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Faculty of Science

Characterisation of novel serpin TILIr and its relatives from superfamily of serine protease inhibitors from *Ixodes ricinus* tick

Master thesis

Bc. Hana Slabá

Supervisor: Nataliia Rudenko, PhD.

Marina Golovchenko, MSc.

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Annotation:

Novel gene, named TILIr (**TIL *I*xodes *r*icinus**), that encoded yet unknown protein with TIL domain characteristic to proteins that belong to trypsin inhibitors family was discovered and isolated from hard tick *Ixodes ricinus*. Two isoforms of novel gene were identified and studied in this project. As the result of this study recombinant TILIr protein was produced in bacterial expression system and analysis of its functional characteristics was conducted. The protein belonging to family of trypsin inhibitors represent a potential candidate for anti-tick vaccine component.

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V Českých Budějovicích, datum …….

Hana Slabá

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Content

1. Introduction

1.1 Differential expression of genes in ticks

Every cell nucleus has the same DNA information that means they all contain complete genome. Only small part of the genome is expressed in each cell and that part varies in different cell types. The cell is defined by the genes that are transcribed into mRNA´s and translated into proteins. In other words, cells regulate gene expression and use great variety of mechanisms to increase and/or decrease gene production. Genes that are not expressed remain potentially active (Developmental biology, S. F. Gilbert, 2000).

Differentially expressed genes play important role in the immune response of the tick to blood meal or infection. Down and upregulation of genes in responce to tick feeding is a commonly observed phenomenon (Carvalho et al., 2010; Nene et al., 2002). Also the presence of pathogen in the vector is a known reason of differential expression of genes. Macaluso et al. (2003) observed upregulation of genes in ovary of *Ricketssia* infected *Dermacentor variabilis* but downregulation of most transcripts in salivary glands and midgut. Expression of 27 mRNA was specifically induced by blood meal in *Ixodes ricinus* ticks (Leboulle et al., 2002). Rudenko et al. (2005) found upregulation of several genes in salivary glands of *I. ricinus* after feeding with pathogen-free bloodmeal and differential expression of *I. ricinus* genes induced by *Borrelia burgdorferi* infected bloodmeal.

1.2 Tick immune system

Ticks are significant arthropod vectors of various pathogens. The diversity of pathogens transmitted by ticks exceeds the amount of pathogens transmitted by any other bloodsucking arthropod. During blood meal tick can be infected with various microorganisms such as protist, viruses, bacteria, fungi or nematodes and they further transmit these pathogens to their vertebrate host (Sonenshine, 1993). Effective immune system is therefore crucial for the tick survival (Fogaca et al., 2004).

Immune defence system of invertebrates is very different from the vertebrate immunity, particularly, because of the absence of memory cells and antigen-antibody complex (DeMar, 2006). The pathogen has to overcome tick´s humoral and cellular defence which form tick innate immune response. The major site of function of humoral and cellular

immunity is hemolymph, but also midgut and salivary glands. But the first barrier for the pathogen comprises cuticula that presents chemical and mechanical barrier followed by other body tissues such as salivary glands or midgut (DeMar, 2006).

Cellular immunity is primarily presented by hemocytes, granulocytes and plasmatocytes. The most significant cell-mediated response is phagocytosis, nodulation and encapsulation (Sonenshine and Roe, 2014). On the other hand, humoral response includes the complement related molecules, haemaglutination and antimicrobial proteins.

Although, the interest to the study of tick immune mechanisms was lately significantly raised, there is still a lot to discover. Antimicrobial peptides in particular are of great interest because of their potential in vaccine development.

1.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are oligopeptides of rather small size ranging from 20 to 50 amino acid residues (Yi et al., 2014). AMPs can be found in both procaryotic and eukaryotic cells (Bahar and Ren, 2013). They are significant part of humoral response of great variety of organisms. Specifically, they affect bacteria, viruses, fungi, protists, yeast as well as nematodes (Ganz, 2003).

Antimicrobial peptides also play an important role in tick immune defense. They are essential for elimination of diverse pathogens present in tick and represent multiple members of defensins', lectins', proteases' and protease inhibitors' families, enzymes and products related to oxidative stress (Sonenshine and Hynes, 2008).

AMPs are present in tissues and organs such as gastrointestinal and respiratory system or skin that most likely gets into contact with airborne pathogens (Zasloff, 2002). Eukaryotic cells producing AMPs are: besides lymphocytes (Oppenheim, 2003) and phagocytes (Hancock and Scott, 2000) of the immune system, also epithelial cells of gastrointestinal and genitourinary system (Ganz, 2003; Niyonsaba, 2002). In invertebrates AMPs are often expressed in fat body or in midgut and then released to hemolymph (Nakajima et al., 2002).

Structure

Most AMPs include α-helices or β-sheets or both motifs as part of their structure. They are also typical for their low molecular weight and positive net charge at physiological pH (Bulet et al., 1999). Cationic and amphipathic peptides represent the vaste majority of AMPs (Ganz, 2003).

Mechanism of action

Antimicrobial peptides kill by disrupting membrane integrity, further they inhibit proteins, DNA and RNA synthesis (Bahar and Ren, 2013). According to Ganz (2003), there are three major hypotheses about how the disruption of membrane integrity kills pathogen. Firstly, the disruption of the membrane destroys the equilibrium of intra- and extracellular ion concertation that affects the microbe. Secondly, the AMP can enter the cell after the destruction of membrane and bind to so far unknown molecules and interfere with their function. And finally, several AMPs can generate pores permeable for water but not for other substances resulting in high osmotic pressure and bursting of the microbial membrane. Exposure to high concentration of antimicrobial peptides leads to irreversible damage.

Some AMPs are able to inhibit intracellular pathways (Cudic and Otvos, 2002). The intracellular action of AMPs consists of ability to inhibit DNA and protein synthesis of microbes (Nicolas, 2009; Nishikata et al., 1991). The mode of action of different AMPs differs regarding their specific target.

AMPs as natural antibiotics

Being considered the natural antibiotics, AMPs represent a great potential as replacement of synthetic antibiotics to which resistance of pathogens is emerging gradually. AMPs differentiate in their target. Antibiotics target specific cellular activities, but the main aim of AMPs is lipopolysaccharide layer of cell membrane (Jenssen et al., 2006).

The main classes of antimicrobial peptides present in ticks are discussed below.

Defensins

Defensins are one of the major groups of antimicrobial peptides present in ticks. They are cyclic antimicrobial peptides containing three or four disulphide bridges pairing six cysteine residues and forming α-helix and β-sheet structural motifs. The molecular weight of

defensin is around 4 kDa. The majority of defensins are cationic (Sonenshine and Hynes, 2008).

Lately, significant number of defensins were isolated and characterised from various species of ticks, including defensin from *Dermacentor variabilis* (Johns et al., 2001), defensins A, B, C, D from *Ornithodoros moubata*, (Nakajima et al., 2001), microplusin from *Rhipicephalus microplus* (Fogaca et al., 2004), scapularisin from *Ixodes scapularis* (Hynes et al., 2005), def1 and def 2 from *Ixodes ricinus* (Rudenko et al., 2005; Rudenko et al., 2007), persulcatusin from *Ixodes persulcatus* (Saito et al., 2009), DefMT from *I. ricinus* (Tonk et al., 2014) and other.

Cystatins

As Abrahamson et al. (2013) describe them, cystatins are reversible and tight-binding inhibitors that interact with papain-like cysteine proteases and legumains. They are classified into 4 subfamilies on the basis of their structure.

The main focus is devoted to type 2 tick cystatins because of their secretory nature. They are essential part of vector control research (Schwarz et al., 2012). Several cystatins were isolated from *Ixode*s genus ticks such as sialostatins from *Ixodes scapularis* (Valenzuela et al., 2002, Ribeiro et al., 2006) or cystatin from *Ixodes ricinus* (Jacot, 2003).

1.4 Serine protease inhibitors

Serine protease inhibitors also called serpins are the largest superfamily of protease inhibitors that can be found in all multicellular eukaryotes (Irving et al., 2000; Rawlings et al., 2004). More than 1500 serpins have been found in various organisms and more than 40 of them in humans (Huntington, 2011, Heit et al., 2013). Serpins take an important part in diverse range of processes such as blood coagulation, fibrinolysis, apoptosis, development or inflammation (Carell et al., 1987). Serpins may play a significant role in tick parasitism as they obstruct the host immune reaction (Gulley et al., 2013).

Organization

Serpins have been found in various organisms starting from prokaryotes and unicellular eukaryotes and going to humans (Irving et al., 2002; Irving et al., 2003).

However, the majority of prokaryotes do not possess serpin-like genes and no serpins have been found in fungi (Irving et al., 2002).

Serpin superfamily is divided into 16 clades. Each clade is named with a letter from A to P (Irving et al., 2000). "Proteins are named SERPINXy, where X is the clade and y is the number within that clade" (Law et al., 2006).

Structure

Serpins are relatively large molecules about 330-500 amino acids long with weight around 40-50 kDa (Ruhlmann et al., 1973). The serpin structure contains three β-sheets and 8-9 α-helices. Important part of the structure forms the reactive center loop (RCL) that is responsible for interaction with target proteases (Huntington, 2011).

Native serpins are metastable. However, by a conformational change they can become more stable or completely relaxed. During this transition an additional strand is formed by amino-terminal portion of the RCL and inserted as fourth strand in β-sheet A. These conformational changes are called the stressed (S) and relaxed (R) transition. Transition from S to R form is the main step of the protease inhibition. Non-inhibitory serpins do not have the ability to insert RCL into β-sheet A (Stein et al., 1989; Hood et al., 1994).

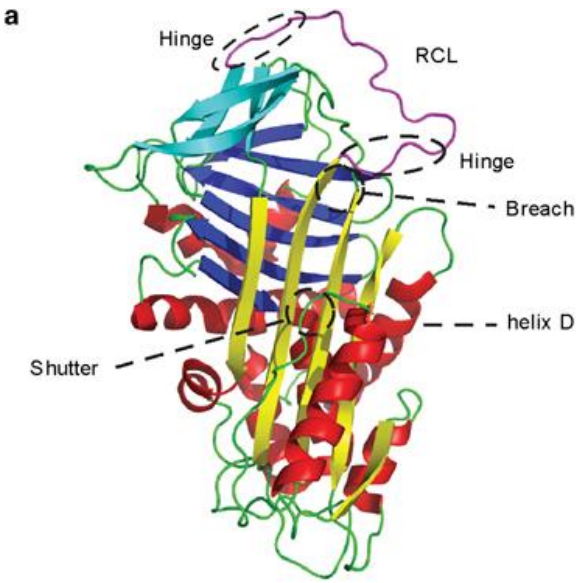

Figure1: Serpin structure (Kaiserman and Bird, 2010).

Functions

The main function of serpins is the inhibition of serine proteases, but some also inhibit caspases (Ray et al., 1992) and papain-like cysteine proteases (Schick et al., 1998; Irving et al., 2002). Besides their inhibitory functions serpins also perform non-inhibitory functions such as tumour suppression (Zou et al., 1994), molecular chaperons (Ishida and Nagata, 2011) or hormone transport (Pemberton et al., 1988).

Serpins can be extracellular or intracellular or in some cases they can be both extra- and intracellular (Mikus et al., 1993). Intracellular serpins are included in cell protection and prevent excessive release of cytotoxic proteases when extracellular serpins control proteolytic cascades such as coagulation (Whisstock, 2005).

Serpins are activated in the presence of specific co-factors that are usually glycosaminoglycans. Typical serpin cofactor is, for example, heparin (Rau et al., 2007). The role of co-factors is to connect serpin and the protease and to accelerate the complex formation.

Mechanism of action

For efficient protease inhibition the RCL cleavage followed by insertion is essential. The protease stays linked to the serpin in the final complex and both the protease and active site of the serpin are extensively disordered after the conformational change (Huntington et al., 2000). Distortion at the active site means that the protease cannot be released. Cleavage of cryptic sites within the protease results in enzyme inactivation. As the RCL site is also cleaved, the reaction is irreversible and destructive for the serpin (Law et al., 2006).

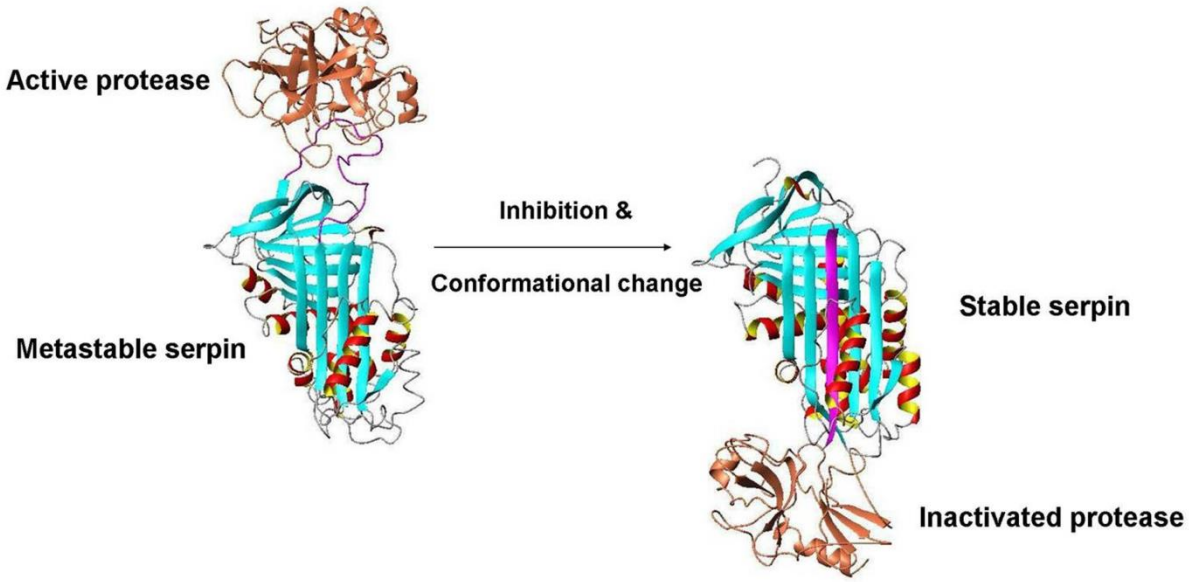

Figure 2: Serpin conformational change and inactivation of protease (http://faculty.rx.umaryland.edu/pwintrode/research-projects/, 5.11.2016).

Inactivation of serpins

Serpins are very susceptible to mutations due to their flexibility in conformational changes. These mutations can lead to loss of inhibitory function (Kaiserman et al., 2006; Gooptu and Lomas, 2009). For example, after insertion of RCL into the β-sheet A, the reactive center loop cannot interact with the protease and it becomes inactive. This can be observed, for example, in SERPINE1 (plasminogen activator inhibitor-1) after becoming latent due to absence of vitronectin (Law et al., 2006).

At higher temperatures serpins often form polymers. This process is very similar to the conformational changes needed for protease inhibition. Polymer is formed by insertion of RCL of one molecule in β-sheet A of another molecule. Created polymer is inactive (Lomas et al., 1992; Huntington et al,, 1999). Serpin polymerization can lead to several diseases (serpinopathy) or deficiencies such as emhysema or liver cirrhosis (Lomas et al., 1992; Lomas and Carrell,, 2002).

1.4 Trypsin inhibitors

Trypsin inhibitors are a type of serine protease inhibitors that inhibit the production of trypsin or reduce its activity.

TIL domain

Trypsin inhibitors contain trypsin inhibitor-like cysteine rich (TIL) domain that is important for the protease inhibition. Peptides with TIL domain play significant role in immune response or anticoagulation (Sasaki et al., 2007; Chen et al., 2013). For some of their attributes such as small size or compact rigidity they have great potential in development of pharmaceutical drugs and vaccines.

Not only trypsin inhibitors possess TIL domain but also many other extracellular proteins. Peptides containing TIL domain are usually smaller than other serine protease inhibitors containing around 56-84 amino acid residues (Zeng et al., 2014).

The TIL domain consists of 10 cysteine residues connected by five disulphide bridges. The disulphide bridges are present in following order: C1-C7, C2-C6, C3-C5, C4-C10 and C8-C9 (Bania et al., 1999). Zeng et al. (2014) observed intron gain in the genes of the TIL peptides that can indicate dynamic evolution of these genes.

Only limited number of peptides containing TIL domain is identified. So far, five TIL family peptides were described. Some of the most known trypsin inhibitor like cysteine rich proteins are Ixodidin (Fogaca et al., 2006) and BMSI-7 from *Boophilus microplus* (Sasaki et al., 2007), Api m-6 from *Apis mellifera* (Michel et al., 2012), BmKAPI from scorpion *Mesobuthus martensii* (Zeng et al., 2002) and SjAPI from scorpion *Scorpiops jendeki* (Chen et al., 2013).

1.5 Tick serpins

Need for anti-tick vaccine has brought the attention to serine protease inhibitors as potential candidates for vaccine mainly because of their ability to regulate blood coagulation, food digestion, inflammatory and immune responses (Mulenga et al., 2001). Imamura et al. (2005, 2006) reported reduction of engorgement rates and increased mortality of *Haemaphysalis* and *Rhipicephalus* ticks after using combination of different serpins to vaccinate cattle.

Serpins were studied in one of the most economically important ticks of livestock *Rhipicephalus microplus*. The first molecule with proteinase activity found in tick, identified by Fogaca et al. (2006), is ixodidin. Ixodidin inhibits elastase and chymotrypsin and also growth of *Escherichia coli* and *Micrococcus luteus*. Boophilin, is another serpin, described

by Macedo-Ribeiro et al. (2008) as thrombin inhibitor with two canonical Kunitz type domains that additionally inhibits trypsin-like serine proteinases such as trypsin and plasmin. At the same time article about novel subtilisin inhibitors BMSI-6 and BMSI-7 was published and BMSI-7 was found to be a first subtilisin inhibitor with activity toward fungus *Metarhizium anisopliae* (Sasaki et al., 2007). Eighteen cDNA sequences encoding serpins were identified in cattle tick *R. microplus* by Tirloni et al. (2014). Followed studies of Rodriguez-Valle et al. (2015) added 4 other novel serpins to this family. Serpins were named RmS (1-22) (*Rhipicephalus mircroplus* Serpin) and the study showed most of them are expressed in ovaries or salivary glands, nevertheless RmS-1 and RmS-2 were expressed in all tissues analyzed. The first commercially used anti-tick vaccine based on Bm86 protein from *R. microplus*, localised in gut by Gough and Kemp (1993), encouraged further studies on potential vaccine candidates, including Andreotti et al. (2012) testing recombinant trypsin inhibitor from *R. microplus*.

Although not so advanced as studies of *R. microplus*, studies of African *Rhipicephalus appendiculatus* are slowly emerging. Due to the economical impact of *R. appendiculatus* on African cattle, research such as Mulenga´s et al. (2003), who described four serine proteinase inhibitors from *R. appendiculatus*, aspire to vaccine candidate search.

Another two serpins, named RHS-1 and RHS-2, were identified in tick *Rhipicephalus haemaphysaloides*. RHS-1 is an extracellular protein differentially expressed in salivary glands, on the other hand RHS-2 is intracellular protein specifically expressed in midgut. Both proteins have shown anti-chymotrypsin and anticoagulant activity (Yu et al., 2013). In addition, Nabian et al. (2014) described serpin with trypsin inhibitory activity from *Rhipicephalus annulatus* larvae.

Mulenga et al. (2007) characterized 17 serine protease inhibitors in *Amblyomma americanum* tick that were named Lospins. Lospins were expressed in salivary glands, ovaries and midgut (Mulenga et al., 2007). Further research revealed anti-hemostatic and anti-complement functions of AamAV422 serpin (Mulenga et al., 2013).

A thrombin inhibitor isolated from *Amblyomma hebraeum* hemolymph termed Amblin was identified by Lai et al. (2004). Analysis of *Amblyomma maculatum* sialotranscriptome has uncovered 32 coding sequences for members of serpin superfamily

(Karim et al., 2011). An insight into *Amblyomma variegatum* sialome has shown 6 serpin expressed tag sequences (Ribeiro et al., 2011).

Two serpins expressed in midgut were identified by Anderson et al. (2008) while exploring the mialome of *Dermacentor variabilis*. Subsequently, Sonenshine et al. (2011) obtained and analysed transcriptome of *D. variabilis* and identified 10 serpins in male reproductive system and spermatophore.

Immunization of Japanese white rabbits by rHLS1 serpin from *Haemaphysalis longicornis* that is only expressed in midgut has been performed by Sugino et al. (2003) and showed significant anti-tick immunity. Nevertheless, the efficiency of single molecule was reported as too low, but the use of cocktail vaccine seems to be promising. Consequently, Imamura et al. (2005) continued by analysing HLS2 as potential anti-tick vaccine. Unlike HLS1, HLS2 is only present in haemolymph of feeding ticks. The results have shown great potential of HLS2 in anti-tick immunization and HLS2 became an important candidate for component in vaccine cocktail.

Ixodes scapularis genome revealed the presence of at least 45 serpin genes. Eighty four percent of these serpins were found to be expressed in salivary glands and midgut (Mulenga et al., 2009). Previously, 2 serpins were found in *I. scapularis* sialome (Valenzuela et al., 2002), the sialome was then updated in 2005 (Ribeiro et al., 2006). Ibelli et al. (2014) analysed *I. scapularis* saliva serpin IxscS-1E1 and found that it inhibits thrombin and trypsin. Kim et al. (2016) found several novel serine protease inhibitors in tick saliva and studied their secretion profiles.

Iris is an elastase inhibitor found in *Ixodes ricinus* tick (Prevot et al., 2006). Iris is upregulated during blood meal with maximal expression at 4th day (Leboulle et al., 2002). The analysis of anti-hemostatic properties showed interference with blood coagulation and fibrinolysis. Unlike any other ectoparasite serpin, Iris inhibits also hemostasis and therefore interferes with both immune response and hemostasis (Prevot et al., 2006). Iris has been studied as a potential vaccine candidate on rabbits and mice infested by nymphs and adults *I. ricinus* ticks. The test showed higher mortality of female ticks and reduced weight gain in nymphs after feeding on vaccinated rabbits. However, ticks were affected by the vaccine only at the end of their blood meal. The immunization by Iris was not found sufficient but in

case of failing in search for efficient vaccine antigen, it could be used in cocktail of antigens (Prevot et al., 2007).

IRS-2, isolated from *I. ricinus* tick saliva, has been characterised. Chmelař et al. (2011) revealed inhibitory activity of IRS-2 toward cathepsin G, chymase and thrombin leading to inhibition of platelet aggregation and interference with inflammation and wound healing. Furthermore, the crystal structure of IRS-2 was presented making it the first crystal structure of serpin isolated from parasitic organism (Kovářová et al., 2010). Páleníková et al. (2015) extended the study and described the mechanism of inhibition of Th17 differentiation by impairment of IL-6/STAT-3 signalling pathway.

Although, mixed salivary gland and midgut transcriptome from partially fed *I. ricinus* ticks was published in 2014 (Schwarz et al., 2014) and midgut transcriptome from naive *I. ricinus* ticks in 2015 (Cramaro et al., 2015), Iris and IRS-2 remain the only two *I. ricinus* serpins functionally characterised up to date.

2. Project objectives

1. Using different techniques of molecular biology to find and characterize new members of *Ixodes ricinus* serpin family and to provide their molecular analysis.

2. To characterize new tick serpins at the genomic and transcriptional level.

3. To provide a close comparison of nucleotides and amino acids with purpose to analyse the divergence within this family of proteins.

4. To produce recombinant protein(s) and to determine their functions by conducting antimicrobial and blood clothing assays.

5. To interpret the obtained results.

6. To clarify the function of serine protease inhibitors in ticks using TILIr as an example.

3. Materials and methods

3.1 Materials and Chemicals

Table I: Used kits.

Kit	**Manufacturer**
NucleoSpin® RNA II Kit	Macherey-Nagel
Enhanced avian HS RT-PCR-100 kit	Sigma
QIAquick®Gel Extraction Kit	Qiagen
QIAprep® Spin Miniprep Kit	Qiagen
TOPO TA Cloning® Kit	Invitrogen
Champion™pET100 Directional TOPO® Expression Kit	Invitrogen
5'/3' RACE Kit, 2nd Generation	Roche
DNeasy® Blood and Tissue Kit	Qiagen

Table II: Used PCR primers.

Primer (Generi Biotech)	**Annealing temperature used (°C)**	**Source**	**Sequence (5´- 3´)**
vWf Forward 1	50	this work	ATG AAG GCG CTT CTG CTC TCC GG
vWf Forward 2	50	this work	CAC CAG AAT TGC GGC CGA CTT GC
vWf Reverse	50	this work	CTA ACA AGC CTT TGG CAT GGG T
Forw expr 101 tilir	55	this work	CAC CAT GAG AAT TGC GGC CGA CTT GC
Rev tilir Orig	50	this work	AGC CTT TGG CAT GGG TTT GAG
Rev Isoform 1	55	this work	AGC CTT AGG CAT GGG CTT GAG
Rev Isoform 2	55	this work	AGC CTT GGG CAT GGG CTT GAG

Actin Forward	55	Chrudimská et al., 2011 (GenBank - HQ682101)	CGT CTG GAT CGG CGG CTC TAT
Actin Reverse	55	Chrudimská et al., 2011 (GenBank - HQ682101)	ACG CGC ACT CTT TTC CAC AAT CTC
M13 Forward	50	TOPO TA Cloning® Kit - Invitrogen	GTA AAA CGA CGG CCA
M13 Reverse	50	TOPO TA Cloning® Kit - Invitrogen	CAG GAA ACA GCT ATG AC
T7 Forward	50	Champion™ Expression Kit - Invitrogen	TAA TAC GAC TCA CTA TAG GG
T7 Reverse	50	Champion™ Expression Kit - Invitrogen	GCT AGT TAT TGC TCA GCG G

Table III: Used chemicals.

50x TAE (for 1x TAE)	200mM Tris-HCl, 50mM EDTA
Agarose	1 % agarose (SERVA) v 1xTAE buffer
2x PCR Premix	Promega Taq DNA polymerase, 400μM: dATP, dGTP, dCTP, dTTP; 3mM MgCl2
6x Loading Dye	10mM Tris-HCL (pH 7.6), 0.03% bromophenol blue, 0.03% xylen cyanol FF, 60% glycerol, 60mM EDTA, 500x SYBR Green
100 bp DNA ladder	Thermo Scientific GeneRuler 0.5 μg/μl
LB medium	1.0% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, pH 7.0
LB agar	1.5% bacto-agar in LB medium
Antibiotics	Ampicillin (stock solution 50mg/ml)
1M IPTG	Isopropyl β-D-thiogalactoside, stock solution

30% Acrylamide/Bis-Acrylamide	Acrylamide and Bis-acrylamide, stock solution
TEMED	N, N ,N´ ,N´-Tetramethylethylenediamine
10% APS	ammonium persulfate, stock solution
10% SDS	sodium dodecyl sulfate, stock solution
Staining solution	PageBlue^{TM} (MBI Fermentas) containing Coomassie Brilliant Blue G-250
C1 buffer	1.5M Tris-HCl; pH 8,8
C2 buffer	0.5M Tris-HCl; pH 6,8
10x SDS-PAGE electrophoresis buffer	25 mM Tris, 192 mM glycine, 0.1% SDS stock solution
1x PBS	137mM NaCl, 2.7mM KCl, 10mM NaH_2PO_4, 1.8mM KH_2PO_4; to 1l ddH_2O; pH 7.4; sterile
Protein Ladder	Spectra^{TM} Multicolor Low Range Protein Ladder
20x Reducing agent	2M DTT, stock solution
Blotting buffer	10% methanol, 25mM Tris-base, 192mM glycine
Blocking buffer	3% BSA (w/v) in 1x TBS
Antibodies	Ni-NTA conjugate; TBS-Tween; 1:1000
1x TBS	10mM Tris-Cl (pH7.5), 150mM NaCl
1x TBS Tween	20mM Tris-Cl (pH 7.5), 500mM NaCl, 0.05% (v/v)Tween 20, 0.2% (v/v) Triton X-100
10x Tris-saline	9% (w/v) NaCl, 1M Tris-Cl; pH 8.0; stock solution
Staining solution (HPR)	Prepared fresh just before using: 18mg 4-chloro-1-naphthol, 6ml methanol, 24 ml 1x Tris-saline, 60µl 30% hydrogen peroxide(H_2O_2)
Ni-NTA agarose	Qiagen

Lysis buffer	10mM imidazole, 300mM NaCl, 50mM NaH_2PO_4; pH 8,0
Washing buffer	20mM imidazole, 300mM NaCl, 50mM NaH_2PO_4; pH 8.0
Elution buffer	250mM imidazole, 300mM NaCl, 50mM NaH_2PO_4; pH 8.0
1% Casein	In 1.0M NaOH
Trypsin	200μg/ml
Oxoid agar	OXOID
3% Acetic acid	
Skimmed milk	
Heparin	SERVA
Rabbit thromboplastion	Sigma Aldrich
0,025M $CaCl_2$	

Table IV: Used bacterial cultures.

Staphylococcus aureus	G+
Escherichia coli	G-

3.2 Methods

3.2.1 Samples collection - ticks, tick organs and haemolymph collection

Ixodes ricinus ticks of all development stages (larvae, nymphs and adult males and females) were obtained from institutional tick collection (Institute of Parasitology, Academy of Sciences of the Czech Republic).

Salivary glands, midgut, ovaries and malpighian tubules were collected from the ticks. The ticks were glued to the bottom of a Petri dish with wax and incised along the dorsal–lateral margin, and the dorsal integument was removed. The organs were excised and collected into RNAlater® solution (Ambion, USA) and kept at -80°C for later use.

In addition, hemolymph from engorged female ticks was collected. The forelegs of the ticks were cut and gentle pressure applied on the tick's body, the clear hemolymph was drawn into a plastic micropipette, collected into RA1 buffer (NucleoSpin® RNA II, Macherey Nagel, Germany) and stored at -80°C for later use.

3.2.2 RNA extraction

The RNA was extracted using the Nucleo Spin® RNA II kit (Macherey-Nagel). The manufacturer´s instructions were followed and finally RNA was eluted with 40µl RNaseFree water. RNA concentration was measured and the quality of the RNA assessed on NanoPhotometer at 260/280 nm wavelength. Extracted RNA was stored at -80ºC.

3.2.3 Complementary DNA (cDNA) synthesis

Single stranded cDNA was synthesized using Enhanced avian HS RT-PCR-100 kit (Sigma). Synthesis was performed according to manufacturer´s instructions for Two-Step RT-PCR using the previously extracted RNA. The cDNA concentration and the quality was determined by measuring on NanoPhotometer at 260/280 nm wavelength. Synthesized cDNA was stored at -20ºC for further use.

3.2.4 Genomic DNA extraction

Total DNA was extracted from 1 unfed and 1 fed female *Ixodes ricinus* tick following the protocol of DNeasy® Blood and Tissue Kit (Qiagen). DNA was eluted with 50µl of water. The concentration of the DNA was measured on NanoPhotometer at 260/280 nm wavelength. DNA was stored at +4ºC.

3.2.5 Polymerase chain reaction

The polymerase chain reaction was used for gene amplification. For each PCR reaction cDNA or gDNA was used as a template. Specifically designed primers, 2x PCR Master Mix and distilled water were collected in 20µl PCR mixture. Negative control was prepared to detect potential contamination by replacing the template with dH_2O. The reaction was carried out in Eppendorf Mastercycler Personal (Eppendorf).

Reaction mixture (1 reaction in 20µl):

2x PCR Master Mix……..10 µl

0.1mM primer Forward……..1 µl

0.1 mM primer Reverse……..1 µl

dH_2O……...…….7 µl

cDNA.....…….…....1µg

PCR reaction parameters:

1. 96°C…….5 min – denaturation of DNA
2. 96°C…….30 s – denaturation DNA
3. 50°C…….30 s – depending upon primers (annealing temperature)
4. 72°C…….1 min – synthesis
5. 72°C…….20 min – final elongation
6. Final temperature was set for 4°C.

Steps 2-4 were repeated in 30-35 cycles.

3.2.6 Agarose gel electrophoresis

The products were run on 1-2% agarose gel/1x TAE buffer at 100 V for 25-30 minutes. For visualization of the sample 6x concentrated Loading Dye buffer supplemented with SYBR® GreenER™ was applied to the samples. 100bp GeneRuler (MBI Fermentas) was used as DNA ladder. The results were visualized with UV trans-illuminator.

3.2.7 RACE PCR 3´/5´

RACE PCR 3´ was used to track the sequence to the polyA end. 5'/3' RACE Kit, 2nd Generation (Roche) was used according to the manufacturer´s protocol. The obtained product was visualized using agarose electrophoresis.

3.2.8 PCR product purification

The PCR product was purified using QIAquick®Gel Extraction kit (Qiagen) following the protocol of manufacturer. DNA was eluted with 30 µl ddH_2O.

3.2.9 Cloning and cell transformation

The purified PCR product was cloned into the pCR™4-TOPO® vector using the TOPO®TA Cloning® Kit for Sequencing (Invitrogen). Four µl of purified DNA were added to the reaction mixture containing 1 µl of vector and 1 µl of salt solution according to the protocol. The reaction mixture was incubated at room temperature for 30 minutes.

Transformation was performed using One Shot®TOP10 Competent Cells. Two µl of the reaction mixture from cloning reaction were added to the *E. coli* competent cells and the mixture was incubated on ice for 30 minutes, then heat-shocked for 30 s at 42°C and immediately transferred on ice. After addition of S.O.C. medium (250 µl), the cells were incubated at 37°C in shaking incubator for 1 hour. Transformation reaction was spread on LB agar plates containing ampicillin (50µg/ml) and incubated overnight at 37°C.

3.2.10 Plasmid DNA purification, screening

Individual colonies were picked from the plates and transferred to 1 ml of LB/ampicillin medium each. After the incubation at 37°C overnight in shaking incubator, the cell cultures were centrifuged and the plasmid DNA was purified from the cell pellet according to the protocol of QIAprep® Spin Miniprep Kit. The plasmid DNA was eluted from SpinColumn with 50 µl ddH_2O.

The presence of the insert in the plasmid was checked by PCR amplification and following electrophoresis using gene specific primers and vector specific primers M13 forward, M13 reverse. Plasmids with present insert were sent for sequencing.

3.2.11 Sequence analysis

The nucleotide sequences were aligned using BLAST (Zhang et al., 2000). Nucleotide sequences were translated to amino acid sequences. The signal peptide cleavage sites of the deduced amino acid sequence were predicted with SignalP 4.0 (Petersen et al., 2011). Distribution of disulphide bridges, estimated charge of the molecule, 3D structures of

the proteins were predicted using publicly available software online. Preliminary biological functions of the protein were predicted as well.

The following web sites were used for the sequence analysis:

3D structure modelling – http://swissmodel.expasy.org

Signal peptide cleavage site prediction - http://www.cbs.dtu.dk/services/SignalP/

Protein charge calculation - http://protcalc.sourceforge.net

Protein function prediction - http://www.sbg.bio.ic.ac.uk/~mwass/combfunc/

3.2.12 Cloning into ChampionTMpET100 TOPO® expression vector and BL21 Star™ (DE3) OneShot® Cells transformation

PCR product was cloned into expression vector pET 100/D-TOPO® following the manufacturer protocol. After transformation of One Shot TOP10 *E. coli* Competent Cells the presence of the insert in recombinant construction was checked using PCR. Plasmid containing the insert was sequenced with T7 promotor primer for control of the correct reading frame encoded.

BL21 Star™ (DE3) One Shot® expressing competent cells were transformed with recombinant expressing plasmid containing insert according to manufacturer protocol.

3.2.13 Recombinant protein production (pilot experiment)

Transformed BL21 Star™ (DE3) One Shot® competent cells were inoculated to LB medium containing ampicilin (50µg/ml). Samples were incubated at 37°C. After 2 hours 500µl of the culture was taken as zero un-induced sample. IPTG (1mM - final concentration) was added to the rest of the culture and sample was taken every hour during 6 hours. Results of pilot expression were evaluated using SDS-PAGE electrophoresis.

3.2.14 SDS-PAGE: Recombinant protein expression control

Cell pellets obtained in pilot expression were resuspended in 250µl 1x PBS and sonicated at mini-sonicator Bandelin Sonoplus. Sonicated samples were centrifuged at maximum speed and supernatant was collected. Fourteen µl of each sample were mixed with 5µl of 4x Dual Color Protein Loading Buffer and 1 µl of Reducing agent. Samples

were heated up to 99°C for 10 minutes and then immediately transferred on ice, cooled and briefly centrifuged.

The samples were loaded at SDS-PAGE gel consisting of 5% stacking gel and 15% resolving gel. Electrophoresis ran approximately 1 hour at 120V on Hoefer SE 250. After the electrophoresis the gel was washed twice in distilled water and then stained in PageBlue^{TM} (MBI Fermentas).

3.2.15 Protein purification under native conditions, Western blot

Cell pellet was resuspended in 5 ml of lysis buffer and lysosym (1mg/ml) was added. The reaction was incubated on ice for 30 minutes and then sonicated 10x10 seconds at 20Hz. Lysated cells were centrifuged and supernatant was collected for purification under native conditions on Ni-NTA agarose.

One ml of Ni-NTA agarose was applied to 10 ml column. The column was washed with water and equilibrated with lysis buffer. For efficient binding to agarose, 5ml of supernatant was applied to the column and left overnight on Bio RS-24 (Biosan) rotator at 4°C. Next day, a sample of the flow through the column was taken. Column was washed 6x with 10 ml of washing buffer. Sample from each washing were stacked away for SDS Page analysis. Eventually, the protein was 5x eluted from the column with 500µl elution buffer. All samples were controlled with SDS Page.

Samples divided on SDS-PAGE gel were transferred to PVDF membrane activated in methanol according to manufacturer´s protocol (Millipore). The transfer ran approximately 1.5 hours at 17V on Idea Scientific Company apparatus. Recombinant protein was detected with anti-His Ni-NTA HPR conjugates following the manufacturers protocol.

3.2.16 Antitrypsin activity test

The assay was conducted as described by Nabian et al., (2014) with slight modifications. Agar plates containing 1% casein (in 1.0M NaOH) and skimmed milk were prepared. Four wells were cut in the agar after hardening. Twenty five µl of TILIr protein (1.97mg/ml) with 25µl of trypsin were applied to the first well, 50µl of TILIr (1.97mg/ml) with 25µl of trypsin - to the second. Two remained wells were filled with 25µl of trypsin (200µg/ml) only as positive control and 50µl of PBS as negative control. Plates were incubated at room temperature for 12 hours.

3.2.17 Antimicrobial assay

Escherichia coli (G-) and *Staphylococcus aureus* (G+) bacteria cultures were used in antimicrobial assay. Bacterial cells were grown at LB agar on Petri dishes. Purified recombinant TILIr protein (1.7mg/ml) was added to LB agar plate with bacteria in two different volumes. Ampicilin was used as positive control and lysate buffer as negative control. Cultures were then incubated for 8 hours at 37°C.

3.2.18 Anticoagulation test

The assay was made by prothrombin-time method of Grasset et al. (1955). Blood was collected from rabbit to 3.8% sodium citrate tubes. Immediately after collection, blood samples were centrifuged and separated plasma was collected and stored for further analysis.

The coagulation was induced by thromboplastin and $CaCl_2$. Graded concentrations of TILIr were added to 0.5ml of plasma. Mixtures were kept for 1 minute at 37°C. Subsequently, 0.1ml of thromboplastin was added to mixtures and kept additional 3 minutes at 37°C. After the incubation, 0.1ml of 0.025N $CaCl_2$ was supplemented. Samples were mixed by inversion. The presence of clot was observed using glass hook in up and down movement.

4. Results

4.1 DNA isolation

Genomic DNA was isolated from unfed and fed female *Ixodes ricinus* tick.

Table I: Concentrations of isolated genomic DNA.

	DNA(ng/µl)
Female unfed	48
Female fed	59

4.2 RNA isolation and cDNA synthesis

RNA was isolated from different developmental stages and different organs of *Ixodes ricinus* tick.

Table II: Concentrations of RNA isolated from different tick developmental stages.

	RNA (ng/µl)
Larvae fed	33
Larvae unfed	194
Nymph fed	57
Nymph unfed	582
Female fed	96
Female unfed	232
Male	10

Table III: Concentrations of RNA isolated from different tick organs and hemolymph.

	RNA (ng/µl)
Salivary glands	20
Midgut	31
Ovaries	62
Malphigian tubules	25
Hemolymph	22

4.3 Race PCR 3´ and RACE PCR 5´

The whole TILIr sequence until polyA end was obtained using 3´RACE PCR.

TILIr

signalsequenceAGAATTGCGGCCGACTTGCCCTGGGTGTGTAGTCCCAGAGAGGTCTTCAAGGAATGCGTG
AGCAGCAGCTGTGCCGAACTGAAGTGCGGCATGGAACGCATGCCACTCGCTTGCACTAAGGACTGTGCCAG
CGGCTGCTTTTGCGCTCCAGGCTTCTACCGCAAGGGGCACAGGGAATGCGTTCCCCGGAGCGAGTGCCAACT
CAAGCCCCTCAAACCCATGCCAAAGGCTTGT**TGA3'UTR**
ACGGATTTCAAGGCCGTTCCGGACCTTACTTAAGAGAGTGAGCTGCTCCTAAATAAAACCTATATTGTAGGCG
AAGAAAAAAAAAAAAAAGTCGACATCGATACGCGTG

Isoform 1

signalAGAATTGCGGCCGACTTGCCCTGGGTATGTGGTCCCAGAGAGGTCTTCAAGGAATGCGTGA
GCAGCAGCTGCGCCGAACTCAAGTGCGGCATGGAAGGCATGCCAATCGGTTGCACCAAAGACTGCGTCAGC
GGCTGCTTCTGCGCTCCAGGCTTCTACCGTAAGGGGCACAGGGAATGCGTTCCCCAGAGCGAGTGCGAAGTC
GAGCCCCTCAAGCCCATGCCCAAGGCT**TGA 3'UTR**
ACGGATTTCAAGGCCGTTCCGGACCTTACTTAAGAGAGTGAGCTGCTCCTAAATAAAACCTATATTGTAGGCG
AAGAAAAAAAAAAAAAAGTCGACATCGATACGCGTG

Isoform 2

signalAGAATTGCGGCCGACTTGCCCTGGGTGTGTGGTCCCAGAGAGGTCTTCAAGACATGCGTGA
GCAGCACCTGTGCCGAACTCAAGTGCGGCATGGAAGGCATGCCAGAAGCCTGCACCATGGACTGCGCCAGC
GGCTGCTTTTGCGCTCCAGGCTTCTACCGTAAGGGGCACAGGGAATGCGTTCCCTGGAGCGAGTGTCAAATC
GAGCCCCTCAAGCCCATGCCTAAGGCT**TGA 3'UTR**
ACGAATTTCAAGACCGTTCCGTTTCTCACTGGCGAGAGTGAACTGCTCCTAAATAAAACCTAGATTGTAAGCG
AACAAAAAAAAAAAAGTCGACATCGATACGCGTGGTC

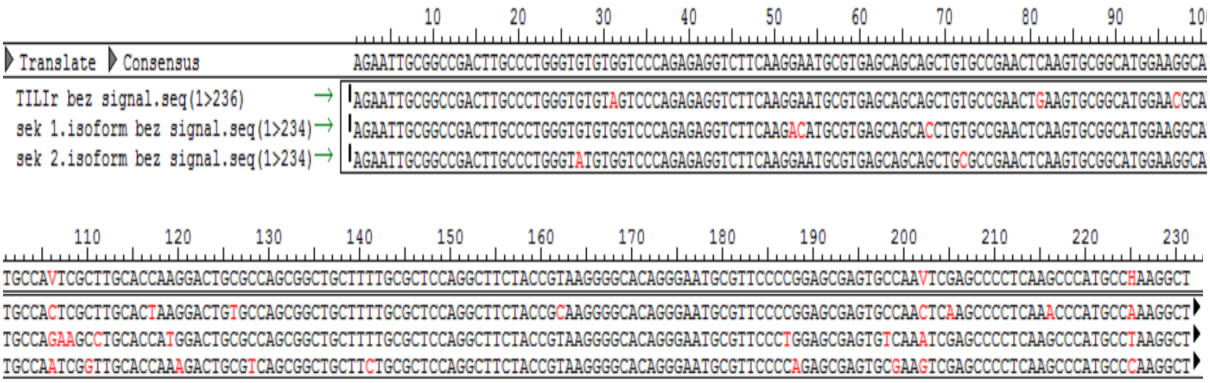

Figure 1: Alignement of TILIr nucleotide sequence with nucleotide sequence of isoform 1 and 2.

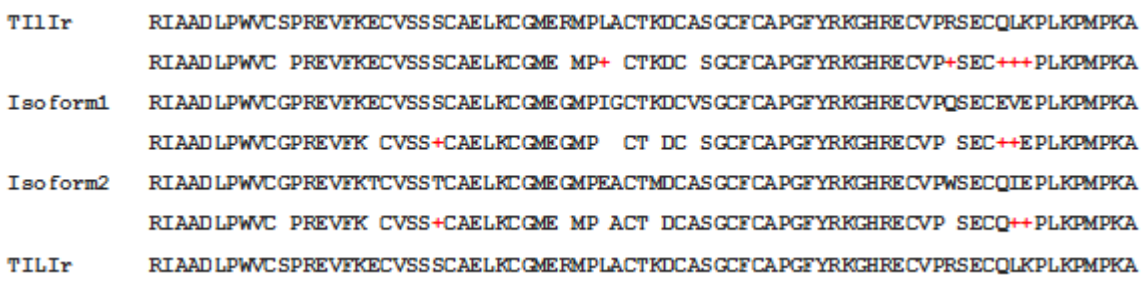

Figure 2: Alignement of TILIr amino acid sequence with amino acid sequence of isoform 1 and 2.

4.4 Genomic DNA analysis

Genomic DNA was extracted and analysed in order to detect presence of introns in the TILIr sequence. Genomic DNA was extracted from unfed female and fed female *I. ricinus* tick. Annealing temperatures were adjusted to 55^0C and number of cycles reduced to 20. No introns were detected in gDNA sample.

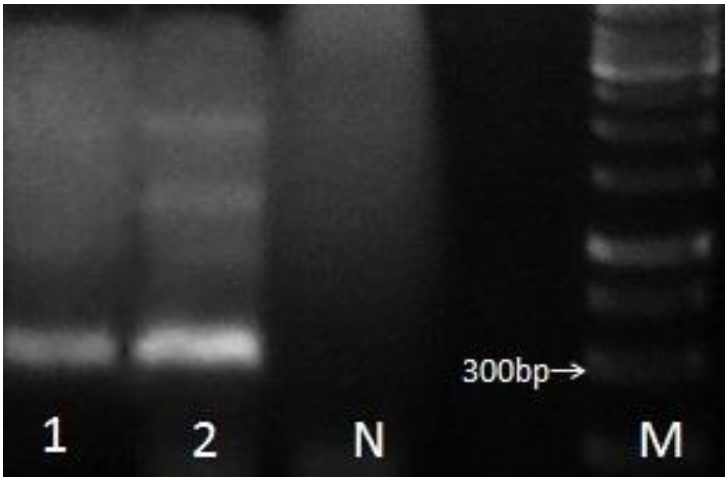

Figure 3: Genomic DNA from unfed female (1) and fed female (2).

4.5 Gene expression in different tick developmental stages

Gene expression was analysed using RT PCR. For RT PCR, RNA (50 ng/µl) from all developmental stages was used. Each method included control reaction with actin primers.

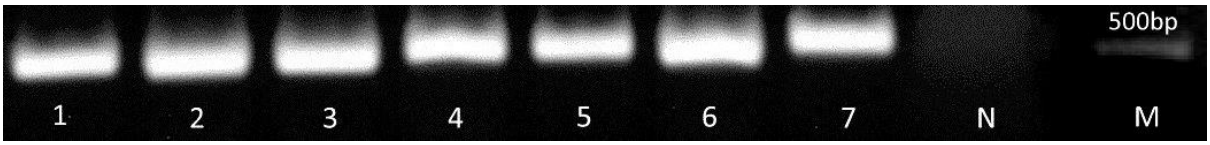

Figure 4: Control of presence of actin in fed female (1), unfed female (2), fed nymphs (3), unfed nymphs (4), fed larvae (5), unfed larvae (6) and male (7) with negative control (N) and marker (M).

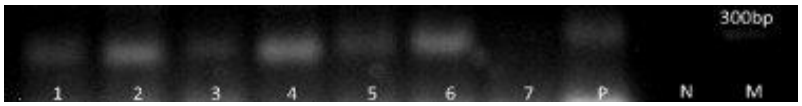

Figure 5: Gene expression in fed female (1), unfed female (2), fed nymphs (3), unfed nymphs (4), fed larvae (5), unfed larvae (6) and male (7) with positive control (P), negative control (N) and marker (M).

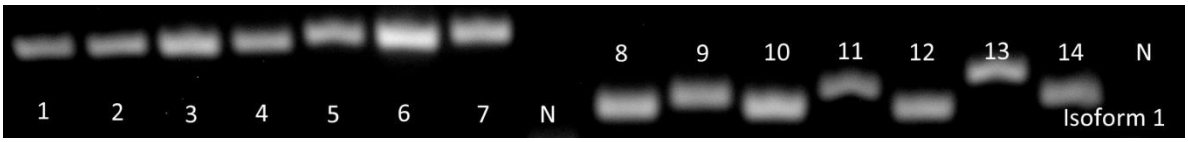

Figure 6: Gene expression of isoform 1 in developmental stages (1-7 actin control, 8-14 specific primers, N- negative control, order of samples viz. Figure 4 and 5)

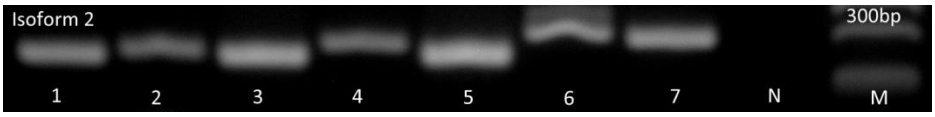

Figure 7: Gene expression of isoform 2 in developmental stages (1-7 actin control, 8-14 specific primers, N- negative control, order of samples viz. Figure 4 and 5)

TILIr was found to be mostly expressed in unfed females, nymphs and larvae. Both isoforms are expressed in all developmental stages.

4.6 Gene expression in different tick organs

Gene expression was also analysed in tick organs, namely salivary glands, midgut, malphigian tubules, ovaries and hemolymph. For RT PCR the RNA was equalized to 25ng/µl. Control reaction with actin primers was performed.

The results showed gene expression of TILIr and both its isoforms in midgut only. No sign of expression was found in other organs.

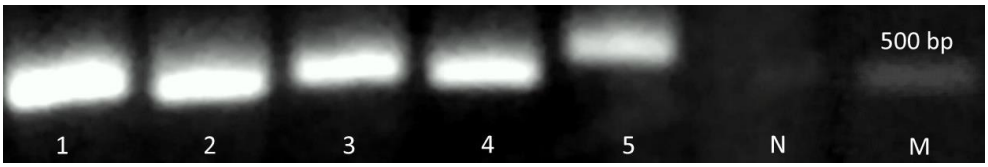

Figure 8: Actin control of salivary glands (1), midgut (2), malphigian tubules (3), ovaria (4), hemolymph (5), negative control (N) and marker (M).

Figure 9: Gene expression of TILIr in midgut (2).

Figure 10: Gene expression of isoform 1 in midgut (6) (Samples 1-4 actin control – (1) salivary glands, (2) midgut, (3) malphigian tubules, (4) ovaria; 5-8 specific primers – same order of samples, N- negative control).

Figure 11: Gene expression of isoform 2 in midgut (6) (Samples 1-4 actin control, 5-8 specific primers, N- negative control, order of samples viz. Figure 10).

4.7 Recombinant protein production, pilot expression

Pilot experiment showed that the TILIr protein is expressed from the first hour (Figure 10). Molecular mass of the recombinant protein is 12 767.80 Da.

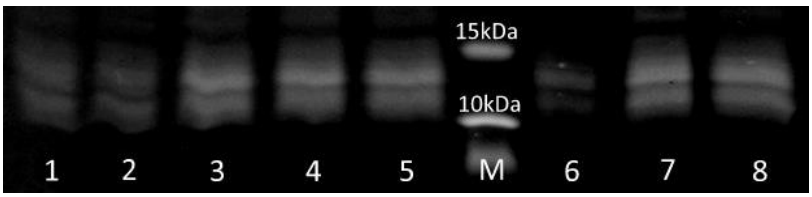

Figure 12: Pilot expression of TILIr.

4.8 Western blot

TILIr was purified under native conditions on Ni-NTA agarose. Obtained elution fractions were checked on SDS gel. Subsequently, the elution fractions were transferred to membrane by Western blot method and the proteins were detected using anti-His antibodies. Antibodies proved the presence of TILIr with His-Tag in fractions 1, 2, 3 and 4.

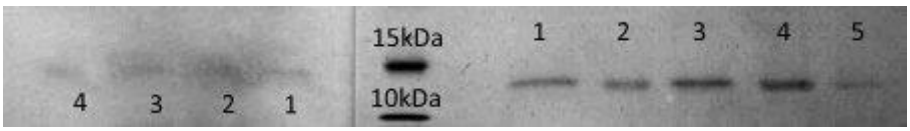

Figure 13: Elution fractions transferred to PVDF membrane.

4.9 Antitrypsin activity test

TILIr (1.97mg/ml) was tested for inhibitory activity towards trypsin on agar plates containing 1% casein. Trypsin activity towards casein and inhibitory activity of TILIr was after 12 hours incubation at room temperature visualised by addition of 3% acetic acid.

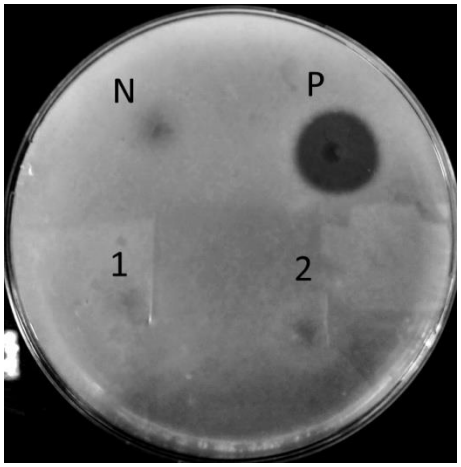

Figure 14: Casein agar plates activated with acetic acid with TILIr and trpysin 1:1 (1), TILIr and trypsin 2:1 (2), trypsin as positive control (P), PBS as negative control (N).

4.10 Antimicrobial activity

Representatives of G- (*Escherichia coli*) and G+ (*Staphylococcus aureus*) bacteria were grown on LB agar plates without antibiotics for following antimicrobial test. Even though *in silico* analysis of protein sequence predicted antimicrobial activity of TILIr, TILIr did not show any activity against tested gram positive and gram negative bacteria (*Staphylococcus aureus* and *Escherichia coli*).

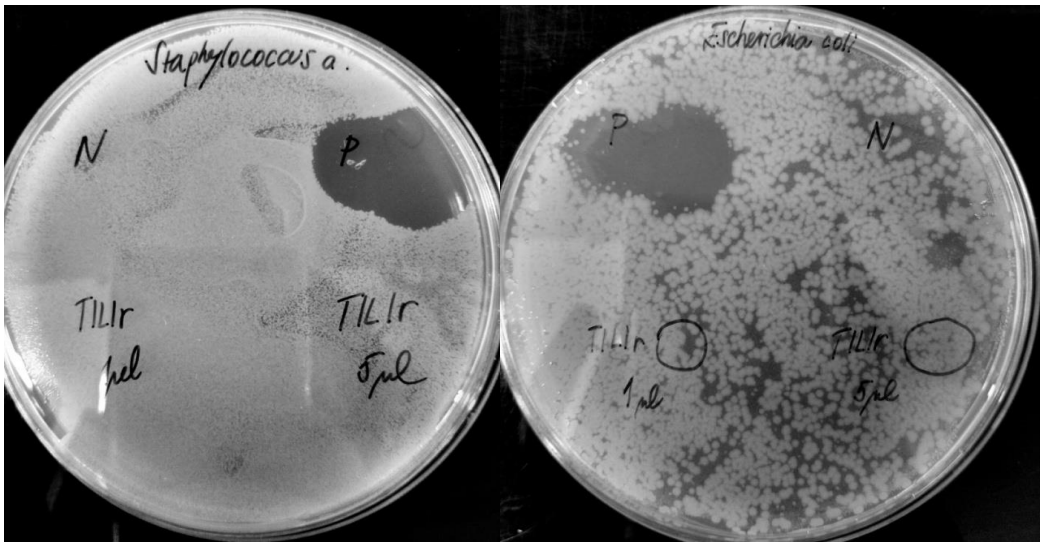

Figure 15: Petri dish with *Staphyloccocus aureus* and *Escherichia coli.*

4.11 Anticoagulation assay

Anticoagulation activity of recombinant TILIr was confirmed by anticoagulation assay. Blood clotting was induced by thromboplastin and $CaCl_2$. Formation of clots was observed in negative control sample, positive control with heparin did not form any clots. Three samples with different doses of TILIr protein were prepared and in all three samples only minimal formation of clots was observable (Figure 17).

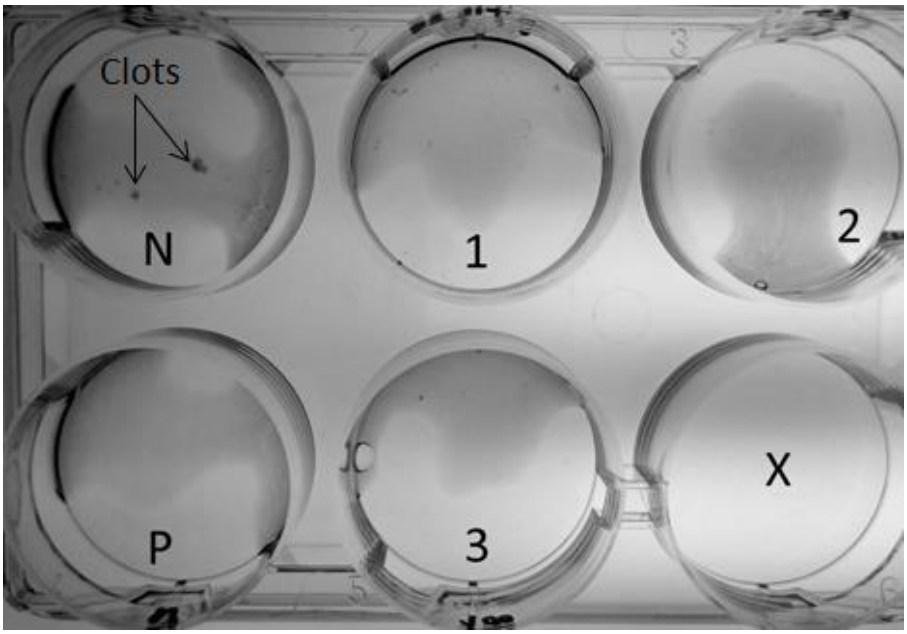

Figure 16: Anticoagulation test with TILIr 0.25mg/ml (1), TILIr 1mg/ml (2), TILIr 1.97mg/ml (3), positive control with heparin (P) and negative control (N).

4.12 *In silico* analysis

TIL domain

Analysis of TILIr amino acid sequence using NCBI databases confirmed the presence of TIL domain in it, domain that is typical for trypsin inhibitors. TIL domain consist of 10 cysteine residues binding in fixed pairs (C1-C7, C2-C6, C3-C5, C4-C10 and C8-C9), so as recombinant TILIr and its isoformes.

TILIr

Isoform 1

Isoform 2

Isoelectric point

Using Protein Isoelectric Point Calculator we found the isoelectric point of TILIr and its isoforms. Protein Isoelectric Point Calculator compares results from 16 different databases (Figure 18). Average pI of TILIr was calculated to be 7.29. At pH 7.29 TILIr has zero net charge. Isoform 1 has pI 6.15 and pI of isoform 2 is 6.14.

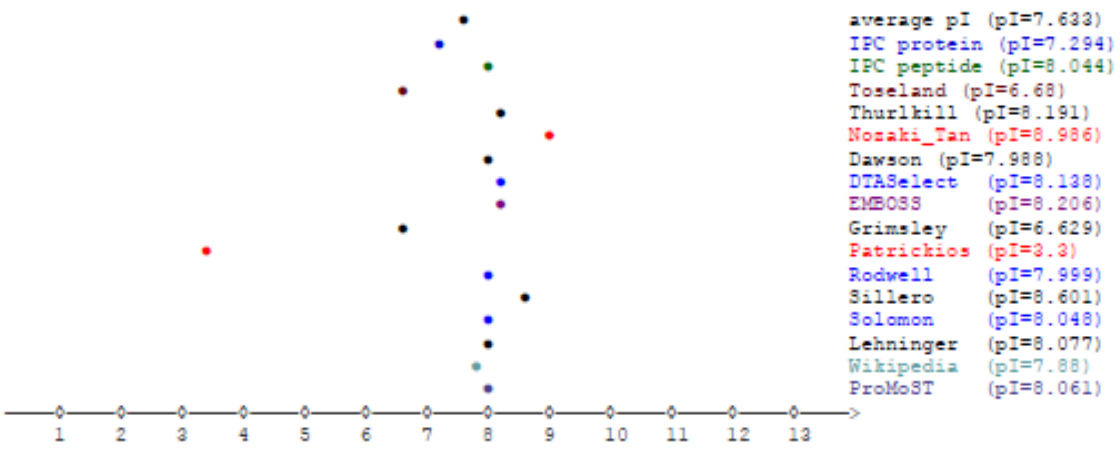

Figure 18: Comparison of isoelectric point calculations from different databases.

Protein charge calculation

Charge of TILIr at pH 7.00 was calculated by Protein Calculator v3.4 to be 4.8. Charge of isoform 1 is -0.1 as well as the charge of isoform 2.

Signal peptide prediction

Signal sequence was predicted with CBS databse (Figure 19). The amino acid sequence of TILIr contains signal sequence of 20 amino acids that cleaves off the peptide between 20th (A) and 21st (R) aminoacid. The lenght of mature TILIr without signal sequence is 80 amino acids. Signal sequence of both isoforms is cleaved off at the same position.

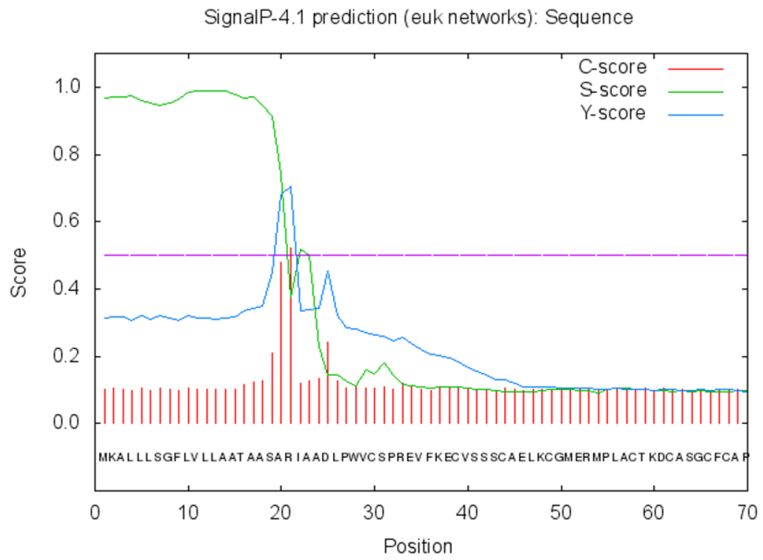

Figure 19: Prediction of signal peptide in amino acid sequence of TILIr.

3D structure

3D structure of the TILIr protein and of amino acid sequences of the two isoforms was predicted using Expasy Swiss-Model (Biasini et al., 2014). Model of TILIr was built mainly using anticoagulant protein 5, nematode anticoagulant protein C2 and chymotrypsin/elastase isoinhibitor and the remaining non-matching part was build using the fragment library. Model of isoform 1 and 2 was built partially from nematode anticoagulant protein C2 and chymotrypsin/elastase isoinhibitor, the remaining non-matching part was also constructed using the fragment library.

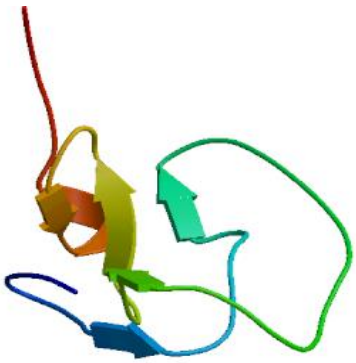

Figure 20: 3D structure of TILIr.

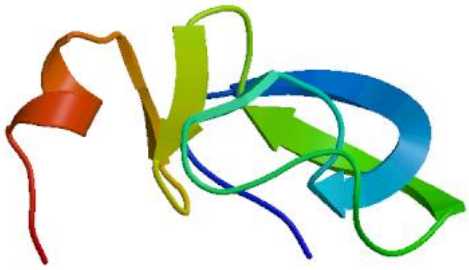

Figure 21: 3D structure of isoform 1 and 2.

5. Discussion

Blood sucking animals are a rich source of proteinase inhibitors mainly those that interfere in their host hemostatic systems. Ticks are arthropod blood feeders. They have to deal with host defense mechanisms in order to successfully feed. They can modulate both innate and acquired host defense. The first line of host defense that ticks have to overcome is the coagulation cascade. Inhibition of blood coagulation is not only important for blood meal, but also for efficient transmission of pathogens into the host´s system (Mulenga et al., 2001). Tick saliva as well as other tissues comprises many factors that are able to counteract the blood clotting. Consequently, current anti-tick vaccine development is focused on prevention of tick blood meal that at the same time prevents the pathogen transmission. Serpins are among the tick proteins that are injected into the host to regulate tick feeding. They are integral part to tick fight with host immunity and those of them that possess anti-coagulant, anti-inflammatory capabilities as well as other immunomodulatory functions represent significant candidates for anti-tick vaccines and therapeutics (Huntington, 2011; Imamura et al., 2004). By blocking the functions of proteins such as TILIr, we will interfere with the ability of ticks to initiate feeding and/or to prepare conditions that are favorable to the transmitted tick borne pathogens to colonize the host. Several serpins mentioned above have already been confirmed to be efficient in anti-tick control or suitable for use in cocktail of agents in potential future vaccine (Sugino et al., 2003; Andreotti et al., 2002; Prevot et al., 2007).

Large and variable families of serpins were already described in ticks. As mentioned above *I. scapularis* genome revealed the presence of 45 putative serpin genes (Mulenga et al., 2009), 17 serpins were found in *A. americanum* (Mulenga et al., 2007), 5 in *R. microplus* (Fogaca et al., 2006; Macedo-Ribeiro et al. 2008; Sasaki et al., 2007). Recently, Tirloni et al. (2014) added 18 full-length ORFs with similarity to tick serpin sequences, four in *R. appendiculatus* (Mulenga et al., 2003), two in *R. haemaphysaloides* (Yu et al., 2013), two in *H. longicornis* (Sugino et al., 2003 and Imamura et al., 2005), 32 in *A. maculatum* (Karim et al., 2011), four in *A. variegatum* (Nene et al., 2002 and Ribeiro et al., 2011), nine in *A. cajennense* (Batista et al., 2008) and one in *A. hebraeum* (Lai et al., 2004) and finally six in *I. ricinus* (Leboulle et al., 2002a and Chmelar et al., 2011).

This study was performed to characterize novel trypsin inhibitor called TILIr and two of its isoforms with significant level of identity (95%) from *Ixodes ricinus* tick with *in silico* predicted inhibitory and anticoagulant activity. The amino acid sequence of gene encoding TILIr as well as isoforms was found to possess TIL domain typical for trypsin inhibitors. Trypsin inhibitors are smaller serine protease inhibitors with, according to Zeng et al. (2014), 56-84 of amino acid residues. TILIr and its isoforms consist of 74 amino acids (98 with signal sequence). Importantly, small size of TIL peptides related with the well-known structure and compact rigidity are assets in search for vaccine (Zeng et al., 2014).

TILIr and both isoforms contain signal sequence suggesting they present extracellular secreted proteins. In the literature, the designation of a tick protein being injected into the host during tick feeding has been based on a candidate gene possessing the signal peptide (Valenzuela et al., 2002; Ribeiro et al., 2006). Eighty two percents of 45 serpins from *Ixodes scapularis* are extracellular as well as 13 out of 17 *Amblyomma americanum* serpins (Mulenga et al., 2009; Mulenga et al., 2007). More than half of 18 serpins isolated from *R. microplus* were also found to own signal peptide. This proportion differs from other arthropods, where the majority of known serpins exist as an extracellular form (Reichhart, 2005, Mulenga et al., 2007 and Mulenga et al., 2009). Further, RAS-3 and -4 unlike RAS-1 and -2 from *R. appendiculatus* are also extracellular that is only desirable for vaccine candidate as Mulenga et al. (2003) remarks.

Many serpins appear to have introns in their structure. Mulenga et al. (2009) who studied 45 *I. scapularis* serpin genes reported 13 out of them possess introns in their genomic structure. However, the remaining 32 serpins were intronless. The analysis of TILIr genomic DNA did not prove the presence of introns. Zeng et al. (2014) suggested that dynamic evolution of the TIL peptide genes is dominated by intron gain.

The gene expression profile analysis provides an insight into the biological relationship of serpins concerning tick feeding biology. Expression of serpin genes appear to be the most common in either tick salivary glands or midgut or both, as it is important to keep the host blood liquid at the beginning of feeding as well as later in the gut for successful digestion (Mulenga et al., 2001). Analysis of 45 *I. scapularis* serpins revealed that 40 serpins are expressed in salivary glands and/or midgut of unfed or partially fed ticks (Mulenga et al., 2009). However, some serpins from *R. microplus* (RmS serpins) are expressed in ovaries (Tirloni et al., 2014; Rodriguez-Valle et al., 2015) or Lospins from *A.*

americanum are found in all three tissues: salivary glands, midgut and ovaries (Mulenga et al., 2007). Further, HLS2 from *H. longicornis* is expressed in haemolymph of feeding ticks (Imamura et al., 2005) only. Our RT-PCR results revealed that TILIr preferable site of its expression is midgut only.

The site of gene expression plays a key role in the vaccine development. It is important whether it is the matter of exposed antigen present at a site that gets to contact with host during feeding, or it is a concealed antigen that does not interact with host directly (Willadsen and Kemp, 1988). Antigens expressed in gut are example of concealed antigens. The advantage of concealed antigens is their chance to simplify the development of immunity and enhance the immune reaction to parasitic feeding. That is why the only commercially available ectoparasite vaccine at the moment is the tick vaccine derived from a midgut membrane-bound protein of the cattle tick, *Boophilus microplus*. But on the other hand, host immune response to concealed antigens is unlikely to be increased after natural exposure to ticks. For that reason, repeated vaccination would be required (Willadsen, 2004).

Almost all known serpins are glycosylated, N-glycosylation is considered to be general feature of serpins and important for biological activity (Rau et al., 2007). Some already described serpins do not have glycosylation sites, for example, 2 out of 18 serpins described in *R. micropulus*. TILIr and neither one of its isoforms contain no asparagines to which nitrogen atom glycan could link.

Tick serpins inhibit proteases of the host immunity system and thus facilitate the tick feeding and the evasion of the host´s defense response (Mulenga et al., 2001). Thus, an important goal in this study was to determine the functional capabilities of the TILIr. Except for a limited number of serpins from *I. ricinus* (Leboulle et al., 2002a; Prevot et al., 2002, 2006; Kovářová et al., 2010; Chmelar et al., 2011), *R. haemaphysaloides* (Yu et al., 2013), and *A. americanum* (Mulenga et al., 2013) that have been functionally characterized, the majority of tick serpins in databases remain uncharacterized.

There are two classes of serpins, those that inhibit serine or cysteine proteases and those without inhibitory activity (Gettins, 2002; Huntington, 2006; Silverman et al., 2010; Whisstock et al., 2010). Data in this study demonstrated that TILIr inhibited activities of thrombin and trypsin. In order to inhibit the protease, serpins trap the protease in stable complex and subsequently destroy it (Gettins, 2002; Huntington, 2006). Kinetically the

consequence of serpins trapping the protease is reducing maximum enzyme velocity of the target protease in a dose-responsive manner as was observed with TILIr in this study.

Several lines of evidence have demonstrated that both thrombin and trypsin play important roles in mediating the host's anti-tick defense pathways, inflammation, platelet aggregation, wound healing and blood clotting. From this perspective, the observation that TILIr inhibited thrombin and trypsin is significant. Besides its important role in blood clotting activation cascade, thrombin is also essential in the host´s first line of defense to tick feeding such as inflammation, cell proliferation, platelet function and angiogenesis (Huntington et al., 2003; Siller-Matula et al., 2011; Colucci and Semeraro, 2012; Chambers and Scotton, 2012; Hunt et al., 2012; Rothmeier and Ruff, 2012; Heemskerk et al., 2013).

Alike mammalian blood clotting system, serine proteases and serpins are also part of arthropod hemolymph clotting system (Theopold et al., 2002; Iwanaga and Lee, 2005; Dushay, 2009). The observation that TILIr inhibited thrombin, an important protease of the blood clotting pathway prompted us to gauge insight into probable role(s) of this protein at the tick-feeding site. TILIr prevented platelet aggregation and delayed plasma clotting in a dose-responsive manner. This fact suggested that this protein is a part of the tick haemolymph clotting regulatory mechanism and may belong to tick proteins that mediate tick evasion of the host's hemostatic defense mechanism.

APD3 database (Antimicrobial Peptide Calculator and Predictor) suggested potential antimicrobial activity of TILIr. However, the antimicrobial assay did not show antimicrobial activity of TILIr against *Escherichia coli* or *Staphylococcus aureus*, representatives of G- and G+ bacteria. Nevertheless, antimicrobial activity might be found towards different bacteria other than those tested.

In conclusion, this study adds another trypsin inhibitor from *I. ricinus* with inhibitory activity on the list of serpin family in ticks. Studies reviewed here and results of our study strongly suggest a high possibility for TILIr to be among tick proteins that facilitate tick and host interactions.

This work is a starting point for further analyses of TILIr or its isoforms as potential candidates for vaccine development.

6. Conclusion

The complete sequence of gene encoding novel serpin from *Ixodes ricinus* tick, which was named TILIr, was obtained. During the research, two isoforms of TILIr were found and analysed. TILIr with its isoforms were found to be expressed in tick midgut. TILIr was mostly expressed in unfed female ticks, nymphs and larvae, on the other hand Isoform 1 and 2 were found in all developmental stages of ticks including unfed and fed females, nymphs, larvae and unfed male. Functional analysis confirmed the inhibitory activity of TILIr against trypsin and thrombin. The presence of any antimicrobial activity was not revealed by selected methods. However, at for now, TILIr antimicrobial activity was tested only with *Escherichia coli* and *Staphylococcus aureus*. Novel TILIr protein contains TIL domain typical for trypsin inhibitors. The weight of the protein is 12.8kDa. The isoelectric point of TILIr is 7.29, of isoform 1 pI 6.15 and isoform 2 pI 6.14. The charge of TILIr at pH 7 was calculated to be 4.8. Charge of both isoforms is -0.1.

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