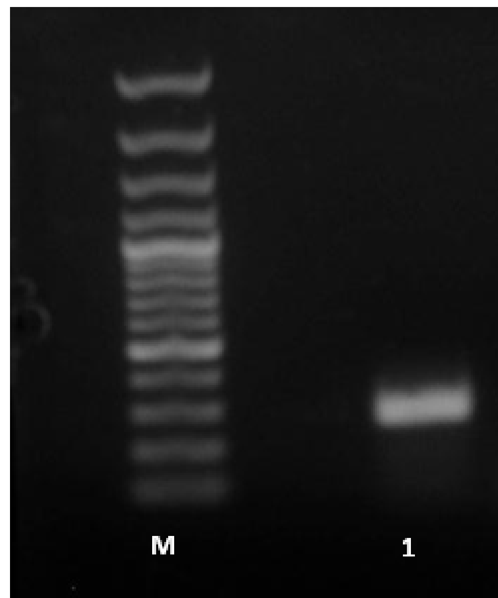

Figure 2: Electrophoresis after PCR of the DNA fragment used for RNAi. M represents molecular weight marker and 1 the amplified gene.

The next step was the restriction of PLL10 plasmid and contig-763 sequence. Enzymes ApaI and XbaI were used because they are both active in Tango buffer (1x) and both cleave to produce sticky ends in the digested DNA.

After that, the ligation of contig-763 to the plasmid was performed; followed by transformation to TOP10 *E.coli* cells. Several single colonies grown on an agar plate were used for further experimentation. 1.5 ml LB medium was subsequently inoculated with one bacterial colony in the presence of antibiotics. DNA was isolated from these colonies using the JET Quick-Plasmid Miniprep kit and PCR with M13 primers was done. Plasmids with a gene insert were successfully detected to all the tested bacterial clones except of number 3 as shown in *Figure 3*.

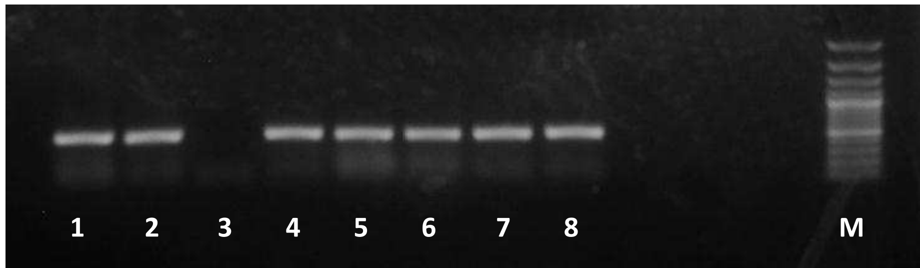

Figure 3: PCR as a control of successful ligation and transformation

The samples in wells 1, 2 and 7 were used for preparation of pure DNA (minipreps). Plasmid DNA was isolated using the JET Quick-Plasmid Miniprep kit, the DNA concentration was determined using Nanodrop and three 7 µl samples containing 150 ng DNA were diluted using water. These 3 samples were sent for sequencing. Colonies 2 and 7 contained the desired sequence; all further work was done with the colony number 7.

A 100ml bacterial culture was inoculated with bacteria containing the contig-763 construct. After overnight incubation the plasmid DNA was isolated using the JET Star LFU/Plasmid purification MAXI Kit/20 (maxiprep). The DNA concentration was measured to be 2259.9 ng/µl.

The circular plasmid DNA had to be cleaved to be linearized. For this purpose, the enzymes ApaI and XbaI were used. The linearized plasmid was purified using the JET Quick PCR product purification spin kit/250 and the concentration was measured again.

conc. plasmid cleaved with ApaI = 494ng/µl

conc. plasmid cleaved with XbaI = 332 ng/µl

Both strands were used for synthesis of ssRNA. All traces of DNA were eliminated using DNAse and pure ssRNA at conc. 3µg/µl was hybridized to produce the desired final product – dsRNA.

Figure 4 demonstrates the success rate of the hybridization reaction. It also demonstrates the molecular weight of all DNA/RNA stages used.

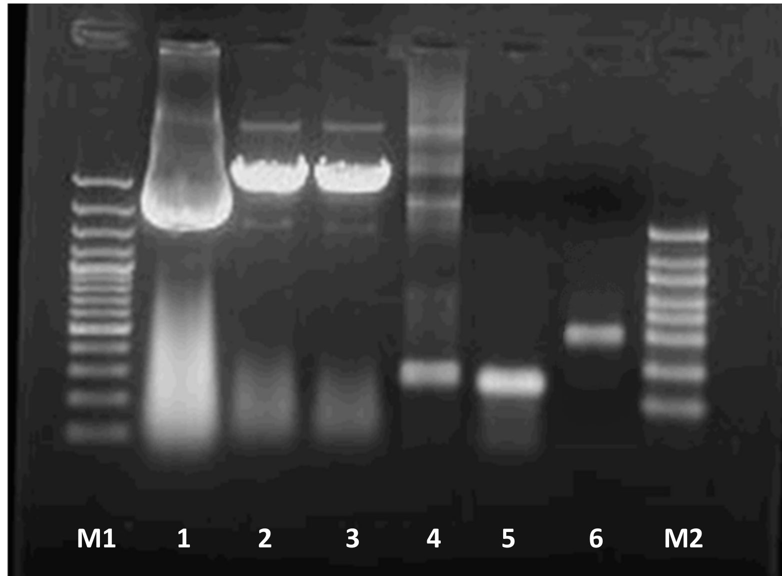

Figure 4: End of dsRNA synthesis.

M1-molecular weight marker for DNA, 1-undigested plasmid, 2-linearized plasmid with ApaI, 3-linearized plasmid with XbaI, 4-ssRNA created after cleavage with ApaI, 5- ssRNA created after cleavage with XbaI, 6-dsRNA, M2-molecular weight marker for RNA

3 tick populations, each consisting of 25 ticks, were injected with ds RNA; two of them with dsRNA silencing expression of GFP as a negative control and 1 with dsRNA silencing contig-763. All ticks attached to their host during the 1st day of exposure. The length of tick's feeding varied from 7 to 10 days; their mortality, body mass, percent of laying ticks and egg mass are shown in *Figures 5-8* for the 1st and also 2nd exposure. In all figures "cyrich" stands for the cysteine rich protein contig-763.

Figure 5: Mortality of ticks after feeding

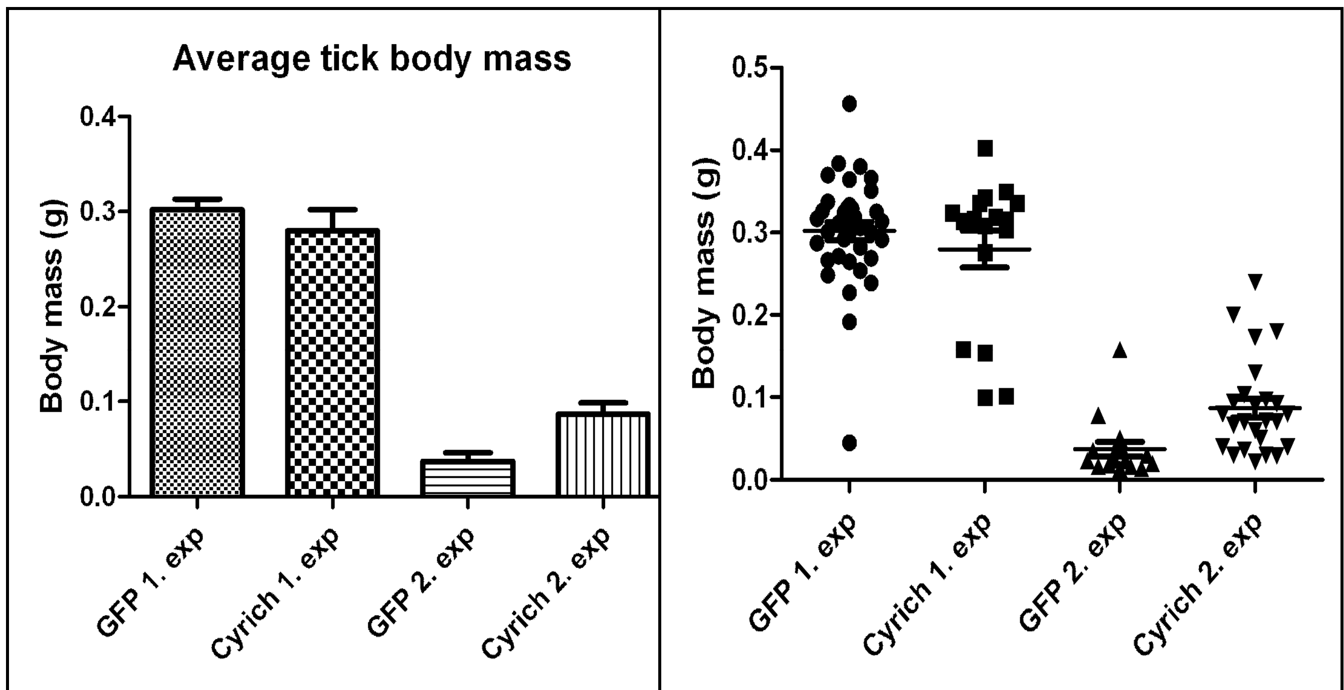

Figure 6: Average tick body mass

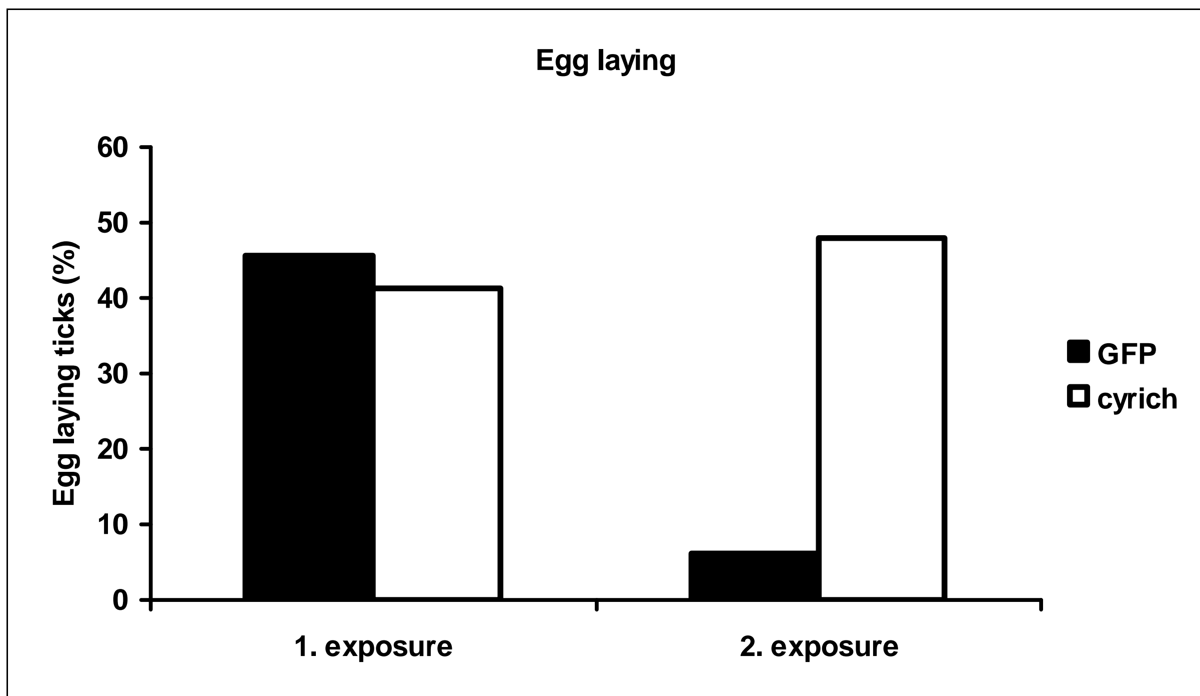

Figure 7: Percentage of egg laying ticks

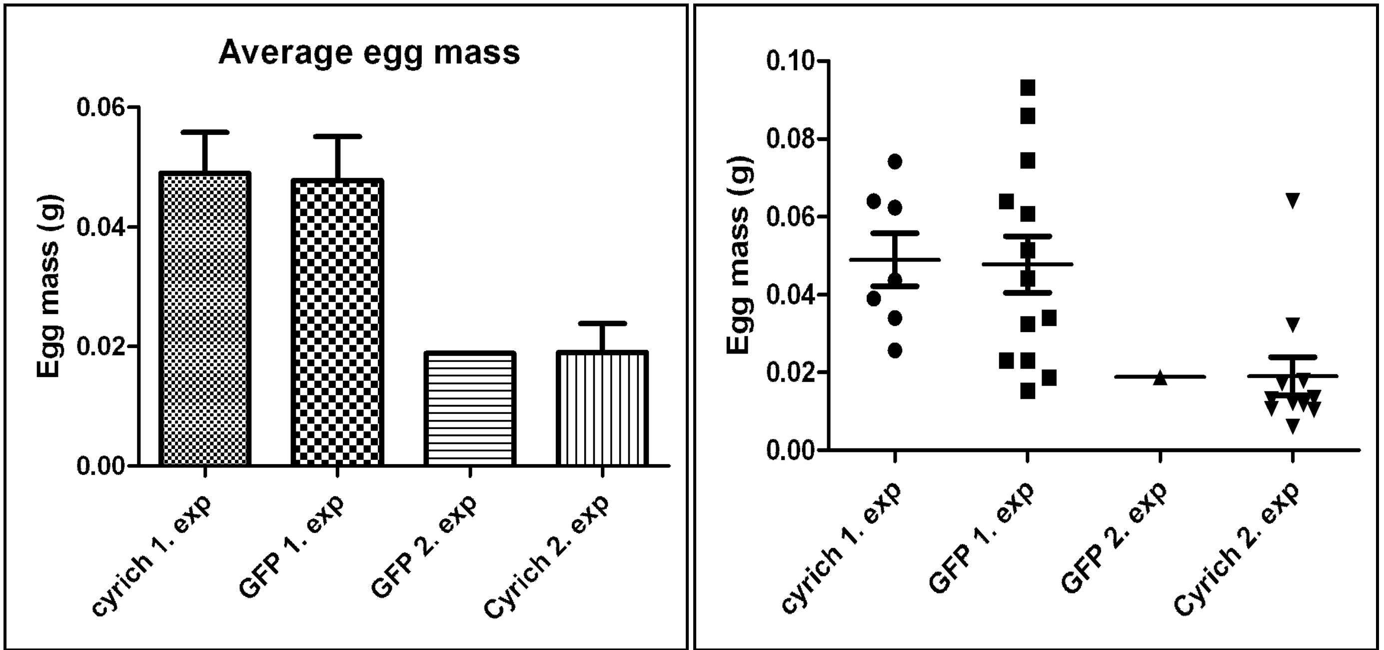

Figure 8: Average mass of eggs

The success rate of RNAi was controlled by PCR using cDNA from the two different tick populations (control and cyrich silenced) as a template. First several ticks were dissected and their ovaries, gut and salivary glands were isolated. Isolated RNA was translated to cDNA using reverse transcriptase. PCR using the same primers as for the RNAi experiment was done, followed by electrophoresis. The results are shown in *Figure 9.*

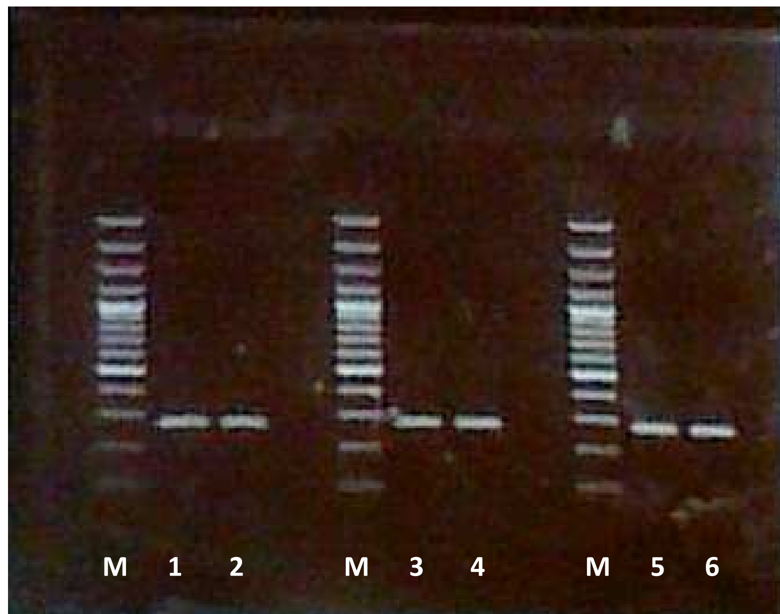

Figure 9: Control of RNAi success.

M represents molecular weight marker; 1-gut contig-763; 2-gut GFP; 3-saliv.glands contig-763; 4-saliv.glands GFP; 5-ovaries contig-763; 6-ovaries GFP

3.2. Protein expression + serine protease inhibition assay

The expressed and folded protein was tested using a series of serine protease inhibition assays. Its effect on different serine proteases of human origin that play a role in host defence against the ticks was measured. To achieve this, the sequence of contig-763 was amplified using PCR, like in the case of RNAi, but using different primers to amplify the whole reading frame of the gene. The amplified DNA was again separated using agarose gel electrophoresis and isolated using JET Quick-Gel Extraction kit. The enzymes NdeI and XhoI were used for the digest of the contig-763 gene and the vector PET17B plasmid (Novagen). This plasmid contains a strong promoter for protein expression, but different cloning restriction sites -than the vector PLL10 that was used for the RNAi experiments. After the ligation, the plasmid was transformed in TOP10 bacterial cells. Bacteria from several distinct colonies were then grown in 1.5ml LB medium, DNA was isolated by doing minipreps and PCR using T7 primers was done. Three 7µl samples containing 150ng from successfully transformed bacterial clones DNA were sequenced to verify the integrity of the frame in the cloned inserts. The isolated DNA from a chosen clone with the correct sequence was transformed into a new bacterial strain appropriate for the gene overexpression – BL21-pLysS.

Next a pilot expression experiment was done in order to find the best time point that protein expression peaks. One day before the experiment a 1.5ml LB medium bacterial culture in the presence of the appropriate antibiotics was prepared in order to have enough bacteria for the pilot expression. 0.5 ml of this overnight bacterial culture was added to 20 ml LB medium with 20 µl ampicilline and 20 µl chloramphenicol. After 2h incubation at 37°C 0.5 ml was removed, centrifuged and pellet was stored at 5°C. After that IPTG was added, the sample was incubated at 37°C and each hour 0.5 ml was again removed and the bacterial pellet was stored. Totally 8 samples were collected - 0 to 6 and 24 hours after IPTG addition. Each of 8 pellets was dissolved in 500 µl Lysis buffer and in four cycles cooled in liquid N_2 and heated at 50°C. After centrifugation, the pellet was dissolved in NuPAGE Sample buffer, NuPAGE Sample reducing agent and water. All 8 samples were analyzed using SDS-PAGE. *(Figure 10).*

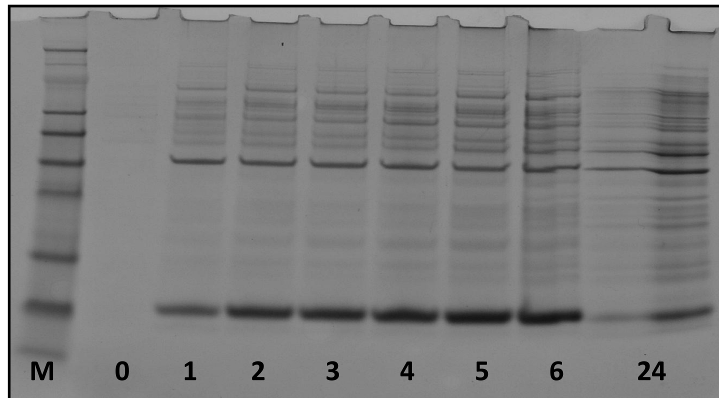

Figure 10: Pilot expression. M - Molecular weight marker, 0-24 – hours after IPTG addition

Next, and based on the results of the pilot expression, the protein overexpression was up scaled. A 10ml LB medium culture was produced overnight and the protein overexpression was done using a 1l culture in LB medium. Cytosolic fraction, membrane fraction and inclusion bodies were separated according to the methods described above. The abundance of contig-763 was determined using SDS-PAGE and the results are shown in *Figure 11.*

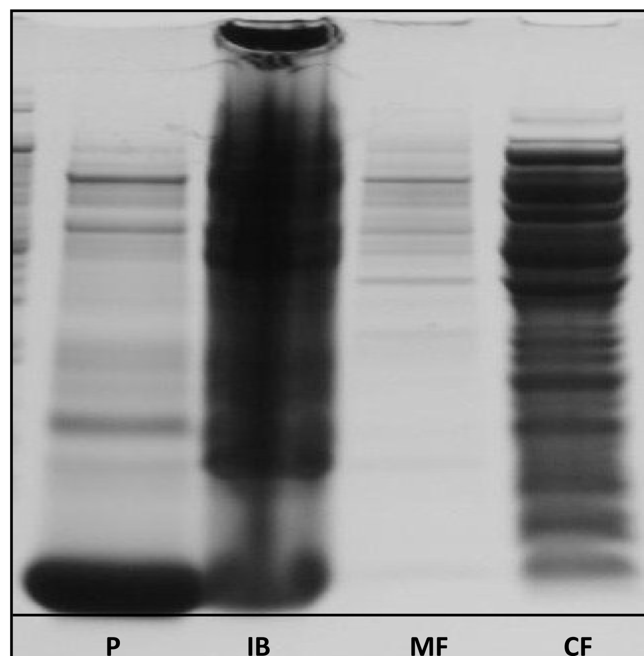

Figure 11:Presence of contig-763 protein in different fractions.
P-isolated protein from the inclusion bodies, IB-inclusion bodies, MF-membrane fraction, CF-cytosolic fraction

The overexpressed protein was isolated from the inclusion bodies, folded in the Refolding buffer and purified using Fast protein liquid chromatography *(Figure 12).* The resulting separated peaks were analyzed using SDS-PAGE *(Figure 13)* and the protein was consequently concentrated using PREP/SCALE-TFF Cartrige (Millipore) with 3kDa membrane.

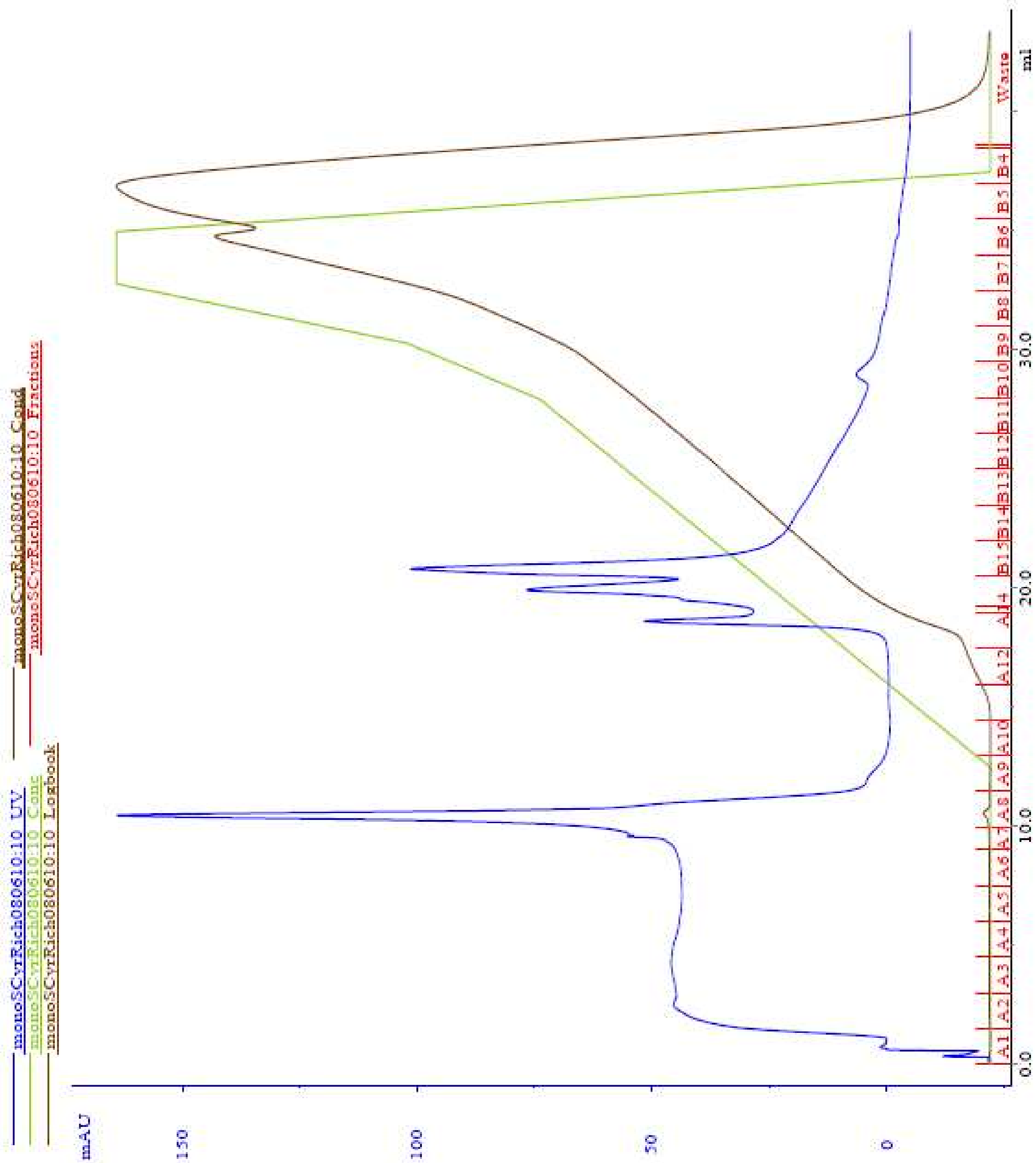

Figure 12: FPLC chromatogram, using column MonoS 5/5 was used. Blue line – UV absorbance signal of each fraction (protein concentration), Green line – NaCl concentration, Brown line – conductivity, Red lines – collected fractions

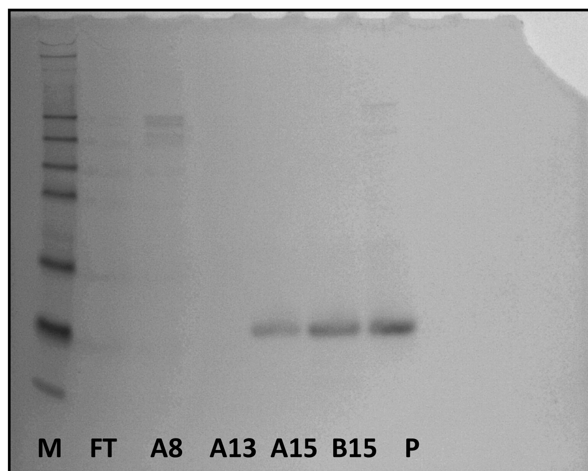

Figure 13: Gel from SDS-PAGE after FPLC. M-molecular weight marker, FT-flowthrough of the column (unbound proteins in the column), A8-B15-collected fractions, P-proteins before separation

Fractions A15 and B15, both containing contig-763 protein were analyzed again using SDS-PAGE without a reducing agent to see possible monomer/dimmer formation. However two bands appeared in both fractions suggesting that there is not any disulfide bond mediated dimmer formation *(Figure 14).*

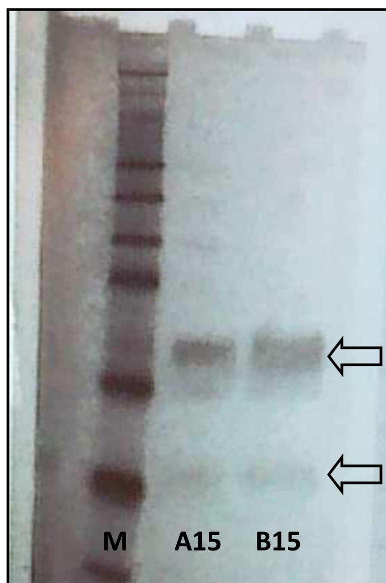

Figure 14:SDS-PAGE without a reducing agent

Finally both fractions, A15 and B15, were tested in the serine protease inhibition assays to check whether the contig-763 protein affects the activity of different human serine proteases. This step was done by my supervisor Michail Kotsyfaki, while I followed him in all the experiments. The results are presented in *Figures 15 and 16.*

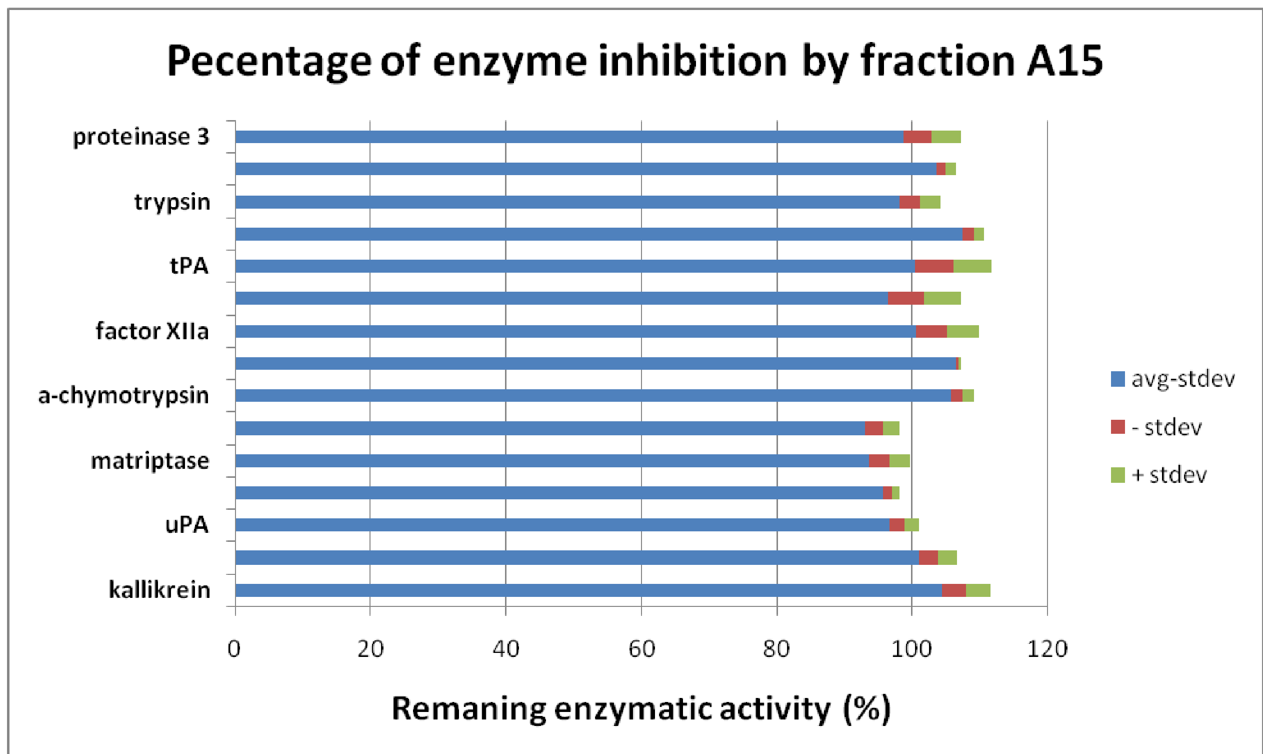

Figure 15: Enzyme inhibition by fraction A15

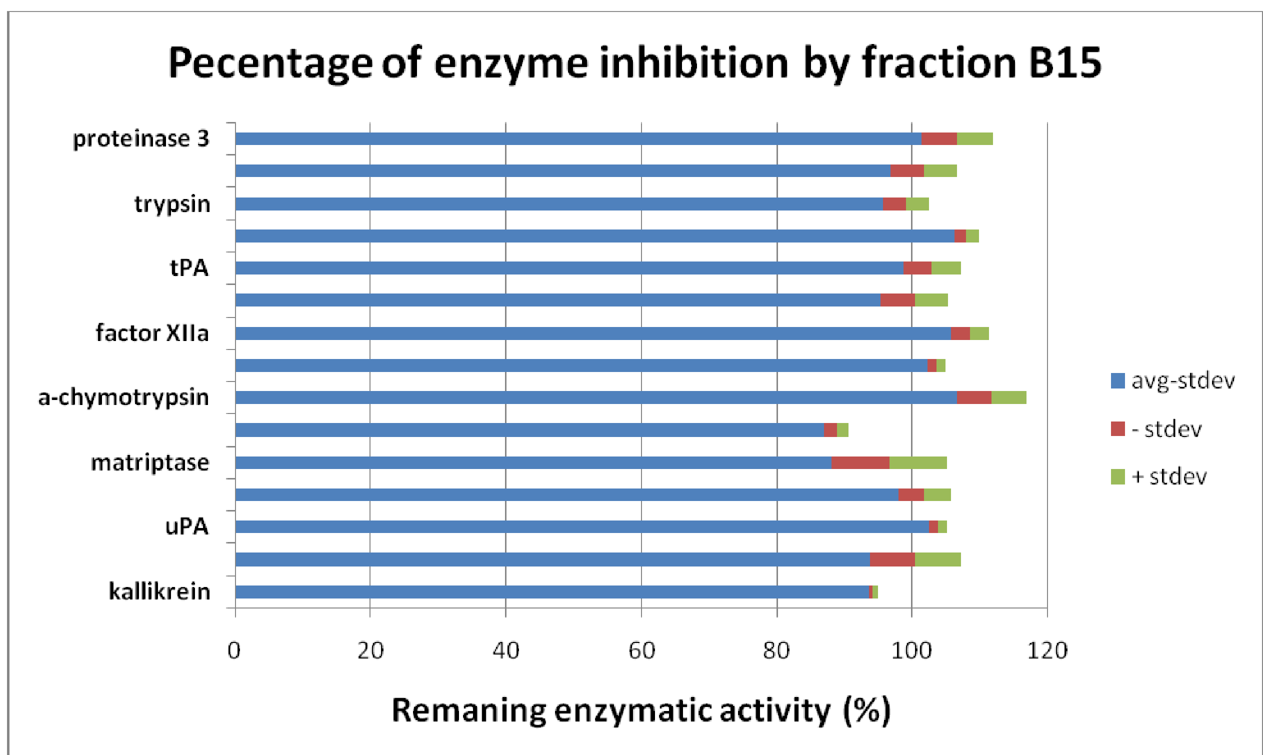

Figure 16: Enzyme inhibition by fraction B15

4. Conclusion and discussion

One of the aims of the thesis was to investigate the role of the contig-763 gene by employing the RNA interference methodology. The gene silencing compound – dsRNA was successfully produced and injected into ticks. Several effects on the tick's feeding ability can be observed, unfortunately none of them shows inhibition of feeding or reproducing ability by contig-763 *(Figures 5-8)*. Mortality of GFP ticks was ca 2x higher after the second exposure than in all other cases (GFP on the first exposure or contig-763 in both exposures). Average mass of ticks and also mass of laid eggs was significantly lower after the second exposure; however this phenomenon could have been caused by feeding in October which is quite late for tick's feeding. The most interesting result was observed in the comparison of egg laying ticks. Almost no ticks with silenced GFP after the second exposure laid eggs; this was of course at least partially caused by high mortality of this studied group.
Contig-763 either doesn't influence tick's feeding and reproducing abilities or the RNAi was not successful. *Figure 9* shows that quite a lot of cDNA was transcribed from the isolated RNA which would mean poor gene silencing. However PCR for this control step was done once again with 28 cycles instead of 30 and no bands were visible at all.

Another way of testing contig-763 function was the protein expression and purification followed by serine protease inhibition assays. The protein was overexpressed using bacteria and it was successfully folded and purified using FPLC. Two fractions containing contig-763 were obtained *(Figure 13)*. The results show that there is no difference between the physicochemical properties of the two protein fractions, although further experimentation is necessary to definitely conclude on the reason that the protein is purified in two different peaks. Finally both fractions were used in serine protease assays. Nor A15 fraction, neither B15 fraction has shown significant enzyme inhibition.

Protein contig-763 seems to have no or minimal influence on tick's feeding ability. It doesn't inhibit the function of human serine proteases involved in the defence against ticks and its silencing doesn't lead to any changes in tick's mortality, feeding length, size or egg laying ability. Further future experimentation is necessary to uncover the function of this gene in ticks.

5. References

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