Faculty of Science of University of South Bohemia

Institute of Chemistry and Biochemistry

IrAM9 – a member of a thioester-containing protein family from the hard tick *Ixodes ricinus*

Petr Rathner

Bachelor thesis

Supervisor: RNDr. Petr Kopáček, CSc.

Supervisor, consultant: RNDr. Lenka Grunclová, Ph.D.

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Bachelor thesis

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Annotation:

The thioester-containing proteins (proteins of α_2-macroglobulin family) are constituents of innate immunity, which comprise (i) universal protease inhibitors of α_2M type, (ii) C3, C4, C5 components of the complement system, (iii) insect thioester-contaning proteins (iTEPs) and (iv) macroglobulin complement related proteins (MCR). In this work, the partial structure of the thioester-containing protein from the hard tick *Ixodes ricinus* (IrAM9) which belong to the MCR was determined by sequencing and cloning of PCR products. The IrAM9 message is expressed in the ovaries and salivary glands of partially engorged females. Received recombinant protein was used to get polyclonal serum which was obtained by repeated rabbit immunization. The obtained antibodies were used in an attempt to detect the native protein in tick tissues by immunoblotting method.

Affirmation:

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagerism detection system.

In České Budějovice, 20.05.2011 .....

Petr Rathner

Aknowledgment

I am heartily thankful to my supervisors, RNDr. Petr Kopáček, CSc. and RNDr. Lenka Grunclová, PhD. whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

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1 Introduction

1.1 Ticks

Parasitic ticks are very important vectors causing a variety of diseases in host organisms; including humans. The most well known pathogens transmitted by ticks and causing serious diseases are for example *Borrelia burgdoferi*, *Ehrlichia*, *Babesia* and many others (Sonenshine, 1993). Transmitted pathogens are capable of surviving the vector immune system which, as in other invertebrate organisms involves components of humoral as well as of cellular immunity.

1.2 Immune response

The humoral immunity in ticks as well as in other organisms is represented by following system. One of the virulent factors are the proteases secreted by the pathogens. The host immune system developed special molecules which are called protease inhibitors to defend itself from the invading pathogen. Two types of protease inhibitors are involved in humoral component of the immune system. The first types are the active-site molecules which bind the active site of the protease thus destructing it. The second class of the protease inhibitors comprises of α_2-macroglobulins. These molecules apply a special trapping mechanism to deliver protease to lysosymes (Armstrong, 2006). The first α_2-macroglobuline isolated from the group of invertebrate organisms was *Limulus*-α_2M from horseshoe crab, *Limulus polyphemus* (Quigley et al., 1982). The first α_2-macroglobulin described in a tick species was isolated from soft tick *Ornithodoros moubata* in 2003 (Saravanan T., 2003).

1.3 Proteins of a thioester-containing family

The proteins of a thioester family, also known as proteins of α_2-macroglobulin family (F-α_2M), can be found in all animal species. α_2-macroglobulins serve as protease inhibitors and there are important components of humoral and also cellular immunity (Blandin and Levashina, 2004). The main function of F-α_2M is to inhibit large spectrum of proteases, especially proteases from invading pathogens, to prevent the damage of surrounding tissue (Sottrup-Jensen et al., 1989). The majority of the α_2-macroglobulin family of protease inhibitors have special thiol-ester bond. This bond links the thiol group of a cysteine residue with the γ carbonyl of a glutamyl residue (Salvesen et al., 1981; Salvesen and Barret, 1980;

Sottrup-Jensen et al,. 1980). The *β*-cysteinyl-*γ*-glutamyl thiol ester sequence (GCGEQBM) is conserved in those members of the α_2-macroglobulins that contain internal thiol ester (Armstrong et al., 1996). The proteins of F-α_2M comprise three subfamily members; C3/C4/C5 components of the complement system, the thioester containing proteins of the insect and the universal protease inhibitors of the α_2M type (Armstrong et al., 2006). The complement system is based on the recognition of pathogen surface and their binding to it via thioester bond. A special member of the F-α_2M family is MCR (macroglobulin complement related) like protein group originally described as TEP6 from *Drosophila (*Stroschein-Stevenson at al., 2005). The phylogenetic tree of F-α_2M members present in the *I. ricinus* is shown in Figure 1.

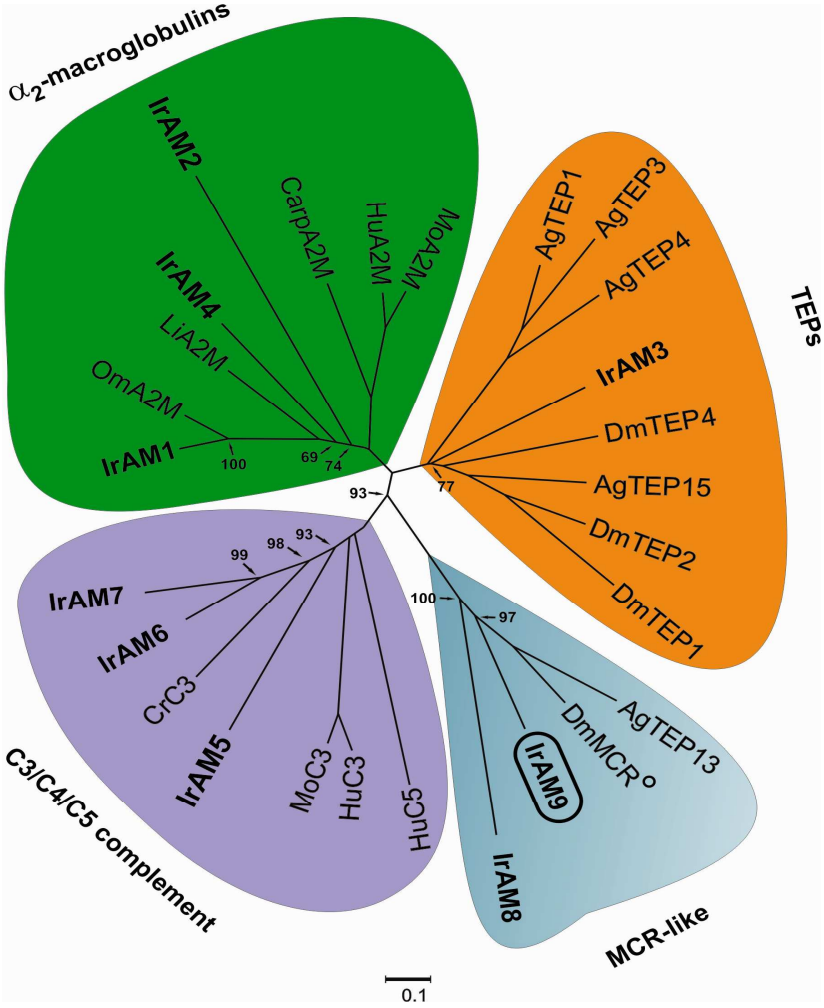


Figure 1: Phylogenic tree of α_2-macroglobulin family *(according to Burešová, 2009)*

1.4 Structure of α_2-macroglobulins

Nowadays, a great diversity in the α_2-macroglobulins superfamily is known. Concerning the structure of these protease inhibitors, only some of them are described. Such proteins with a known sequence are for instance some of the α_2-macroglobulins, TEP and complement group from *Ixodes scapularis* (IsAM1, IsAM3, IsAM4, IsAM6). Sequence of one representative of MCR like group from *Ixodes ricinus*, IrAM8, was determined (Burešová et al., 2011, in press).

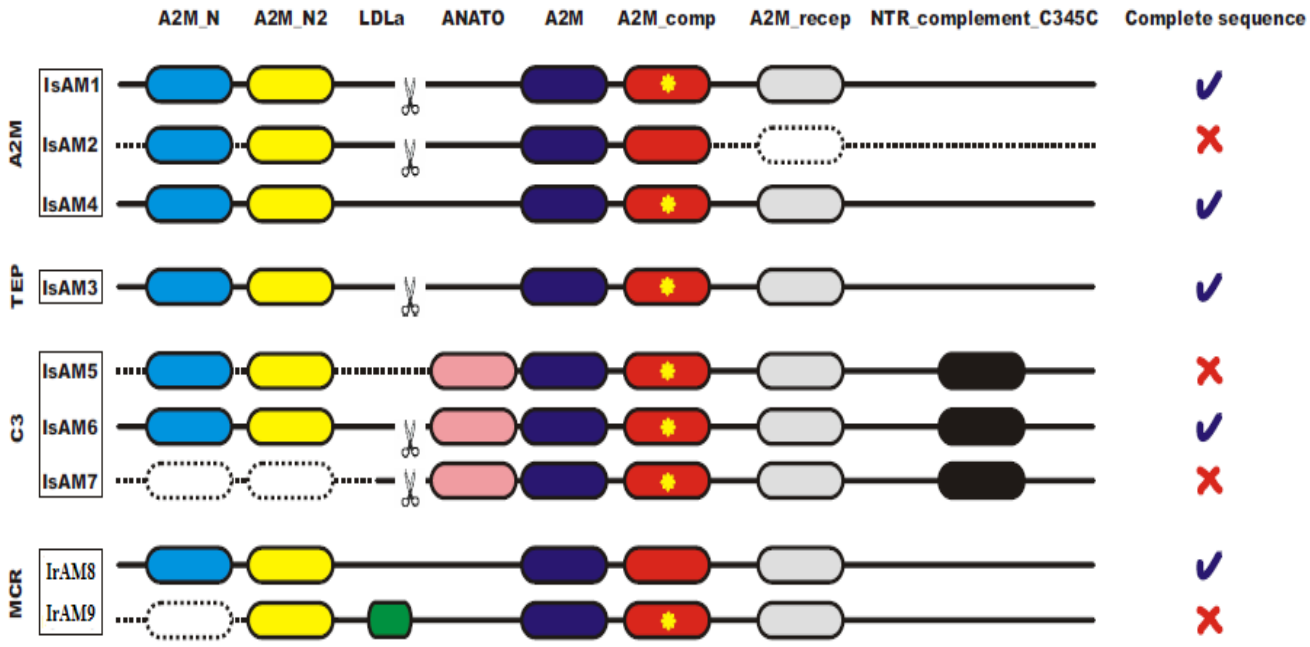


Figure 2: Overview of α_2-macroglobulin family from Ixodes ricinus and Ixodes scapularis *(Burešová, 2011). Abbreviations and nomenclature are according to the NCBI-conserved domain database (http://www.ncbi.nlm.nih.gov/cdd).* ***A2M_N*** *- MG2 (macroglobulin) domain of alpha-2-macroglobulin,* ***A2M_N_2*** *- Alpha-2-macroglobulin family N-terminal region,* ***ANATO*** *- Anaphylatoxin homologous domain,* ***LDLa*** *- Low Density Lipoprotein Receptor Class A domain,* ***A2M*** *- Alpha-2-macroglobulin family,* ***A2M_comp*** *- A-macroglobulin complement component,* ***A2M_recep*** *- A-macroglobulin receptor,* ***NTR_complement_C345C*** *- NTR/C345C domain. Scissors indicate post-translational cleavage; Yellow asterisks indicate the presence of thioester bond.*

2 Materials and Methods

2.1 Material

2.1.1 Primers (Generi Biotech™)

Primer	Sequence	Annealing temperature
IrAM9-RT S	5´-TGT TGG TGA TGT GGT GGG-3´	55°C
IrAM9-RT AS	5´-GTA ATA TCC GTC ACA TCC CTCG-3´	55°C
IrAM9pET100S	5´- CAC CTT TTG CGC CCG GGT GTT TCA -3´	55°C
IrAM9pET100AS	5´- TTA CTG CAT CGCCAT CAG GGT GTCC -3´	55°C
T7 S	5´- TAA TAC GAC TCA CTA TAG GG -3´	50°C
T7AS	5´- GCT AGT TAT TGC TCA GCG G -3´	50°C
M13 S	5´- GTA AAA CGA CGG CCA – 3´	50°C
M13 AS	5´- CAG GAA ACA GCT ATG AC -3´	50°C

2.1.2 Chemicals and Commercial Kits

PCR product purification from agarose/EtBr gel:
QIAquick PCR Purification Kit (250) (QIAgen)
Plasmid isolation:
High pure Plasmid isolation Kit (Roche)
cDNA synthesis:
Transcriptor High Fidelity cDNA Synthesis Kit (Roche)
Restriction enzyme:
EcoRI Fastdigest® restriction enzyme (Fermentas)
Chemicaly competent cells for cloning:
One Shot® TOP 10 Chemically Competent *E. coli* (Invitrogen)
BL21 Star™ (DE3) One Shot® Chemically Competent *E. coli* cells (Invitrogen)
Expression and Cloning Vectors:
pET100/D-TOPO® expression vector with ampicilin selection (Invitrogen)
pCR 2.1 TOPO Plasmid vector with ampicilin selection (Invitrogen)

DNA electrohoresis:	
50x TAE buffer	200mM Tris-HCl, 50mM EDTA(pH 8.0)
1x TAE buffer	50x TAE buffer 50x diluted in dd H_2O
Agarose	1% agarose (Serva^{TM}) in 1x TAE buffer
5x Loading dye	10mM Tris-HCl (pH 8.0), 20% Ficoll 1mg/ml G-Orange, 500x SYBR green
DNA Ladder	Gene Ruler^{TM} 1Kb DNA Ladder Plus
EtBr	Ethidium bromide 1 mg/ml
RNA electrophoresis:	
10x TBE buffer	20mM Na_2EDTA, 0.89M boric acid, 0.89M Tris-HCl
1x TBE buffer	10x TBE buffer 10x diluted in DEPC H_2O
Agarose	1,2% Agarose (Serva^{TM}) in 1x TBE buffer
6x Loading dye	0,25% Bromphenol blue, 0,25%xylene cyanol, 30% glycerol, 1,2% SDS, 60mM NaH_2PO_4, 500x SYBR green; pH 6.8
High Range RNA Ladder	Fermentas
DEPC H_2O	Diethylpyrocarbonate (Sigma^{TM})
SDS PAGE:	
1% - 30% AA	Acrylamide and bisacrylamide, gradient
buffer 1	1.5M Tris-HCl; pH 8.8
buffer 2	0.5M Tris-HCl; pH 6.8
10% SDS	Sodium dodecyl sulphate ($C_{12}H_{25}SO_4Na$)
10% APS	Ammonium persulphate $(NH_4)_2S_2O_8$
TEMED	Tetramethylethylenediamine $(CH_3)_2NCH_2CH_2N(CH_3)_2$
5x reducing sample buffer	250mM Tris-HCl (pH 6,8), 500mM dithiothreitol, l0% SDS, 0.2% bromophenol blue, 50% glycerol
1x reducing buffer	5x reducing buffer 5x diluted in H_2O
10x ELFO buffer	250mM Tris-HCl, 1.92mM glycin, 1% SDS
Staining solution	Coomassie Brilliant Blue R 250
LMW protein marker	GE-HealthCare^{TM}

Bacterial media and agars:	
SOC medium	2% w/v bacto-tryptone , 0.5% w/v bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$ or 20mM $MgSO_4$, 20mM glucose
LB medium	10g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl pH 7.0
LB agar	1.5% bacto-agarv LB medium
IPTG	1M Isopropyl β-D-1-thiogalactopyranoside
Antibiotics	Ampicilin (stock solution 50mg/ml H_2O)
Isolation of recombinant protein:	
Resuspension buffer	20mM Tris-HCl, pH 8.0
Izolation buffer	2M Urea, 20mM Tris-HCl, 0,5M NaCl, 10mM imidazol, pH 8.0
Solubilization buffer	6M guadinine hydrochloridum, 0.5M NaCl, 10mM imidazol, 1mM mercaptoethanol, 20mM Tris-HCl
Chelate column chromatography:	
Buffer A	8M urea, 50mM Tris-HCl, pH = 7.8
Buffer B	8M urea, 50mM Tris-HCl, 0.5 NaCl, 0.5 M imidazol, pH = 7.8
Co^{2+} column	5ml HiTrap™ IMAC FF coulm (GE Healthcare) loaded with 0.1M $CoCl_2$
Recombinant protein refolding:	
Refold buffer I	4M urea, 50mM Tris-HCl, 0.5M NaCl, 20% glycerol, 2 mM mercaptoethanol
Refold buffer II	2M urea, 50mM TRIS, 0.5 M NaCl, 20% glycerol, 2mM mercaptoethanol
Refold buffer III	1M urea, 50mM TRIS, 0.5 M NaCl, 20% glycerol, 2mM mercaptoethanol
Refold buffer IV	150mM Tris-HCl, 150mM NaCl, 10% glycerol
Western blot:	
Blotting buffer	20% methanol, 25mM Tris-Base, 150mM glycin, 0.4% SDS
PBS tween solution	8 g/l NaCl, 0.2 g/l $KH_2PO_4 \cdot 12H_2O$, 0.2 g/l KCl, 0.05% Tween 20
Secondary antibody	SwAR–Px, Swine-antirabbit-peroxidase 1:1000 (Sevapharma)
Developing solutions	0.1M T- Diaminobenzidine chlorid, 30% H_2O_2
Blocking buffer	5% dry milk solution in PBS tween

2.2 Methods

2.2.1 Tick tissue isolation

Females of *Ixodes ricinus* were fed for five days on guinea-pig (*Cavia porcellus)* at 24 ^{o}C and atmospheric humidity about 95 %. Salivary glands and the gut were extracted from six subjects and washed in a physiological buffer (phosphate buffered saline, PBS). Washed samples (gut and salivary gland) were separately placed into two microtubes. Samples for RNA isolation were filled with 500µl of TRI reagent (Invitrogen) and stored for a short time on the ice. Samples further used for SDS PAGE and immunodetection method were stored at -80°C until use.

2.2.2 RNA Isolation

Gut and salivary gland tissues were homogenized in the mictrotubes filled with 500µl of TRI reagent (Invitrogen) by using plastic homogenizator. To each tissue samples was added 100µl of chloroform (200µl chloroform per 1000µl of TRI reagent). The microtubes were shaked using vortex and consequently centrifuged for 15 minutes at 12000 rpm at the temperature of 4^{o}C. After the centrifugation the supernatant containing RNA was transferred into new 1.5 ml microtubes. Following purification of the RNA samples was provided using TRI Reagent^{TM} protocol. Resultant RNAs qualities were measured electrophoreticaly on 1% aragose gel in 1x TAE buffer. The concentration and purity of the RNAs were determined from the light spectra absorbance at λ = 260 nm.

2.2.3 Preparation of complementary DNA

The isolated RNA samples from the gut and salivary gland tissues were diluted to the final concentration of 5 µg/µl. Following steps were provided via Transcriptor High Fidelity cDNA Synthesis Kit (Roche^{TM}) using an anchored-oligo(dT)$_{18}$ primers.

2.2.4 Amplification of the DNA

Chosen DNA sections were PCR-amplificated using annealing temperature of 50°C and Taq DNA polymerase (Invitrogen) for 35 cycles. Final PCR products were placed on the 1% agarose gel with EtBr and electrophoretically separated by U = 125V, t = 30 min. Amplification was provided using gene-specific primers IrAM9-RT S and IrAM9-RT AS (*I. ricinus*). As the template samples was used cDNA from salivary glands of female *I. ricinus*. PCR products were isolated from 1% agarose gel using QIAquick PCR Purification Kit (250) (QIAgen).

2.2.5 DNA cloning and sequencing

PCR products were cloned by TOPO TA Cloning manual (Invitrogen) into the pCR 2.1-TOPO® Plasmid vector with ampicilin resistance and white/blue selection (Invitrogen) were inserted by heat shock method (42^{o}C/ 45 s) into the TOP 10 Chemically Competent *E. coli* (Invitrogen) and kept on ice. To this bacterial suspension 250µl of SOC medium was added and cultivated at 230rpm/ 30 min. /37^{o}C. The bacteria culture was spread out on LB/agar media with ampicilin (50µg/ml), X-Gal (40µg/ml) and kept overnight at 37^{o}C. Positive colonies were replanted into LB medium with ampicilin (50µg/ml) and cultivated overnight at 230rpm/ 37^{o}C. Plasmids from bacterial culture were purified using the High pure Plasmid isolation Kit (Roche). Positive clones were confirmed by restriction analysis with EcoRI Fastdigest® restriction enzyme (Fermentas) and used for DNA sequencing in the laboratory of genomics (Biologické centrum AVČR, v.v.i., České Budějovice) using M13 S and M13 AS primers.

2.2.6 Tissue profile of IrAM9 expression

For the differential expression location the RNA from ovaries, salivary glands and gut tissue was isolated and use for preparation of complementary DNA using Transcriptor High Fidelity cDNA Synthesis Kit (Roche^{TM}). The extent of the expression was approved via RT-PCR method using gene-specific primers IrAM9-RT S and IrAM9-RT AS (*I. ricinus*).

2.2.7 Recombinant protein expression

For the protein expression the bacterial system *E. coli* (Champion ™ pET Directional TOPO® Expression Kit, Invitrogen) was used. The construct for recombinant protein with 6His-tag terminus expression was prepared using pET100/D-TOPO® (Invitrogen) and using the PCR product with specific IrAM9pET100S and IrAM9pET100AS primers. As the template, salivary gland cDNA from *I. ricinus* was used. The expression construct was transformed into the TOP 10 Chemically Competent *E. coli* (Invitrogen) and sequenced using T7 S and T7 AS primers (Laboratory of genomics; Biologické centrum AVČR, v.v.i., České Budějovice). The vectors containing the insert in right reading frame were used for the transformation into BL21 Star™ (DE3) One Shot® Chemically Competent *E. coli* cells.

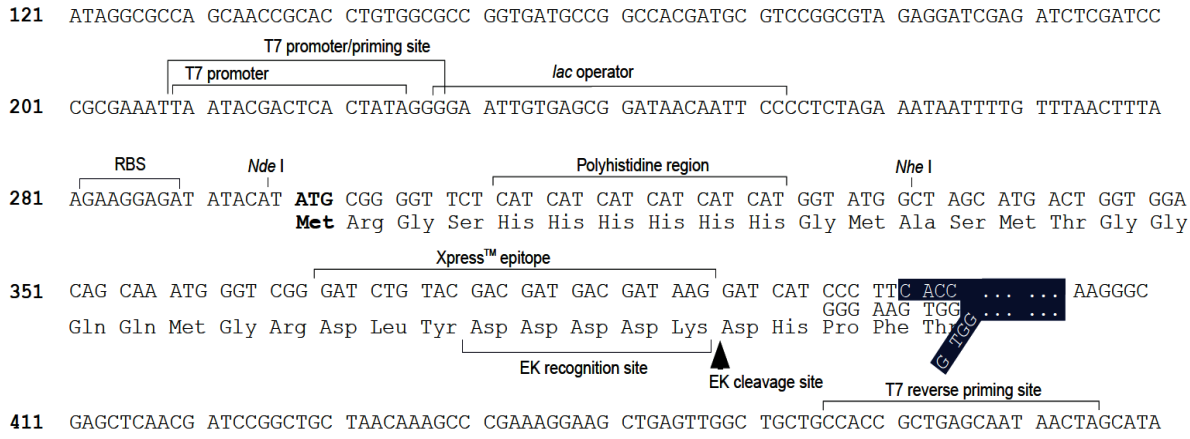


Figure 3 : Scheme of expression vector pET100/D-TOPO® (Invitrogen)

The transformed bacteria of BL21 Star™ (DE3) One Shot® Chemically Competent *E. coli* cells were added to 250µl of SOC medium and kept at 37°C at 200 rpm for 30 minutes. Accrued bacterial culture was added to 15 ml of LB medium with ampicilin (50µg/ml), incubated at 200 rpm/ 90min/ 37°C and transferred to 200ml of LB medium, 1 ml of the bacterial culture was taken away (‘zero time sample‘ – the check sample of bacterial proteins). The IPTG solution was added into the solution with OD_{600} = 0.6 to final concentration of 0.5mM, the expression proceed for six hours. Every one in 90 minutes the check sample (1ml) was taken from the solution and stored in the refrigerator at 4°C. The expression samples were centrifuged at 13000 rpm/ 10 min/ 4°C and the pellets were re-suspended in 0.5ml of homogenization buffer. The samples were frozen and de-frozen in

liquid nitrogen for three times. The check of the recombinant protein expression was provided by 20µl of every sample (mixed with 8µl of DTT and boiled for 5 minutes) via SDS-PAGE electrophoresis (150V, 90 minutes). The rest of the bacterial culture was centrifuged at 6000 rpm/ 10min/ 4^{o}C and pellet stored at -20^{o}C.

2.2.8 Recombinant protein isolation

The bacterial pellet was resolved in resuspension buffer, sonificated on ice for 4x10 s and centrifuged at 13000 rpm/ 10min/ 4^{o}C. The pellet was dissolved in isolation buffer, the suspension was sonificated on ice at 3x30 s and centrifuged at 13 000 rpm/ 10 min/ 4^{o}C. The purification was repeated with isolation buffer and the final pellet was dissolved in solubilization buffer at room temperature and centrifuged at 13000 rpm/ 20 min/ 4^{o}C. The supernatant was filtrated using 0.22µm membrane filter. The course of the isolation procedure was verified by SDS-PAGE electrophoresis.

2.2.9 The recombinant protein purification

The recombinant protein containing 6His-tag was purified from dissolved inclusion bodies on Co^{2+} chelating column. The purification procedure was monitored spectrophotometrically at λ = 280nm. The purification fractions as well as the first 'flow-through' fraction were verified by SDS-PAGE gradient gel electrophoresis.

2.2.10 Refolding

Chosen purification fractions were filled into dialysis tubing membranes (VISKING®, Serva). The sample was dialyzed in 12h intervals by using refold buffers I – IV, which were cooled down at 4^{o}C. After a complete dialysis the concentration of recombinant protein was determined by the Bradford protein assay method, aliquoted into 1.5ml microtubes and stored at -20^{o}C.

2.2.11 Rabbit immunoglobulin fraction

The polyclonal serum was obtained by repeated rabbit immunization (once in 10 days for total 40 days) with 0.5ml of purified recombinant protein with 0.5ml of adjuvant. The

immunoglobulin fraction was prepared from 6 ml of the serum was mixed with 12 ml of sodium acetate buffer, while stirring 18 x 25µl of caprylic acid was added. After a complete precipitation the sample was centrifuged at 5000 rpm/ 10min /25^{o}C. Supernatant was filtrated and dialyzed in 5mM Na_2HPO_4 solution for 1 hour. The concentration was measured by Bradford protein assay method at λ= 595 nm.

2.2.12 Western blotting and immunodetection

For the SDS-PAGE separation the tissues from 6 ticks were homogenized and boiled at 100°C species in 500µl (guts) 150µl (salivary glands and ovaries). Tick hemolymph was diluted 20 x in reducing and non-reducing (without DTT) sample buffer and boiled for 3 minutes. From these samples 15µl of expressed recombinant protein, 10µl of gut tissue homogenate, 20µl of ovaries, 20µl of salivary glands and 15µl of reduced and non-reduced hemolymph was applied on the gel. The PVDF (polyvinylidene fluoride) membrane (Milipore, 45 µm) was preincubated for 1 min. in 100% methanol solution and transferred into the blotting buffer solution for 10 minutes.

The protein transfer was proceeding for 1.5h at constant current 250mA. After complete blotting procedure, the membrane was 2x washed in TBS solution, incubated for 1 hour in prepared primary antibody obtained from rabbit sera (1:50). The membrane was washed 3x for 5 minutes in PBS solution and again incubated for 1 hour in swine-anti-rabbit antibodies conjugated with horseradish peroxidase (1:1000). The result was after thorough washing in PBS solution visualized by 0.1M Tris-HCl (pH = 7.5) in presence of 0.6% 3,3´-diaminobenzidine and 100µl/100 ml of 30% H_2O_2.

3 Results and Discussion

3.1 RNA Isolation and Preparation of complementary DNA

Total RNA was isolated from tissues of six partially engorged females. Single-strand complementary DNA was synthetized from isolated RNA using an anchored-oligo$(dT)_{18}$ primers. The concentrations of the RNAs and DNAs were determined by light spectra absorbance at λ = 260 nm.

tissue sample	**RNA (μg/ml)**	**ss cDNA (μg/ml)** dilution 1:10 (cDNA:dH_2O)
salivary glands	1128	1874.8
gut	679	1125.2
ovaries	723	1314.7

Tab. 1: Isolated RNA and synthetized cDNA concentrations.

3.2 Isolation of gene fragment for IrAM9

Amplification was provided by complementary DNA from salivary glands using gene-specific primers IrAM9-RT S and IrAM9-RT AS. Amplificated PCR products were isolated from 1% agarose gel using QIAquick PCR Purification Kit (250) (QIAgen). The size of the gene fragment is approximately 1040 bp. The expected size of the *I. scapularis* genome fragment which is closely related to *I. ricinus* (>95%) was 498 bp. (Burešová et al., 2011).

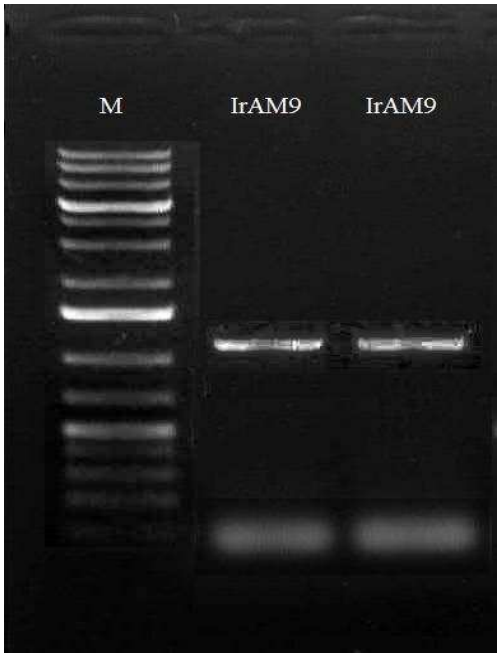


Figure 4: PCR product with specific primers.
(IrAM9-RT-S and IrAM9-RT-AS)
M – DNA molecular weight marker (bp)
IrAM9 – acquired gen for Iram9 (≈ 1040 bp)
(twice for better products isolation)

3.3 PCR product cloning, transformation and sequencing

The amplicon product was cloned into the pCR 2.1-TOPO® Plasmid vector with ampicilin selection (Invitrogen) and transformed into the TOP 10 Chemically Competent cells (Invitrogen). From 12 accrued colonies the plasmid DNA was isolated and the presence of the insert was confirmed using PCR reaction (with M13 S and M13 AS primers). Plasmids with inserts were sequenced in the Laboratory of genomics (Biologické centrum AVČR, v.v.i.., České Budějovice)

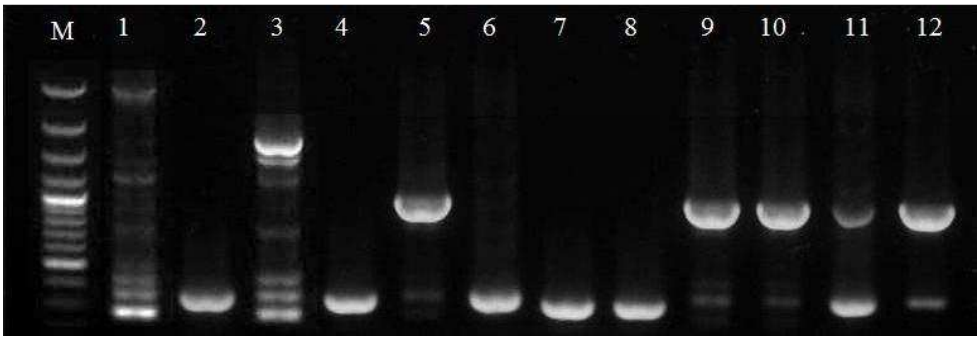


Figure 5: PCR insert verification. M – molecular weight marker, 1 – 12 colonies samples. *Samples 5, 9, 10 and 12 were selected and sequenced using M13 S and M13 AS primers.*

3.4 Tissue profile of IrAM9 expression

The reverse transcription PCR profiling of ovaries, salivary glands and gut tissue from partially engorged females revealed that the message for IrAM9 is expressed in salivary glands and ovaries, but not in the gut.

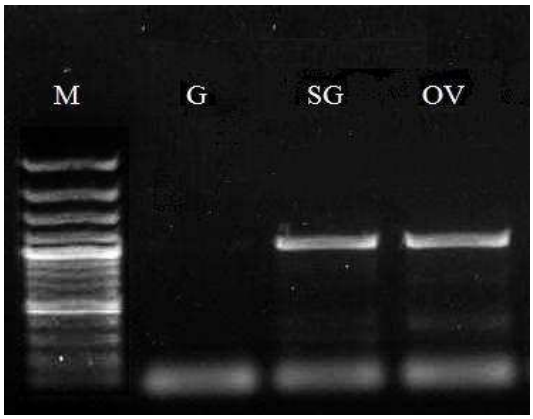


Figure 6: Transcriptional profile of IrAM 9 by RT-PCR analysis*. mRNA of IrAM9 is present only in ovaries and salivary glands.*
M – molecular weight marker, G – gut,
SG – salivary glands, OV – ovaries

A similar expressional profile was reported also for IrAM1 (Burešová, 2009). This observation could indicate that the IrAM9 is secreted to saliva. This could predicate its important role in the immune cascade. This phenomenon is very interesting for the future study of mechanisms which occurs at the tick-host interface.

3.5 Nucleotide sequence

```
IrAM9     1 TGTTGGTGATGTGGTGGGACCGGCCTTTCCAACCATGCCTGTCAATGCCACCAGCCTCTT
IsAM9     1 TGTTGGTGATGTGGTGGGACCGGCCTTTCCAACCATGCCTGTCAATGCCACCAGCCTCTT

IrAM9    61 ATCCAAGCCCTTCTACTGTGGGGAACAGAACATGTTCAGCTTTGCTGCCAACTTGTACAC
IsAM9    61 ATCCAAGCCCTTCTACTGTGGGGAACAGAACATGTTCAGCTTTGCTGCCAACTTGTACAC

IrAM9   121 ATTGTTGTACTTGCGGCTCACTAACCAACGTGACGTTTCCATCGAGAAGCAGGCCTTCAA
IsAM9   121 GTTGTTGTACTTGCGGCTCACTAACCAGCGTGACGTTTCCATCGAGAAGCAGGCCTTCAA

IrAM9   181 GTACCTAAACTTGGGATACCAAAGACAACTCAGTTACCAAAACGACGATGGGAGCTTCAG
IsAM9   181 GTACCTAAACTTGGG----------------------------------------------

IrAM9   241 TGTGTTTCGGTGGCACAGCCAGCCAAGTGTCTGGTTAACATCCTTTTGCGCCCGGGTGTT
IsAM9   196 ------------------------------------------------------------

IrAM9   301 TCACAAGGCAACGTTTCAGGCTTCCGCAACGTGTCTCTAACCGCGCACGTGCTCATCACA
IsAM9   196 ------------------------------------------------------------

IrAM9   361 CTGGCCGAAGTTAGGGATATCCGAGGAGAAATCGGCTCAAGGGCAGCCACGGCCCGCAGG
IsAM9   196 ------------------------------------------------------------

IrAM9   421 TCAGCTGCACGCTACCTGGAGCGCATGCTGCACGATATCGAGAAGTTGGAGGACCCCTAC
IsAM9   196 ------------------------------------------------------------

IrAM9   481 GAACTGGCCATTGTGGCGTATGCCCTCACCCTGGTCAACAGCGTGGAAAGGGGAGACCGC
IsAM9   196 ------------------------------------------------------------

IrAM9   541 ATTCAACCGACTGAGCGAGAAGTTGAGAGAGACAAGCGGCATGAGGTACTGGTCTCGCAG
IsAM9   196 ----------------------------------CGGCATGAGGTACTGGTCTCGCAG

IrAM9   601 CGACCTGCCGGCGCCTCCTGTCCTCATAGAGAACAACCGGCCCTACCTGTACCCGCGCCT
IsAM9   220 CGACCTGCCGGCGCCTCCTGTCCTCATAGAGAACAACCGGCCCTACCTGTACCCGCGCCT

IrAM9   661 GCCCTTCACGAACGACGCCTCCAACGTGGAGACCACATCCTACGGGCTGCTGGTGCACGT
IsAM9   280 GCCCTTCACGAACGACGCCTCCAACGTGGAGACCACATCCTACGGGCTGCTGGTGCACGT

IrAM9   721 GGCTCGTCAAGCGGTCGTGCAGAAGGAGATTGTCGAGTGGCTCAACACACAGAGGCTCTC
IsAM9   340 GGCTCGTCAAGCGGTCGTGCAGAAAGAGATTGTCGAGTGGCTCAACACACAGAGGCTCTC

IrAM9   781 GCATGGTGCCTGGGCGTCCACTCAGGACACCCTGATGGCGATGCAGGCACTGACCGAGTT
IsAM9   400 GCACGGTGCCTGGGCGTCCACTCAGGACACCCTGATGGCGATGCAGGCACTGACCGAGTT

IrAM9   841 TTCGGTGCAATCCCGCTCGAGGGATGTGACGGATATTACG
IsAM9   460 TTCGGTGCAATCTCGCTCGAGGGATGTGACGGATATTACG
```

Figure 7: Nucleotide sequence comparison of two macroglobulin complement related proteins. *Partially sequence of new identified IrAM9 from I. ricinus and known IsAM9 from I. scapularis (GenBank XM_00240261).*

The similarity of macroglobulin complement related protein from *I. ricinus* and *I. scapularis* is apparent. However, the middle region of IsAM9 from *I. scapularis* is missing. This could be caused by missing exon in the nucleotide sequence for *I. scapularis*.

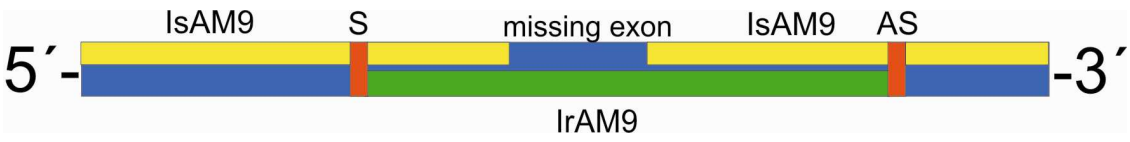


Figure 8: Scheme of known sequence of IrAM9 and IsAM9 with the missing exon.
S – sense primer, AS – antisense primer.

3.6 Expression of recombinant protein – the pilot expression

The PCR construct for protein expression was prepared using PCR product with specific primers IrAM9pET100S and IrAM9pET100AS (cDNA from salivary glands of *Ixodes ricinus* was used as a template). The PCR construct was cloned into pET100/D-TOPO® expression vector (Invitrogen) and transformed into BL21 Star™ (DE3) One Shot® Chemically Competent cells (Invitrogen). Expression of recombinant protein was undertaken in 200 ml bacterial culture by induction with 0.5mM isopropyl-*β*-D-thiogalactopyranoside (IPTG) according to the Champion™ pET Expression System protocol (Invitrogen). The progres of the induction was evaluated by SDS-PAGE electrophoresis. The most suitable expression period was reached after 4.5h with IPTG addition. On the other hand, after 6 hours of cultivation without IPTG addition only reduced ammount of expressed protein was formed.

atgcggggttctcatcatcatcatcatcatggtatggctagcatgactggtggacagcaa
M R G S H H H H H H ***G M A S M T G G Q Q***
atgggtcgggatctgtacgacgatgacgataaggatcatcccttcacctttgcgcccgg
M G R D L Y D D D D K D H P F T F C A R
gtgtttcacaaggcaacgtttcaggagtgggagcactttctgtatatagatcccaccatc
V F H K A T F Q E W E H F L Y I D P T I
atccagaaggccatagggtggctactagaccgccaatctcccgaaggctcgtttcacgaa
I Q K A I G W L L D R Q S P E G S F H E
acatccttttcgcctatgaccgcaagatgtctgcttcgtcggagaatcccgatgatccg
T S F F A Y D R K M S A S S E N P D D P
gtgcgcttccgcaacgtgtctctaaccgcgcacgtgctcatcacactggccgaagttagg
V R F R N V S L T A H V L I T L A E V R
gatatccgaggagaaatcggctcaagggcagccacggcccgcaggtcagctgcacgctac
D I R G E I G S R A A T A R R S A A R Y
ctggagcgcatgctgcacgatatcgagaagttggaggacccttacgaactggccattgtg
L E R M L H D I E K L E D P Y E L A I V
gcgtatgccctcaccctggtcaacagcgtggaaggggagaccgcattcaaccgactgagc
A Y A L T L V N S V E G E T A F N R L S
gagaagttgagagagacaagcggcatgaggtactggtctcgcagcgacctgccggcgcct
E K L R E T S G M R Y W S R S D L P A P
cctgtcctcatagagaacaaccggccctacctgtacccgcgcctgcccttcacgaacgac
P V L I E N N R P Y L Y P R L P F T N D
gcctccaacgtggagaccacatcctacgggctgctggtgcacatggctcgtcaagcggtc
A S N V E T T S Y G L L V H M A R Q A V
gtgcagaaggagattgtcgagtggctcaacacacagaggctctcgcatggtgcctgggcg
V Q K E I V E W L N T Q R L S H G A W A
tccactcaggacaccctgatggcgatgcagtaa
S T Q D T L M A M Q -

Figure 9: Nucleotide sequence and its translation to AA sequence with predicted molecular weight of 31 kDa. *Grey – 6His-Tag, bold and italic – part of the pET100/D-TOPO® expression vector.*

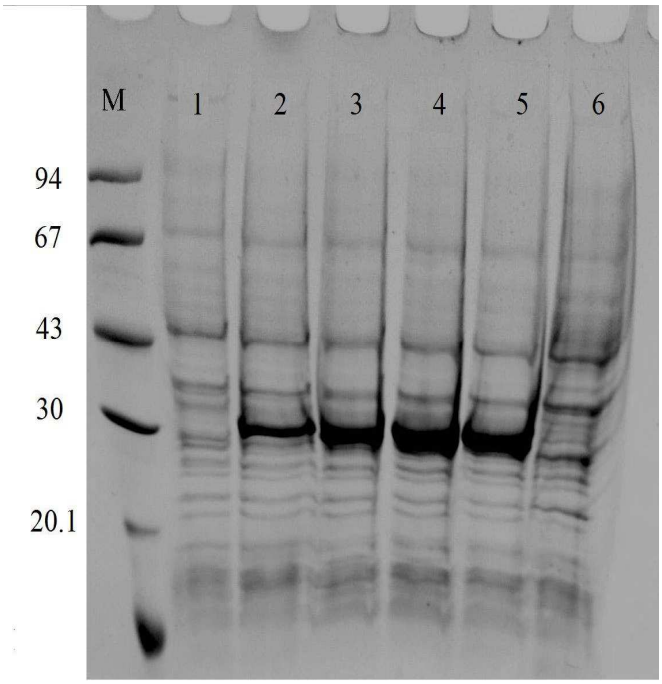


Figure 10: SDS-PAGE of the pilot expression induced with 0.5mM IPTG. *(incubation times at 200 rpm/ 37^{0}C) 1 - 0 h from the expression beginning, 2 - 1.5 h from the expression beginning, 3 - 3.0 h from the expression beginning, 4 - 4.5 h from the expression beginning, 5 - 6.0 h from the expression beginning, 6 - 6.0 h from the expression beginning without IPTG addition, M – marker (kDa)*

3.7 Recombinant protein isolation and purification

3.7.1 Fraction isolation

The bacterial cells were disrupted by sonification method and centrifuged. Supernatants and the sediments (soluble and nonsoluble protein fractions) were separated and examined by SDS-PAGE electrophoresis. The figure 11 shows, that the recombinant protein was mainly present in inclusion bodies. Molecular weight of IrAM9 with 6His-tag is 31,35 kDa (protein calculator).

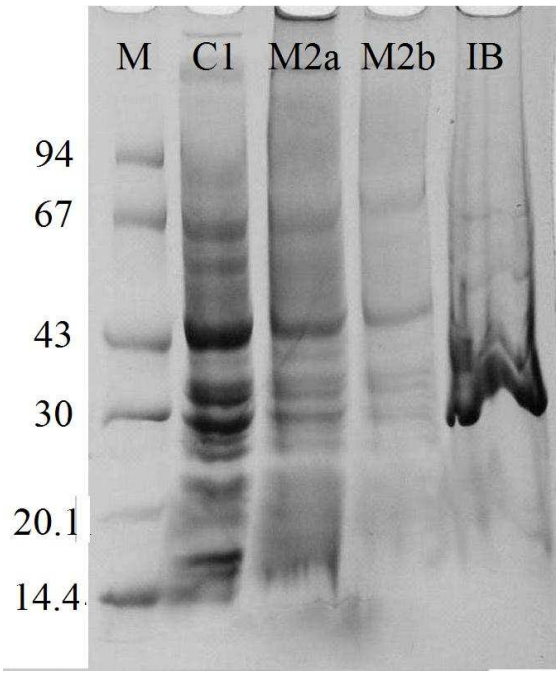
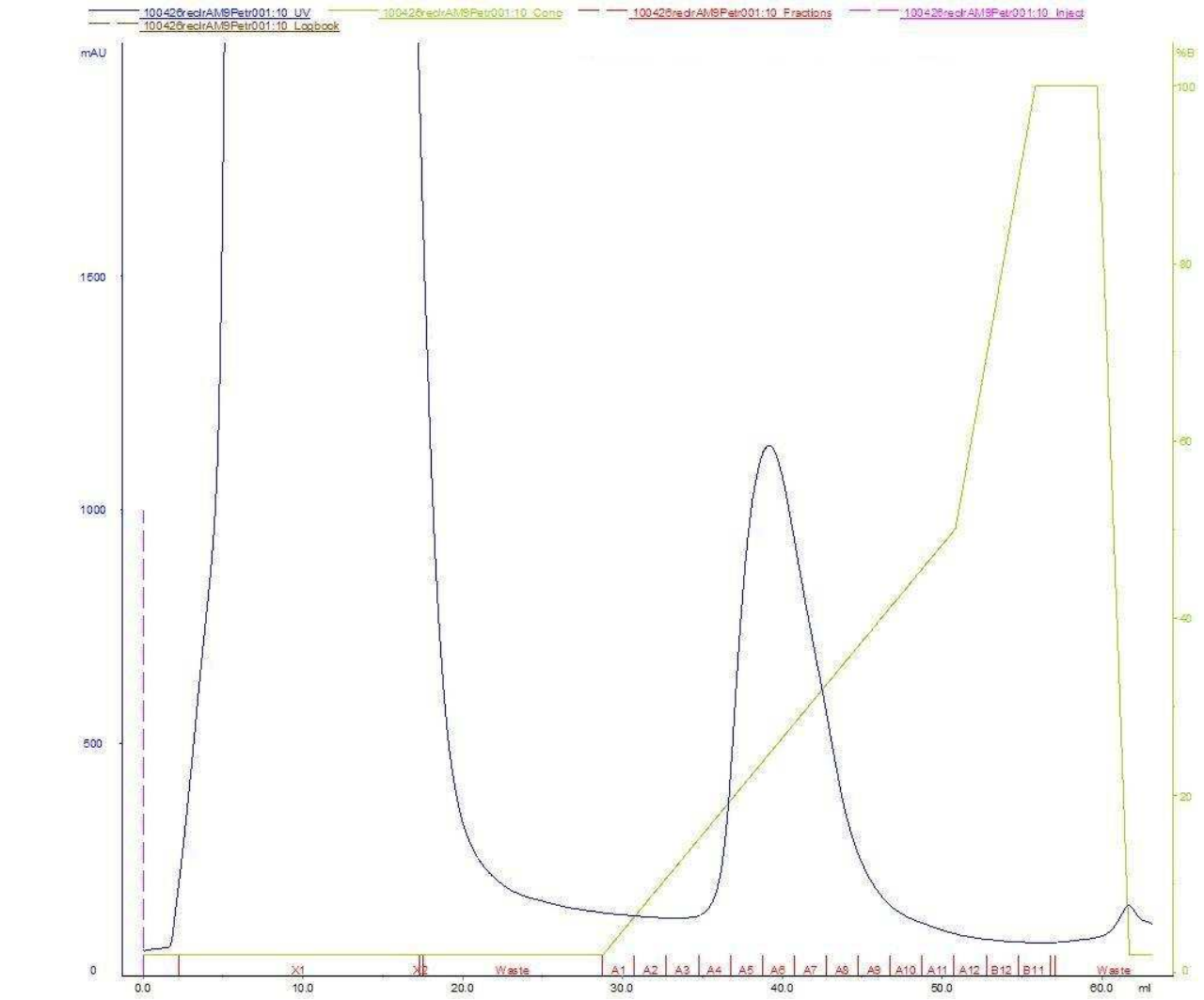


Figure 11 : SDS-PAGE of isolation fractions. *Excessively expressed IrAM9 in inclusion bodies. M – marker (values in kDa). C1 – cytoplasmatic proteins, M2a and M2b – membrane proteins, IB – inclusion bodies.*

3.7.2 Chelate column chromatography

Dissolved inclusion bodies were purified by chelate chromatography making use the affinity of 6His-tag to Co^{2+} ion. The presence of IrAM9 eluate fraction was detected spectrophotometrically and resulting fractions were verified by SDS-PAGE (see Figure 12).



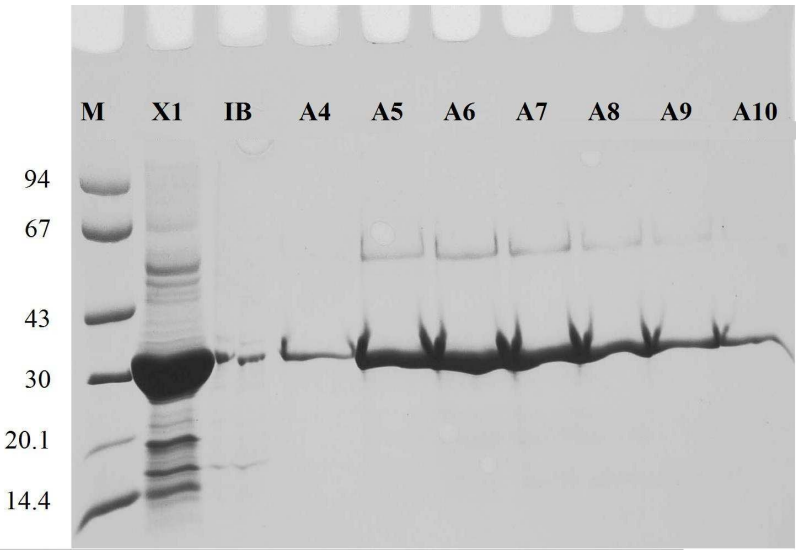


Figure 12: Chromatogram (top) and SDS-page (bottom) of the purification. *M – marker (kDA), X1 – flow through, IB – inclusion bodies, A4-A10 – eluate recombinant protein IrAM9 (~ 31 kDA)*

3.8 Detection and analysis of IrAM9

Separated tissue protein samples were electrophoretically transferred on PVDF membrane and stained with Coomassie Brilliant Blue (Figure 13). Second PVDF membrane was used for immunodetection of IrAM9. The immunodetection was performed using prepared primary antibody (dilution 1:50) obtained from rabbits sera and swine-anti-rabbit antibodies conjugated with horseradish peroxidase (dilution 1:1000). The result was visualized diamobenzidine as a substrate.

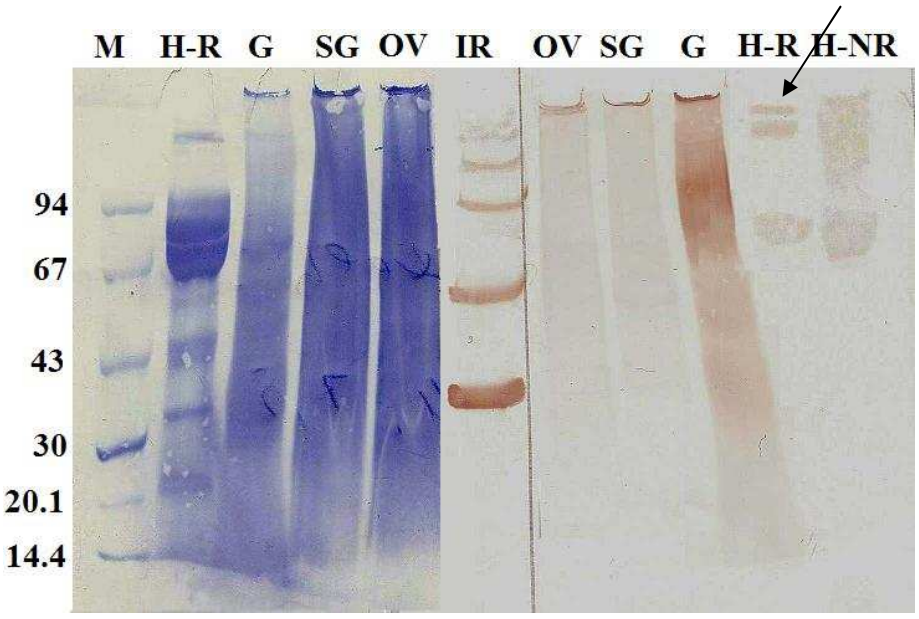


Figure 13: Tick tissue proteins on PVDF membrane stained by CBB (left). Detection and analysis of IrAM9 in tick plasma by immunoblotting (right). *M – marker (kDA), IR – recombinant protein IrAM9, OV – ovarie, SG – salivary glands, G – gut,H-R – reduced hemolymph IR – recombinant protein IrAM9, H-NR – nonreduced hemolymph.The arrow points the position of IrAM9 bands.*

IrAM9 was likely detected as a high molecular weight band in the hemolymph under reducing conditions. The range of the marker (see Figure 13) does not allow the precise determination of the molecular weight of IrAM9. The total expected molecular weight of IrAM9 is about 170 kDa without glycosylation. We estimate the size of detected band to be of about 200 kDa. Under nonreducing conditions the protein could not be detected indicating that it possibly forms disulfide-bond linked dimers as is the case of many α_2M molecules (Armstrong, 2006). Regarding to the recent papers (Burešova et al, 2011) and Figure 1, the structure of IrAM9 belonging to the MCR-like (macroglobulin complement related) protein group is not yet fully known. For the future investigation of exact molecular weight of the protein a native gel should be used. The ultimate verification that the 200 kDa band really belongs to the IrAM9 has to await a successful RNA interference experiment.

4 Conclusion

Using primers derived from IsAM9 from *I. scapularis*, the partial sequence of IrAM9 from *I. ricinus* was obtained. The similarity of macroglobulin complement related protein from *I. ricinus* and *I. scapularis* was apparent. However, the IsAM9 from *I. scapularis* has missing exon in the nucleotide sequence. The reverse transcription PCR profiling of ovaries, salivary glands and gut tissue revealed that the message for IrAM9 is expressed in salivary glands and ovaries, but not in the gut. The recombinant protein IrAM9 from *I. ricinus* was successfully expressed in *E. coli* expression system. The native IrAM9 was likely detected by immunoblotting as a high molecular weight band in the hemolymph under reducing conditions. Under nonreducing conditions the protein could not be detected indicating that it possibly forms disulfide-bond linked dimers as is the case of many α_2M molecules.

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