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MASTER THESIS

IDENTIFICATION AND CHARACTERIZATION OF NEWLY FOUND ANTIMICROBIAL PEPTIDE (IRAMP) FROM HARD TICK *Ixodes ricinus*

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ANOTATION:

Antimicrobial proteins (AMPs) are effector molecules and an important part of the innate immune system. AMPs have a broad antimicrobial spectrum and lyse microbial cells by interaction with biomembranes. Besides direct impact in host defence, AMPs are mediators of inflammation with impact on epithelial and inflammatory cells influencing diverse processes such as cytokine release, cell proliferation, angiogenesis, wound healing, chemotaxis, immune induction, and protease-antiprotease balance. AMPs could replace antibiotics which efficiency has decreased due to extensive clinical use. Therefore knowledge of mechanism of action of antimicrobial peptides, their properties and possible usage is essential for their further use as therapeutics. Ticks are blood-feeding ectoparisites that serve as extremely effective vectors of pathogens. Analysis of the ticks molecules that are involved in immune response to the pathogens invasion represent one of the strategies in searching for new compounds that might be used in future as theurapeutic agents.

This study represents analysis of newly identified antimicrobial peptide form in the hard tick *Ixodes ricinus* (IRAMP). IRAMP revealed the high similarity to the recently described antimicrobial peptide isolated from hard tick *Ixodes scapularis* (protein ISAMP). Analysis and characterization of novel AMP, testing its antimicrobial potential and expression pattern are the main objectives of this study.

DECLARATION:

Prohlašuji, že svoji diplomovou práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své diplomové práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajoby kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

České Budějovice, 14. 12. 2012

Lucie Ouředníková

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1. Introduction

The extensive clinical use of antibiotics has resulted into the development of pathogens' resistance to them at the genetic level. Microbes have developed very successful weapon against different classes of antibiotics in form of plasmid- born resistance genes. Therefore, the efficiency of this therapeutics has decreased. A new solution of this problem, which would increase the success of treatments again, is needed. Many of modern researches try to find novel therapeutic agents which would be effective in killing harmful pathogens without damaging the host.
One of the most promising fields is a study of antimicrobial peptides (AMP) - potent, broad spectrum, gene- encoded natural antibiotics. AMPs are mostly short cationic peptides with antimicrobial and/or immunomodulatory activities that are present in virtually every life form, as an important component of (innate) immune defenses. AMPs are evolutionary conserved and play an important role in the first line of defense against various pathogens. For example, some human defensins can act as chemoattractans (1, 2) not only for nonspecific phagocytes but also towards cells engaged in adaptive immunity (3).

The innate immune system is crucial in pathogen/infection eliminations as well as in controlling these processes until the adaptive immune response takes care of it. The innate immune system helps to localize the infection in the organism.

The discovery of first AMP was done almost at the same time as the discovery of penicillin. In 1939, Rene J. Dubos found out that an unidentified soil bacillus had produced some antimicrobial peptides which were effective against certain Gram-positive species (4). He proved the assumption that some microbial cultures could have antagonistic activities toward other species. During next two decades, the antimicrobial peptides were also found in plants and animals. Nowadays knowledge proved AMPs with the diverse and complex antimicrobial activities are produced by all complex organisms as well as some microbes. Therefore a broad research on this topic is being performed and some AMPs have already entered the clinical trials.

Among the numerous directions in antimicrobial proteins investigations, the one directed on AMPs in arthropods, especially ticks, is of great importance and interest, as ticks are known to transmit

several human diseases such as Lyme disease, human anaplasmosis, human babesiosis or tick-borne encephalitis. While searching for antimicrobial peptides of *Ixodes ricinus*, a main vector of Lyme disease spirochete, we identified and characterized a novel AMP (IRAMP) from this tick.

2. State of knowledge

2.1. Problematic of antimicrobial peptides

2.1.1. Characterization of AMPs

Antimicrobial peptides (AMPs), evolutionarily developed compounds, serve as a first line of host defense and represent important, though poorly understood components of the innate immune system of all living organisms, from bacteria up to animals and plants. Even though they have ancient lineage, they are still very effective in limiting microbial infection of a wide range of pathogens. The AMPs are effective against fungi (5, 6), Gram- positive and Gram- negative bacteria (7, 8), parasites (9, 10) and viruses (11). Some of AMPs have also been shown to exhibit cytotoxic activity against cancer cells (12). Thus, they can also be potential therapeutics against tumor growth (13, 14).

An umpteen number of AMPs has been found in nature and also their synthetic analogs have been proved to have a wide spectrum of antimicrobial activities. There are several databases of the peptides with antimicrobial activities available on the internet right now. These databases are constantly updated (See Table 1).

***Table 1**: Examples of databases reporting the sequences of natural antimicrobial peptides*

Database	Content	Location	Web address
AMSDb	Eukaryotic AMP sequences	University of Trieste	http://www.bbcm.units.it/~tossi/amsdb.html
BAPDb	Bacterial AMP sequences	University of Trieste	http://bioweb.ucr.edu/bapdb/
ANTIMIC	Natural antimicrobial Peptides	Institute of Infocomm Research	http://research.i2r.a-star.edu.sg/Templar/DB/ANTIMIC/
APD	Antibacterial, antifungal and antiviral peptides	University of Nebraska Medical Centre	http://aps.unmc.edu/AP/main.html

At the moment one of the AMP databases (APD) contains 168 bacteriocins, 287 plant AMPs, and 1479 animal host defense peptides with the following activity: antiviral peptides, antifungal peptides, anticancer/tumor peptides, antibacterial peptides, anti-protist peptides, antiparasitic peptides, insecticidal peptides, spermicidal peptides, anti-HIV peptides, AMPs with chemotactic activity.

2.1.2. AMPs classification

AMPs are a broad and diverse group of molecules with wide spectra of activities. Until now their classification is too far to be finalized or to have precise definition. There are numerous ways of AMPs classification. Some examples are presented below.

(a) Based on biological source

Based on the biological source the AMPs can be divided into bacterial AMPs (bacteriocins), plant AMPs and animal AMPs. Animal AMPs are further classified into insect, amphibian, fish, reptile, mammal, and others AMPs. This type of classification is used in Antimicrobial Peptide Database (15). The major and well-studied AMPs families in the animal kingdom are cathelicidins (16), defensins (17), and histatins (18).

(b) Based on biological functions

Based on the biological functions the AMPs can be divided into antibacterial, antiviral, insecticidal, antifungal, antiparasitic, etc.

(c) Based on mechanism of action

Taking into consideration the mechanism of action, the AMPs can be broadly classified into two families: membrane targeting peptides,e.g. temporins (19) and non-membrane-targeting peptides (20). Membrane-targeting peptides can be further classified as cell wall, lipids, and proteins targeted molecules. Likewise, non-membrane targeting AMPs can be further classified based on the specific target molecules (e.g. protein, DNA, RNA).

(d) Based on amino acid sequences

Without any three-dimensional structural information, based on protein sequences only, AMPs can be classified into linear, loops and circular. The linear form is the simplest. The loops can be formed between parts of the peptide chain due to covalently chemical modifications such as disulfide bonds, e.g Rana box (21). In case of circular AMPs, the polypeptide chain is a seamless circle due to the formation of a covalent bond between the N- and C-termini of the peptide. Circular proteins have been found in bacteria, e.g. AS-48 (22), plants, e.g. cyclotides (23), and animals,e.g. theta-defensins (24).

(e) Based on 3D structure

In the recent book edited by Dr. Wang (15), AMPs are classified into four families: alpha, beta, alphabeta, and non-alphabeta based on the types of their secondary structures.

The structural requirements of peptides for antiviral and antibacterial activities are evaluated in light of the diverse set of primary and secondary structures described for host defense peptides. Knowledge regarding the relationship between peptide structure and its function as well as its mechanism of action is being applied in the design of antimicrobial peptide variants as potential novel therapeutic agents.

Thus it can be said that the most frequently used classification of AMPs nowadays is done based on their secondary structures (for illustration see Figure 1, adapted from (25).

I. Alpha family

Alpha family consists of AMPs with helical structures. **Amphipathic α- helical peptides** represent one of the most successful designs in nature, being produced by all types of organisms from bacteria to mammals (26). They are mostly short, 12-37 residues in length, linear and lack cysteine residues peptides. Some of them have a hinge region.

In aqueous solutions many of these peptides are disordered, but in the presence of trifluoroethanol, sodium dodecyl sulphate (SDS) micelles, phospholipid vesicles and liposomes, or Lipid A, all or part of the molecule is converted to α-helix (27). Moreover, it also happens within the membrane mimetic environment (28). Unlike other AMPs they can be easily chemically synthesized. Cecropins, magainins or melittin can serve as examples of α-helical peptides.

II. Beta family

The beta family is composed of AMPs with beta-strands. **β- sheet peptides** such as defensins, tachyplesins, and protegrins, have no or just a few helical domains and a well-defined number of β-strands. Most of them contain cysteine residues linked by disulfide bridges. Thus, they adopt either a β-sheet or β-hairpin fold. It is a highly diverse group at the level of primary structure.

In 2004, the team of Yount and Yeaman (29) found out that all of cysteine-stabilized antimicrobial peptides have a γ-core. This γ-core, composed of two anti-parallel β-sheets and basic

residues along the axis, appears in all major cysteine-stabilized host defense peptides through whole biological kingdom. Therefore, it seems it is the unifying archetype in many AMPs.

III. Alphabeta family

The alphabeta family comprises both helical and beta-strands in the 3D structure (e.g. beta-defensins).

IV. Non-alphabeta family

Non- alphabeta family or other specific residue-rich peptides contain neither helical nor beta-strands. Peptides of this group have one or more particular residues of amino acids in higher occurrence, especially arginine, proline and histidine (30), phenylalanine or tryptophan (31). These peptides lack cysteine residues and they often differ from typical α-helical or β- sheets structures. They are linear, although some can form extended coil (32).

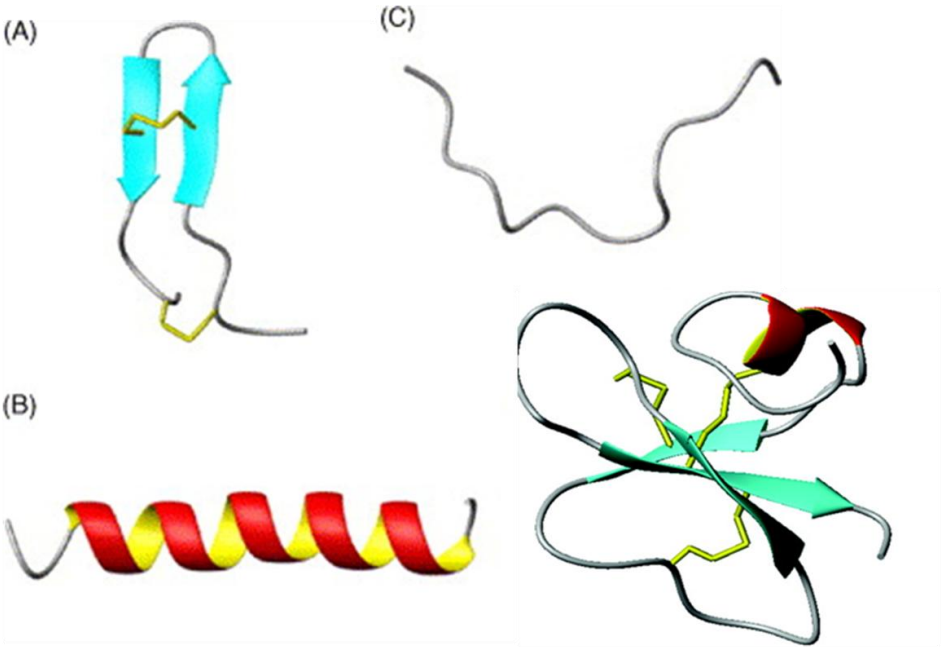


Figure 1: *Structural families of antimicrobial peptides (26): A) β-sheet, B) α- helical, C) Non-alphabeta, D) Alphabeta; the disulfide bonds are indicated in yellow; adapted from (26).*

2.1.3. Mode of action

As mentioned above AMPs are one of the most important parts of innate immunity which is the main defense system of many living organisms. Therefore, they have to sustain a powerful mechanism to deal with pathogens overcoming the physical barriers. Collectively these peptides demonstrate a broad range of antiviral and antibacterial activities and modes of action, but it is important to distinguish between direct antimicrobial and indirect activities against such pathogens (20). Based on this, the host-defense peptides provide a template for two separate classes of antimicrobial drugs:

I. Direct-acting antimicrobial host-defense peptides:

Direct- acting AMPs are the fast-acting compounds that possess an unusually broad spectrum of activity; consequently, they have prospects as new antibiotics, although clinical trials to date have shown efficacy only as topical agents. However, to fulfill their therapeutic promise and overcome clinical setbacks, further work is needed to understand their mechanisms of action and to reduce the potential for unwanted toxicity (33).

II. Cationic host-defense peptides:

In contrast to the first group, the abilities of these peptides are directed into modulating of the innate immune response and boosting infection-resolving immunity. Dampening the harmful pro-inflammatory (septic) responses give them the potential to become an entirely new therapeutic approach against bacterial infections (33).

There is a broad range of action the peptides can perform as they act on bacteria, fungi, viruses and also cancer cells (34). There are many reasons for this variability and one of them is the different sites of action in host, thus different environment, i.e. skin, respiratory system, gastrointestinal tract or oral cavity.

But this is not the only parameter influencing the properties of AMPs. AMPs properties are influenced by many other factors. Some of these factors are presented below:

(a) Size

The size is variable; the vast majority of AMPs have up to 100 amino acid residues**.**

(b) Sequence and structure

There is a strong and complex interaction with many molecular determinants, i.e. membranes, other peptide chains, the bilayer interface, and with water (35). All this together affects the cytolytic activity of AMPs, as was proved by Oren et al. (36). Structure of AMPs helps in binding and permeabilization of the membrane.

(c) Charge

I. Cationic peptides:

Most AMPs are cationic peptides with a positive net charge (from +2 to +9). It helps them to bind to the negatively charged phospholipid bilayers and to form clusters. Actually, this represents the main driving force for the folding of a peptide at the lipid-peptide interface (28). It is also a critical parameter in AMP's mechanism of action. The significant difference in membrane chemiosmotic potential and lipid composition between AMPs from prokaryotes and eukaryotes seem to play a pivotal role on their selective toxicity (37).

Cationic peptides belong to the largest group and the first to be reported, being widely distributed in animals and plants (38).

Taking cationic peptides´ structure into consideration, they can be divided into two broad groups: within the first group, two subgroups can be distinguished: (a) linear peptides that tend to adopt a-helical amphipathic conformation, and (b) linear peptides of unusual composition, rich in amino acids such as proline, glycine, histidine and tryptophan. The second group, encompassing all cysteine-containing peptides, can also be divided into two subgroups corresponding to single or multiple disulfide structures (39).

Important subfamilies of cationic peptides include: defensins, cathelicidins, thrombocidins, cecropins, thionins, amino acid-enriched class of peptides, other natural structural proteins, etc.

II. Anionic peptides

This is a smaller novel group of molecules displaying antimicrobial activity which, up to now, have been mostly isolated from mammals that includes neuropeptide derived molecules, aspartic-acid-rich molecules, aromatic dipeptides and oxygen-binding proteins.

Anionic peptides have a net charge from -1 to -7. They have a broad range of biological activities and it seems that their antimicrobial activity, which is mostly dependent on post-translational modifications, is of a secondary role. Structure is variable: from α-helical peptides to the cyclic cysteine knot structures. Some AMPs use metal ions to form cationic salt bridges with negatively charged membrane components. This, in turn, favors the interaction with target organisms. However in many cases the mechanism is still unclear (40).

(d) Amphipathicity

Amphipathicity reflects the relative abundance and polarization of hydrophobic and hydrophilic domains within a protein. This property is crucial for the functionality of AMPs because most peptides fold into amphipathic structures when interacting with target membranes. The most common structure is amphipathic α-helix with periodicity of three or four residues per turn. This is the optimum for membrane-peptide interaction as well as for folding of monomeric α-helices. The non-polar faces insert into the membrane by hydrophobic and van der Waals interactions. All these aspects result in a barrier loss of function and increased permeability (37).

A high degree of helicity and/or amphipathicity is correlated with increased toxicity toward cells composed of neutral transporters (41).

(e) Hydrophobicity

Hydrophobicity is defined as the percentage of hydrophobic residues within a peptide. For most AMPs it is approximately 50%. Hydrophobicity is an essential feature for AMP biological activity, especially membrane interactions, as it governs the extent to which a peptide can partition into the lipid bilayer (42). Although hydrophobicity is necessary for effective permeabilization, its increased levels have a higher impact on mammalian cell toxicity and also lead to the loss of antimicrobial specificity due to remarkable reduction of antimicrobial activity. Thus, highly

hydrophobic peptides beyond a threshold are related to higher hemolysis. Therefore many AMPs are moderately hydrophobic, otherwise increased hydrophobic levels result in di- or oligo-merization which is energetically less costly but it decreases the antimicrobial activity due to possible weaker interactions with membranes. Moreover, it is more energetically costly to reorganize and assume the correct folding and orientation.

(f) Polar angle

Polar angle is a measurement of the relative proportion of polar against non-polar facets of a peptide conformed to an amphipathic helix. As a reference, polar angle of 180˚ is used, i.e. when one face has hydrophobic residues and the other - charged/polar residues. Changes in structure lead to the change of angle. According to several studies, the smaller the polar angle is, the more increased is the membrane permeabilization (43).

The polar angle also correlates with the overall stability and half-life of peptide induced membrane pores. Uematsu and Matsuzaki (43) showed the effects of polar angle on membrane permeabilization and pore formation. All this indicates that polar angle of AMPs affects the ability of disrupting microbial membranes.

2.1.4. Mechanism of action

As mentioned above, AMPs interact with cell membranes. The ability to associate with membranes is a characteristic feature of antimicrobial peptides. There are two main possible modes of actions:

- **Membrane disruptive activity**
- **Non- membrane disruptive activity**

(a) Membrane disruptive proteins

Antimicrobial proteins can cause a dissipation of the electrochemical potential, lipid asymmetry and loss of important metabolites and cellular components that usually culminate into cell shrinkage and ultimately cell death (42). It is done through membrane permeabilization via formation of stable pores, membrane thinning or detergent-like

micellization. All these mechanisms help to make an intracellular space accessible, which is followed by loss of ions, metabolites and cytosolic components.

There are several mechanistic models of the above described mechanism - the “barrel-stave”, “carpet”, “wormhole or torodial”, and “aggregate channel”.

The “barrel-stave model” is proposed for quite hydrophobic peptides. In this model, small individual peptides bind to the membrane leaving there a lumen (Figure 2, (32). Their hydrophobic α- helical and β-sheet domains interact with acyl chains of the membrane, whereas the hydrophobic surfaces form the pore lining (44). When threshold concentration is reached after peptide attachment, the conformational change may occur and may result in induction of membrane thinning. It is due to insert of protein hydrophobic part to the membrane. Other peptide monomers can be added or lost which can influence pore size. If the monomers are added, it results in higher pore size as well as in phospholipids translocation and it can induce the peptide transport into the cell due to the concentration gradient or due to electrochemical potential.

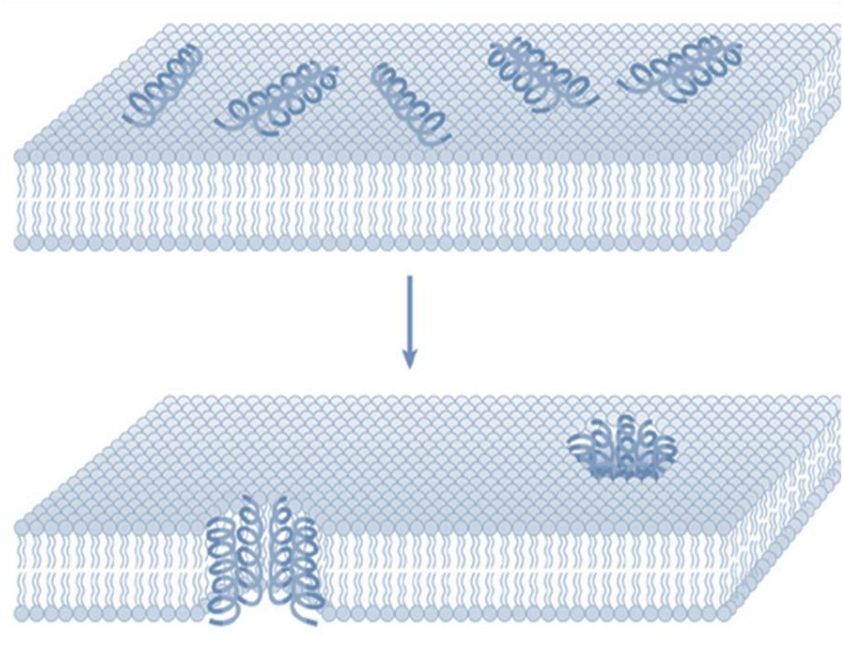


***Figure 2**: The barel- stave model of antimicrobial peptide; adapted from (41)*

In the “carpet model” (Figure 3, (32) proteins bound to the membrane due to the electrostatic interactions with anionic phospholipids head groups. They bind to the membrane at many points and

therefore carpet the membrane. When the threshold concentration is reached, the peptides might form torodial transient holes in the membrane, allowing additional peptides to access it, therefore the membrane disintegrates and forms micelles after disruption of the bilayer curvature (42). Cecropins, ovispirin or some magainins, for example, belong to this group.

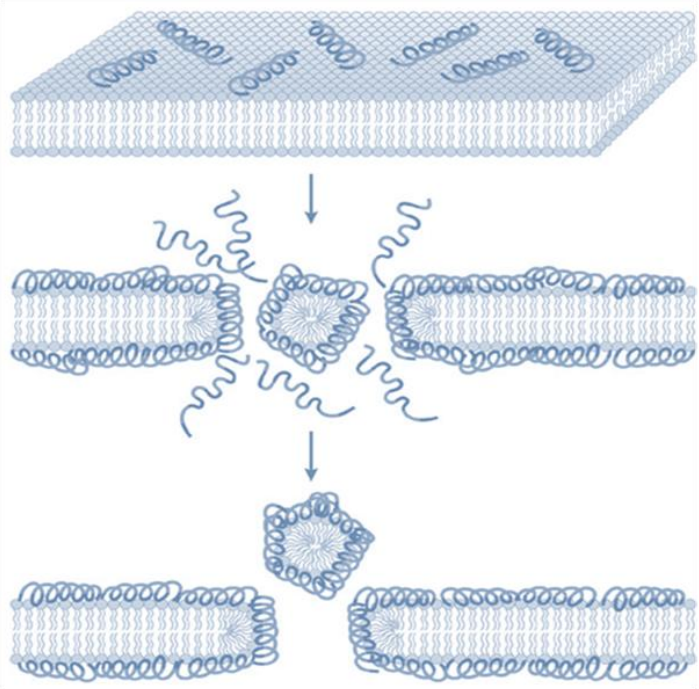


Figure 3: *The carpet model (adapted from (41))*

The "torodial-pore mechanism" (Figure 4) is a mechanism where not only peptides are involved. A pore is made of the lipid head groups. The constitution of a torodial pore implies that the polar faces of peptides associate with the polar headgroups of the lipids due to bending and therefore, the lipids tilt from the lamellar normal and connect the two leaflets of the membrane, forming a continuous bend from the top to the bottom in a torodial pore manner. There is a great advantage in this protein-lipid lining: the negatively charged phospholipids headgroups reduce the repulsive interactions caused by positively charged peptides. This supports peptide aggregation resulting in pore formation.

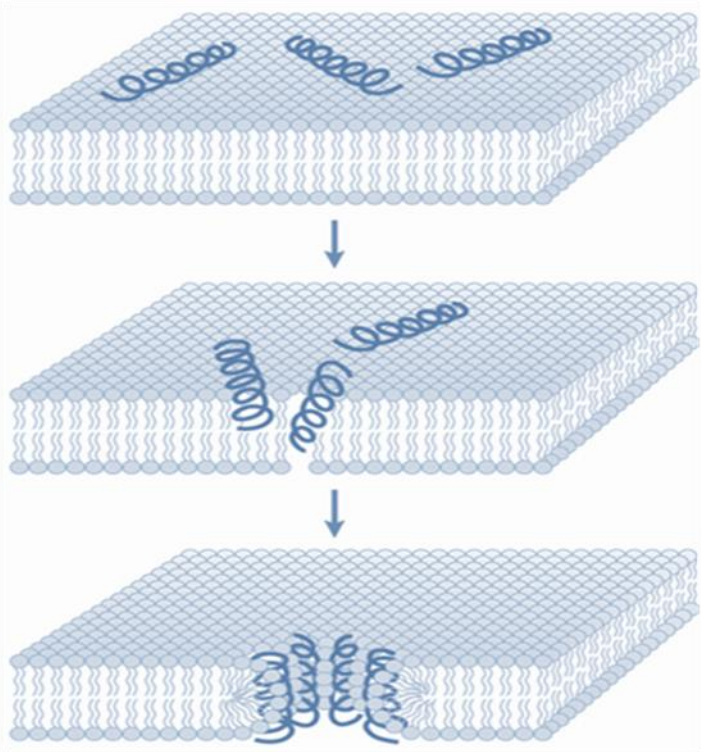


Figure 4: *The torodial – pore mechanism (adapted from (41))*

However, these models cannot be considered as the only occurring mechanisms. Different peptides have different biological responses within the cells and moreover, some studies have shown there is a continuous gradation between all mentioned models.

(b) Non- membrane disruptive activity

It was found out that AMPs do not necessarily have to permeabilize the cell membrane. Several studies have shown they can form the transient defect on the membrane, e.g. pores or ion channels. These transient defects are short-termed and they do not cause any damaging depolarization of membrane or even its disruption.

There are many mechanisms how the peptides can reach the inner cell space. It can be done through: channel aggregates (37), peptide-induced lipid segregation (45), raft sinking (46), leaky-slit model (47) or peptide-mediated non-lamellar phase formation (48), etc.

(c) Other mode of action- Intracellular targets

For a long time the AMPs biological activities were considered to be related to their ability to disrupt cell membranes. However, some studies recently indicate that permeabilization alone may not be enough to explain antimicrobial activity (25). As mentioned before, AMPs can pass cell membranes without their damaging. Once in the cell, the peptides can interact with intracellular targets and affect in this way the viability of the cells at low or moderate concentrations.

Examples of intracellular activity include inhibition of DNA and protein synthesis, inhibition of chaperone-assisted protein folding, inhibition of enzymatic activity, and inhibition of cytoplasmic membrane septum formation and cell wall synthesis (49). However, the interpretation of a huge amount of published data is very difficult due to their variability.

Intracellular targets and AMPs which are involved in interactions with these compartments are summarized in Table 2.

Table 2: *Examples of AMPs interacting with intracellular targets*

Intracellular target	Mode of action	AMPs involved	References
DNA	Binding to DNA	Tachyplesin I, pleurocidin and magainin 2	(50), (51)
	Inhibition of DNA synthesis	Dermaseptin, buforin II, indolicidin	(52), (53), (54)
Mitochondria	Inhibition of F_0F_1-ATPase causing bioenergetics collapse	Histatin-5	(55)
Proteins and enzymes	Affecting ribonucleotide reductase	Purothionin	(56)
	Inhibition of enzymatic activity	Histatins, drosocin and apidaecin	(18), (57)
	Inhibition of protein synthesis	Pleurocidin, indolicin	(52), (54)
RNA	RNA inhibition	RNA III inhibitor-dermaseptin Chimera	(58)
Cell	Inhibition of cell division	Indolicin, microcin	(54), (59)
	Cell wall assembly	Lysozyme, lactoferrin	(60), (61)

2.1.5. Application of AMPs

(a) AMPs as therapeutics

AMPs are excellent candidates for development of novel therapeutic agents and compounds to conventional antibiotic therapy because they generally have a broad range of activity.

Their effectiveness is mainly attributed to their ability to disrupt the cell membrane and to cause the cell lysis. Therefore the AMPs are able to kill a broad spectrum of bacteria even multidrug resistant as well as fungi, viruses, protozoa or tumor cells in a short period of time. The explanation of this miraculous effectiveness is very simple- the targets of AMPs, as already mentioned, are mostly the biological membranes. To become resistant to AMPs it would mean for the cells to redesign their membranes, i. e. to change the composition of the membrane by reorganization and usage of different kinds of its components. Moreover, the sequences of AMPs mostly do not contain any uncommon or unique areas to be marked as typical recognition sites for the concrete peptide in order to be destroyed. This is the main aspect which makes the AMPs so promising in vaccine development.

Recently, the investigation of many naturally occurring peptides as well as their derivatives has been performed. They can be used for diverse therapies from anti- pathogenic through to anti-cancerogenic.

Many of antimicrobial peptides have already entered clinical trials. Their status in the clinical trial can be searched and checked easily (http://clinicaltrials.gov/).

(b) AMPs as biomarkers

Another possible application of AMPs in medicine is biomarkers (especially, human α-defensins). Biomarkers have been detected in malignant cells and in body fluids of patients with tumor, e.g. in case of metastatic colorectal cancers (62), lung tumors (63), renal cell carcinomas (64), and others. Moreover, their increased concentration (cca. 40 ng/ ml) in body fluids could be an indicator of an inflammatory syndrome, or when measured in milligrams, it could be a marker of sepsis (65).

(c) Modified AMPs

Some naturally occurring AMPs have been proved not to be suitable candidates as therapeutics and therefore not suitable for further pharmaceutical development. The reasons for this can be diverse: unfavorable pharmacokinetics, the loss of activity under physiological conditions, low microbiological efficiency, quick degradation etc. To overcome all these problems modified AMPs are used.

AMPs are diverse and they possess various properties as well as structures. Despite this diversity, they do have some common features - all of them are charged and their active regions are generally amphipathic (66). The modified AMPs share these common properties. Moreover, they have to fulfill several requirements: to have the smallest amino acid domain which has full antimicrobial activity, to maintain the same activity in different environment, e.g. biological fluids, to be specific to selected organism as well as to be resistant against degradation.

There are many ways how to modify AMPs but the detailed description of this process is above the frames of this thesis. In short, AMPs can be modified according to following models:

- AMPs hybrids- they consists of active regions of 2 or 3 natural peptides, this increases their activity and decreases their cytotoxicity for host cells, e.g. cecropin A- melittin (67,68);

- AMP conjugates- they are conjugated to some receptor ligand or specific antibody which is situated on the outer surface of a specific pathogen, therefore they can be used in lower concentration and their side effects are decreases, e.g. *Fusarium* spp- specific antibody (69);

- AMP congeners- these compounds are related to another in composition and they have similar/antagonistic effects. A number of disulfide bonds can be reduced in congeners and it results in higher antimicrobial activity, abolishment of chemotactic activity, etc., e. g. SMAP29 (70) or CAP18 (71);

- AMP mimetics- these AMPs are synthetic but not peptide molecules, even though they have the same properties and activities. They have similar chemical and physical properties, e.g. peptoids (72);

- Cyclotides- they are isolated from plants and are characterized by their head-to-tail cyclic backbone. Thanks to their cyclic structure they are more stable in diverse environment and

resistant to degradation by proteolytic enzymes and by heating. Moreover, they are more active. Their application as anti-HIV agents (73), insecticidal, antibacterial etc. is broad (for example, a circularized peptide analogue of rabbit defensin NP-1 (74);

- Immobilised AMPs- AMPs are incorporated into broad spectrum of materials and surfaces through plastics up to films as they are able to kill the pathogens. This method is used in food industry for preservation (75) as well as in medical research, for example, lysozyme, nisin or nisaplin.

(d) Limitations of AMPs

However the AMPs are very promising as new therapeutics there are also several aspects which should be taken into consideration. Thus, as everything, the application of AMPs is also limited in a certain way. The main limitations are:

- High production cost
- Toxicity
- Susceptibility to proteolysis

I. High production cost

Antimicrobial peptides have relatively high molecular weights compared to most antibiotics. To keep prices down they have to be produced as recombinant proteins and in higher yields. The production of recombinant proteins has become common because it is effective and the costs are lowered when produced in a large-scale. However, there are some technical difficulties especially when large amounts are required. At the current production efficiency, for each 100 kg of recombinant peptide one million liters of fermentation mixture is necessary (76). Moreover, it is very important to choose suitable expression system, so-called, fusion partner that is crucial for protein stability, yield and purification.

Another way is to produce peptides synthetically, but its cost is from 5 to 20 times higher as compared to modern antibiotics - the cost per gram can differ from $50 to $400 (77). The only way how to overcome this cost problem is to produce synthetic peptides massively to

lower the cost of all reagents as it was done by Roche Company in case of T-20 peptide, the suppressor of HIV-1 replication, for example.

II. Toxicity

Several studies have shown that some antimicrobial peptides, especially some cationic ones, are toxic for mammalian cells, for example antimicrobial peptides from the venom reservoirs of wild bees (78). However, in spite of the fact that not many studies on AMPs toxicity have been published, it was shown that a lot of antimicrobial peptides have neglectable toxicity.

III. Pharmacodynamic and pharmacokinetic issues

Another aspect to discuss is AMPs susceptibility to proteolysis in the body, peptide aggregation problems, the *in vivo* half-life and required dosage frequency.

AMPs are relatively labile to proteolysis which affects their applicability. In this context, several protection strategies have been proposed. AMPs can be protected by incorporation of negatively charged or lipophilic proteins (79), or by other chemical modifications as insert of uncommon amino acids (i.e. isovaleric acid, hydroxyproline) to prevent binding to the active sites of proteolytic enzymes (80) or to prevent cleavage by alkylation of nitrogen atoms (81).

To increase the stability of peptides, amidation of N-terminus, non-natural amino acids or peptide cyclizations are performed.

Except the already mentioned limitations, significant differences of AMPs effects was noticed under *in vivo* and *in vitro* conditions. Exploration of high antimicrobial activity in *in vitro* tests does not always mean the same results in *in vitro* experiments, as AMP activity can be lost under physiological conditions. Human β-defensin is (82) one of the best confirmation of this observation.

The discovery of antimicrobial peptides itself as well as their presence in almost all living organisms and their importance in defending organism against pathogens have become a milestone of this millennium. AMPs are very important compounds which help to maintain organism alive as well as protected them against microbes without specific immune responses. Their discovery has shown new possible direction in designing new therapeutics.

As it was already mentioned, it has been proved that AMPs are effective against a broad range of microbes, viruses, in some tumor cases. Therefore many research teams pay attention to this topic and try to find solutions how to use the AMPs against the existing pathogens as well as in prevention of pathogen caused diseases.

One of the directions in searching for new effective compounds is the research of AMPs in arthropods in general and in ticks, in particular.

2.2. Ticks

Ticks are blood feeding ectoparasites widely distributed all over the world. Ticks are very efficient vectors of pathogens; they are known to transmit a wide range of microorganisms including protozoans, viruses, bacteria, rickettsia and nematodes (83) to vertebrate hosts, including humans. Different species of ticks transmit different pathogens (Table 3). Obviously their veterinary and medical importance is significant.

Transmitting a wide variety of pathogens, ticks can protect themselves against infection with variety of compounds. The efficiency of ticks as vectors is probably caused by two important aspects: their innate immune system and the length of time they are attached to their hosts.

Table 3: *Examples of diseases transmitted to humans by ticks*

Disease	Affected host	Causative agent	Tick vector
Human babesiosis	Humans	*Babesia microti, B. divergens, B. gibsoni*	*Ixodes scapularis, I. ricinus*
Tularemia	Humans, etc.	*Francisella tularensis*	Heamaphysalis leporispalustria, other tick species
Lyme disease	Humans, dogs, cats, domestic animals	*B. burgdoferi s.s., B. afzelii, B. garinii*	*I. scapularis, Ixodes ricinus, I. pacificus, I. pesulcatus*
Tick- borne relapsing fever	Humans	*Borrelia* spp.	*Ornithodoros spp.*
Tick-borne encephalitis	Human, carnivores	Flavivirus	*I. ricinus, I. persulcatus*
Tick paralysis	Human, cattle, sheep, other mammals	Tick proteins	*I. holocyclus, I. rubicundus, Dermacentor variabilis, D. andersoni*
Rocky mountain fever	Humans	*Rickettsia rickettsii*	*D. variabilis, D. andersoni,* others
Human monocytic ehrlichiotis (HGE)	Humans	*Ehrlichia chafeensis*	*Amblyomma americanum, D. variabilis*
Sweating sickness (and other toxicosis)	Humans, catlle, sheep	Tick proteins	*H. truncatum, O. savignyi, O. lahorensis, A. persicus*

2.2.1. Tick innate immunity

Ticks and invertebrates in general have innate immune system which lacks any adaptive immunity consisting of antibody responses and cell-mediated responses. Therefore, another mechanism to protect themselves against various infections had to be developed.

Despite its importance, our knowledge of tick innate immunity is still inadequate and the

limited number of sufficiently characterized immune molecules and cellular reactions are dispersed across numerous tick species (84). Greater understanding of specific activity and efficiency in innate immune responses of different tick species is also likely to help in providing clues to vector competence and vector efficiency for transmitting the diverse array of microbes associated with ticks (85). Search for the answer why some ticks are efficient competent pathogen vectors, whereas the others are not, is apparently more complicated than simple expectation that it is dependent on the presence or absence of T- lymphocytes, memory cells, etc. It seems that midgut plays an important role in vector competence. The gut content is a hostile environment for ingested microbes, which is mainly due to the antimicrobial activity of hemoglobin fragments generated by the digestion of the host blood as well as other antimicrobial peptides (86). Another possible importance in pathogen transmission may have reactive oxygen species (ROS) (87).

In ticks, we can distinguish cell- mediated responses and humoral responses.

(a) Cell- mediated immunity

Phagocytosis, encapsulation or nodule formation (i.e. trapping pathogens in coagulum of granular cells which can undergo melanization) belong to these nonspecific responses.

At this point, the impact of tick hemocytes should be acknowledged. Several studies have been done on this topic and it has been proved that tick hemocytes are able to phagocyte and to remove foreign cells from the organism (88- 90). However, different studies do not agree in one thing: which of three kinds of hemocytes, plasmocytes, granulocytes or spherocytes, are phagocytic and moreover, if it is just one or all of them. Moreover, the phagocytosis of microbes by tick hemocytes seems to be coupled with a primitive complement-like system, which possibly involves self/nonself recognition by fibrinogen-related lectins and the action of thioester- containing proteins (91).

(b) Humoral responses

Cell- mediated responses cooperate with humoral responses to protect the organism against certain foreign agents. There are three main types of humoral responses- hemagglutination (92), humoral encapsulation (93), and, what is the most important from our point of view - antimicrobial defense.

I. Tick AMPs

Some tick AMPs, such as lysozymes, cecropins, attacins, defensins, proline-rich peptides, glycine-rich peptides and others, have been already identified and characterized. The most significant groups of them are discussed below.

Lysozymes

Lysozymes are activated when the tick is infected. They act as cleavage enzymes of β-1,4 glycosidic bonds of bacteria peptidoglycan cell walls and, thus, in lysis of the cell. Lysozyme functions and its occurrence in the tick body differ from specie to specie. For example, lysozymes are not active in the midgut of hard ticks (94) but are active in soft ticks (95). In addition, in hard tick hemolymph and hemocytes, the lysozymes have been up-regulated but in soft ticks they are not.

Lysozymes have been shown to be active against *Borrelia burgdorferi* (96) *, Rickettsia monacensis, R. peacockii* (97) or *Bacillus subtilis* (98).

Cystatins

Cystatins are naturally occurring inhibitors of the cysteine proteinases with which they form reversible but tight complexes and in this way protect host tissues from destructive proteolysis by its own, bacterial or viral proteinases. Moreover, it has been recently found that in vertebrates they have many other functions, i.e. in development and immune response.

Cystatins have been also found in hard tick salivary glands (99, 100) but it was not shown to be expressed anywhere else. Even though not much information has been found on this topic, cystatins are already very promising potential candidates for tick vaccines.

Defensins

This is the broadest group of antimicrobial peptides in invertebrates and nowadays they are the most studied group of AMPs in ticks. Defensins are mostly cationic, cyclic AMPs with 6 conserved disulfide-paired cysteins (101): Cys1-Cys4, Cys2-Cys5, Cys3-Cys6. Their cationic character helps them in the interaction, the depolarization and the following disruption of the cytoplasmic membranes. Defensins are mainly effective against Gram-positive and Gram-negative bacteria, certain fungi and yeast (102).

It was found that defensin genes are expressed in all tick developmental stages, from eggs to adults.

Obviously, the digestive system of ticks is main gate of pathogen invasion. Therefore the major boost of defensins expression take place in midgut as a reaction to a blood meal (*I. ricinus, I. scapularis, O. moubata),* or in hemolymph (*D. variabilis*) (103).

2.2.2. Systematic classification of ticks

Ticks, Ixodida, are mainly divided into families- Argasidae, Ixodidae and Nuttalliellidae. The first two families are called soft ticks (Argasidae) and hard ticks (Ixodidae), respectively, due to their flexible or nonflexible cuticle. The third family is represented by only one species, *Nuttalliella namaqua,* which is found in southern Africa.

Systematic classification of ticks is described in Table 4 below.

Table 4: *Systematic classification of ticks*

Scientific classification	
Kingdom	Animal
Phylum	Arthropoda
Class	Arachnida
Subclass	Acari
Superorded	Parasitiformers
Order	Ixodida
Superfamily	Ixodoidea
Families	Argasidae, **Ixodidae,** Nuttalliellidae

(a) Ixodidae (hard ticks) and their life cycle

Following information is applied to family Ixodidae only, genus Ixodes because it is important for this diploma thesis.

This hard tick family includes around 243 species. They are the most efficient and important pathogen vectors. Their life cycle includes egg and three developmental stages, i.e. larva, nymph and adult. Most of ticks are three- hosts and it means every stage leaves its host before it moults into the next life stage (Figure 5). Adult fed females lay off eggs on the ground.

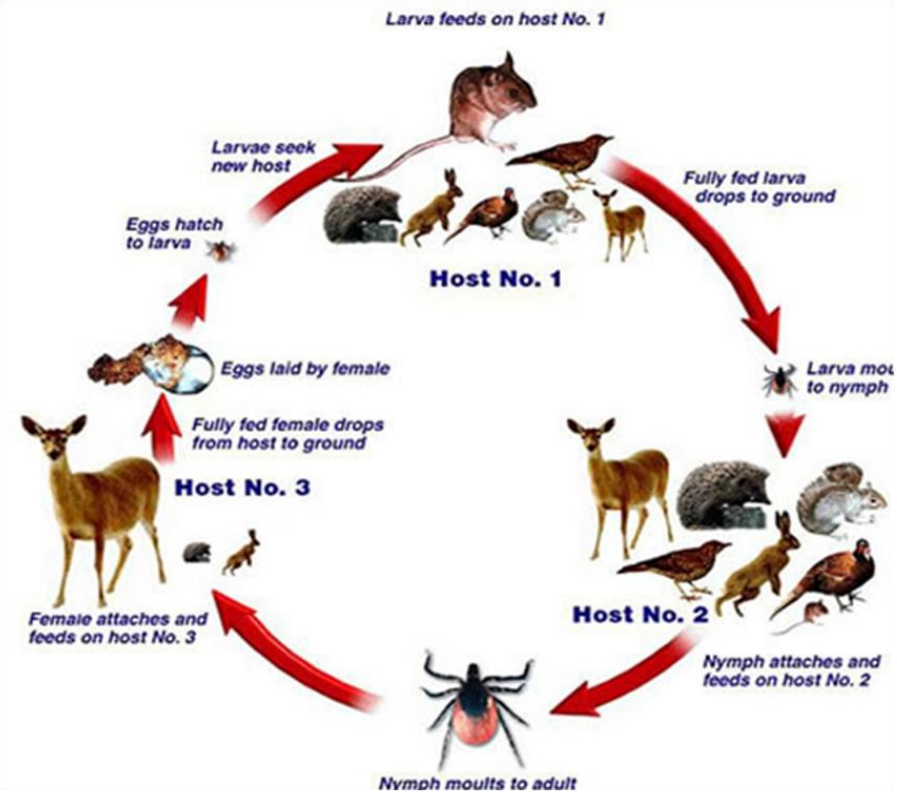


Figure 5: Life cycle of Ixodidae ticks (source: www.medicalecology.org/diseases/lyme/print_lyme.htm)

Ixodid ticks have variable period of feeding from several days up to 2 weeks. After attaching to their host, tick salivary glands start to secrete cement into the bite site and around the wound to make a firm attachment. Therefore it is difficult to remove ticks out of the skin. Except cement, anticoagulants and other agents are also secreted. During the feeding tick body weight can increase 10- 100 times and the new cuticle is synthesized to be able to deal with high amount of blood.

Mating of ixodid ticks is performed on and off the host.

Ticks host specificity is very strict in the majority of tick species, but it is not the case of *Ixodes ricinus* and *Ixodes scapularis* which are known as opportunistic species that can feed on all types of hosts.

Non-adult stages feed on rodents, lizards, small to medium sized mammals and on birds. Adult ticks feed on larger herbivore (including cervids and bovids), carnivore, or human. The three hosts do not necessarily have to be different species, or even different individuals. Also, humans may serve as first, second or third hosts. Host specificity is strongly influenced by ecological and environmental conditions. Interestingly, ticks are able to survive long time between blood feeding. If it is so, their life cycles can be extended for a long time, in exclusive cases for years.

Most of ticks are not active during the whole year. Their seasonal activity starts at the early beginning of spring when the weather is warmer and day length is prolonged and it ends approximately in October. During winter, ticks remain in state of reduced metabolic activity, so-called diapauses.

I. Species *Ixodes scapularis* and *Ixodes ricinus*

Both tick species are important vectors of the causative agent of Lyme disease in North America and Europe (Figure 6, 103).

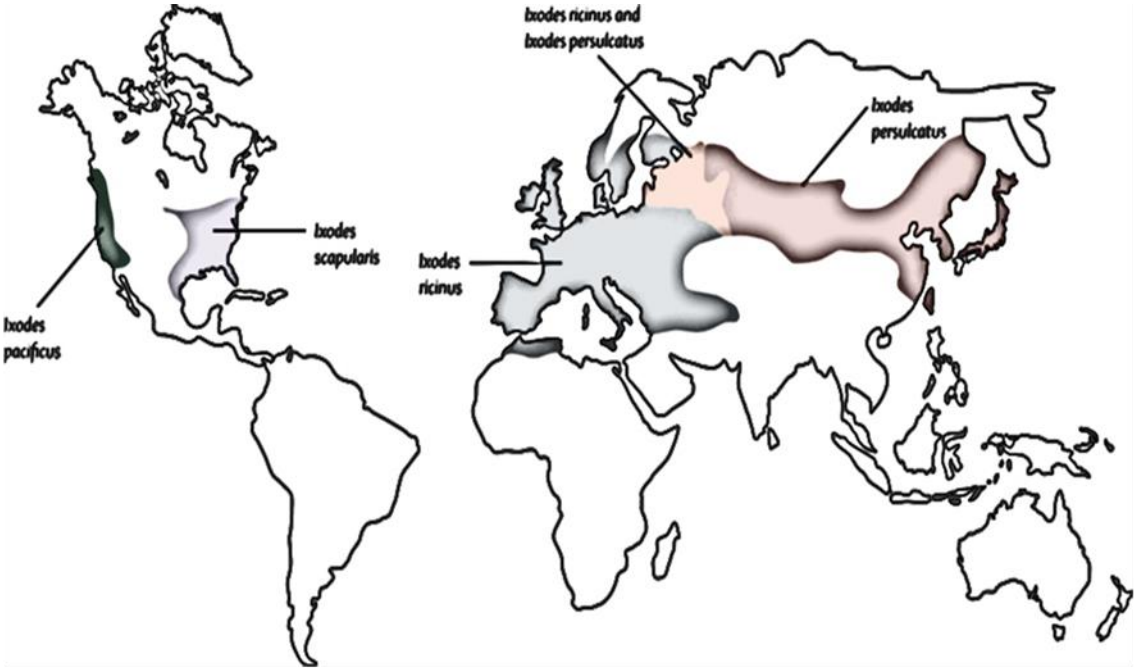


Figure 6: *Distribution of Ixodidae ticks over the world (adapted from (102))*

I I. scapularis, known as deer tick or blacklegged tick, is widely distributed in the eastern, central and northern part of the United States.

I. ricinus is the most important tick species in Europe and a main vector of the majority of tick-borne pathogens.

3. Objectives of the thesis

In 2009 the research team of Dr. Pichu (104) introduced new antimicrobial peptide from *Ixodes scapularis.* This peptide has 69 amino acids with molecular weight of 5.3 kDa. It was shown to be active against both Gram-negative and Gram-positive bacteria.

Because *I. scapularis* and *I. ricinus* are closely related, we assumed that AMPs expressed in *I. scapularis* could be also found in *I. ricinus.* Based on the information available for *I. scapularis* antimicrobial peptide ISAMP, the search of it homolog in European tick *I. ricinus* was conducted.

This Master thesis was designed to analyze a novel antimicrobial peptide from *I. ricinus*, that we named IRAMP (*Ixodes ricinus* antimicrobial peptide). The main goals of the study were to find the full gene sequence that encode IRAMP in *I. ricinus*, to produce a recombinant protein in the bacterial expression systém, to isolate the native peptide from tick hemolymph and to analyze the presence of antimicrobial activity in both, the native and recombinant AMP.

An essential part of this study is devoted to expression and purification of recombinant IRAMP protein, with the following analysis of its antimicrobial potential and to analysis of the protein expression pattern in different tick organs.

The main goals are briefly summarized below:

- To detect and isolate the full gene sequence encoding antimicrobial protein IRAMP in European tick *I. ricinus*, using the data available from APM from *I. scapularis* ISAMP
- To express and purify the recombinant IRAMP in bacterial expression system.
- To isolate the native IRAMP protein from the hemolymph of *I. ricinus*.
- To test the presence of antimicrobial activity in the native and the recombinant protein.
- To find the tissue expression pattern of IRAMP in *I. ricinus*.
- To refold the synthetic analog of IRAMP for following analysis of its antimicrobial activity.
- Using the obtained data on antimicrobial activity of native, recombinant and synthetic IRAMP from *I. ricinus* to evaluate IF this protein belongs to antimicrobial protein family.

4. Materials and methods

4.1. Recombinant protein production

4.1.1. Ticks

Ixodes ricinus ticks were collected in nature around Ceske Budejovice and fed on adult guinea pigs in the internal tick facility of the Biological Centre, Institute of Parasitology, Academy of Science of the Czech Republic. Laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic no. 246/1992 Sb.

All developmental stages of *I. ricinus* showed bimodal seasonal activity. The major peaks of nymphs and adults activities were observed in April - June and September - October (105). Ticks were collected during mentioned periods.

4.1.2. Total RNA isolation and cDNA synthesis

Partially fed ticks (6 days fed) were washed in 10% bleach (for 20 minutes), then rinsed in water and washed in 70% ethanol (5 minutes). After final wash in water and drying on sterile filter paper, the ticks were homogenized in a sterile Falcon tube. Thirty milligram of cleaned ticks were used for total RNA isolation with RNeasy® Mini Kit (Qiagen) strictly according to the manufacturer instructions.

The cDNA was synthesized using the Enhanced Avian HS RT-PCR kit (Sigma) strictly following the manual. Concentration of synthesized cDNA was measured by BioPhotometer at wavelength of 260 nm.

4.1.3. PCR and primers

First round of PCR reactions was made using the primers designed on *I. scapularis* gene encoding ISAMP, the sequence of which was available in the GenBank.

To substitute *I. scapularis*-specific primers by *I. ricinus*-specific primers, Rapid Amplification of cDNA (RACE PCR) method was used. This method is suitable for searching of

whole transcripts within the cells and for gaining the sequences when only a partial cDNA sequence is available, i.e. the amplification of nucleic acid sequences from mRNA between a defined internal site and unknown sequences (106). RACE- PCR can be performed at the 3´ end as well as 5´end, with the slight adjustment of procedure for each case.

RACE- PCR at the 5´end, so called „anchored“ PCR, was performed. Obtained mRNA was converted into cDNA using reverse transcriptase (RT) and IRAMP-specific primer. The sequence of mRNA from 3´ to the 5´end was amplified. Specific single stranded cDNA of interest is directly amplified by PCR using a specific primer and adapter.

RACE- PCR was performed strictly following the protocol of RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit, #K1631, #K1632 by MBI Fermentas Life Science. PCR primers used in all PCR reactions are summarized in Table 5. Conditions for amplification are summarized in Table 6. Steps 2- 4 were repeated as a cycle 35 times.

Table 5: *Primers used in RACE PCR*

Primer	Sequence	Temp. of annealing [°C]
IRAMP FORW$_1$	5´- ATG CGT GCC GTA GCC ATT TTC ATC-3´	55
IRAMP FORW$_2$	5´- AAG GTA CTT CCT AAT AGC CCG CC-3´	48
ISAMP REV$_1$	5´- TTA GCG GCA AGT CCT GTC GCC C-3´	50
ISAMP REV$_2$	5´- TTT ATT ATC CAT TGT TTA TTC GTG-3´	48
IRAMP F$_{EXPRES.}$	5´- CAC CGA GCC CCT TCC TGG ACA AGC-3´	50
UAP	5'- CUA CUA CUA CUA GGC CAC GCG TCG ACT AGT AC -3´	52

Table 6*: *Amplification conditions

Steps	Temperature [˚C]	Time [min]
1. Initial denaturation	96	5
2. DNA Denaturation	95	0,5
3. Annealing of the primers	According to specific primers used, see table 5	0,5
4. Extension step	72	0,5
5. Final extension	72	10
6. Holding	4	≥10

The amplifications were performed in 0.2 ml thin-wall PCR tubes in the Eppendorf thermal cycler (Mastercycler by Eppendorf, Germany).

For reagents, concentrations and volumes used in the PCR reactions see Table 7.

***Table 7:** Reagents per one PCR reaction (20 microliters)*

Composition of reagents/ supplier	Used volume
2x GoTaq Colorless Master Mix (GoTaq® DNA Polymerase,2X Colorless GoTaq® Reaction Buffer (pH 8.5), 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM MgCl2); Promega, USA	10 µl
dH_2O	7 µl
Forward primer (10 µM); Generi Biotech, CZ	1 µl
Reverse primer (10 µM); Generi Biotech, CZ	1 µl
cDNA (200 µg/ml)	1 µl

PCR products (20 µl) were mixed with 3.35 µl of 5x sample loading buffer and analyzed by agarose gel electrophoresis (2% agarose gel in 1x TAE). Five microliters of 100 bp PCR ladder (Thermo Scientific) was loaded on the gel as a size markers. Both PCR ladder and loading dye contain SYBR® GreenER™ (Invitrogen) allowing the visualization on the agarose gel. The electrophoresis was performed at 86 V, 1 hour. For composition of used chemicals see Table 8.

Table 8: *Composition of used chemicals*

Chemical and supplier	Composition
Agarose (SERVA)	2% agarose prepared in 1x TAE buffer
50x TAE buffer	2 M Tris-acetate (Sigma), 50 M EDTA (pH 8,0)
6x loading dye (Promega)	0.4% orange G, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 15% Ficoll® 400, 10mM Tris-HCl (pH 7.5), and 50mM EDTA (pH 8.0)
Marker GeneRuler 100 bp Plus DNA Ladder (ThermoScientific)	10mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03%xylene cyanol FF, 60% glycerol, and 60mM EDTA

4.1.4. PCR- products purification and sequencing

Identification of specific product bands may be complicated by the presence of nonspecific products that are dependent on both reverse transcription and dC-tailing (109). Therefore, the PCR products of the appropriate size (210bp) were cut out of the gel and purified using „QIAquick®Gel Extraction kit“, following the instructions of the producer(Quiagen, USA). The PCR product was verified by bi-directional sequencing with the gene-specific primers (see Table 5). The sequencing was done at the “in-house” sequence facility of the Faculty of Science, University of South Bohemia (FS SBU) and Biology Centre of the Academy of Sciences of the Czech Republic (BC ASCR) in Budweis. Sample preparation is described in Table 9. Acquired nucleotide sequence of PCR product as well as its predicted amino acid sequence was compared to the date available in public database (GenBank).

Table 9: *Sample preparation for sequencing*

Component	Volume used [µl]
PCR Product (120 – 150ng/ml)	3
dH_2O	4
Primer (0.1 µg/µl)	0.5
Total volume	**7.5**

4.1.5. Cloning and transformation

After the sequence of PCR product was verified, the correct primers were designed (Table 10).

Table 10: *The verified primers used*

Primer	Sequence	Temp. of annealing [˚C]
IRAMP $R_{CORRECTED}$	5´- TTA GCG GCA AGT GTT GTC GCC-3´	50
IRAMP $FORW_1$	5´- ATG CGT GCC GTA GCC ATT TTC ATC-3´	55

The PCR product with substituted *I. ricinus*-IRAMP-specific primers was cloned into pCR®4-TOPO® vector for sequencing (Invitrogen, USA) following the transformation of One Shot® TOP10 *E. coli* competent cells with the obtained construct. The procedure was performed strictly according to the manufacturer's instructions. Transformed cells were applied on agar plates with imMedia^{TM} Amp Agar for Amp^{R} recombinant *E. coli* strains by Invitrogen and grew at 37°C overnight. Middle sized colonies were inoculated into 2 ml of LB/ampicillin media and left growing overnight at 37°C with shaking.

The cultures were centrifuged for 5 min at 10 000 rpm and supernatant was discarded. The isolation of plasmid DNA was performed with Qiagen Plasmid Purification Kit Protocol (Qiagen,

USA). The sequence of the insert was verified by sequencing (for sample preparation see Section 4.1.4.) in both directions using M13 Forward as well as M13 Reverse primers at the plasmid (see Table 11).

Table 11: *Primers used for sequencing*

Primer	Sequence	Temp. of annealing (˚C)
M_{13}-Forw	5´- GTA AAA CGA CGG CCA -3´	50
M_{13}-Rev	5´- CAG GAA ACA GCT ATG AC -3´	50

4.1.6. Recombinant protein production

Recombinant protein was produced to gain large quantities of the desired protein for further exploration, e.g. for testing activity or interactions with other compounds.

(a) PCR product ligation into the expression system

Before further steps, the sequence of the product of interest was checked for the presence of signal sequence using website of Centre for biological sequence analysis, Technical university of Denmark (http://www.cbs.dtu.dk/services/SignalP/). Because signal sequence was recognized, the specific forward primer was designed to remove this signal sequence. For sequence of primers see Table 12.

Champion™ pET Directional TOPO® Expression system from Invitrogen was used for the preparation and expression of the recombinant IRAMP protein. PCR product was obtained using the above mentioned construct as a template (section 4.1.5.) with specifically designed primers (Table 12) for directional cloning into expression vector (pET 100/D-TOPO®).

Table 12: *Primer used for PCR product ligation*

Primer	Sequence	Temp. of annealing [˚C]
IRAMP $R_{COR.}$	5´- TTA GCG GCA AGT GTT GTC GCC-3´	50
IRAMP $F_{EXPRES.}$	5´- CAC CGA GCC CCT TCC TGG ACA AGC-3´	50

The fresh purified PCR product was sub-cloned into pET 100/D-TOPO® vector following the transformation of One Shot® TOP10 E. coli Competent cells. The obtained recombinants were checked by PCR for the presence of the insert. The verification of the insert and the proper reading frame of the fusion protein were conducted by sequencing using T7 promoter primer from the plasmid (Table 13).

Table 13: *Primers used for sequencing of expression plasmids*

Primers	Sequence	Temp. of annealing [˚C]
T7-Forw	5´- TAA TAC GAC TCA CTA TAG GG -3´	50
T7-Rev	5´- GCT AGT TAT TGC TCA GCG G -3´	50

The system used allows to produce a fusion protein with N-terminal histidine-tag (6x His) that makes possible the following detection and purification of the recombinant protein.

(b) BL21 Star™ (DE3) One Shot® Cells Transformation

The prepared construct was used for transformation of competent *E. coli* BL21 Star™ (DE3) One Shot® cells by Invitrogen following the instructions of the provider.

Transformed BL21 Star™ were inoculated into 10 ml of fresh LB/ampicillin media and incubated at 37°C for 12 hours with shaking. For composition of LB media see Table 14.

Table 14: *Composition of LB media*

Solution	Composition
LB media (Luria-Bertani Medium)	1 l dH_2O, 25 g LB Broth, Miller, Tissue Culture Grade (Amresco)

(c) Recombinant protein expression (pilot experiment)

Since each recombinant protein has different characteristics that may affect optimal expression, the time course of expression was performed to determine the best conditions for expression of IRAMP.

After overnight incubation, 500µl of bacterial culture was inoculated into 10 ml of fresh LB/ampicillin and after 2 hours (OD_{600}= 1), the expression of the recombinant protein was induced by adding of isopropyl-β-d-thiogalactopyranoside (IPTG) at final concentration 1mM, following the protocol from Champion™ Pet Directional TOPO® Expression Kits by Invitrogen.

Before IPTG adding an aliquot of 500 µl was removed (sample 0, before induction). The incubation of the culture was continued. For each time points 500 µl of the induced culture was removed every hour for 6 hours total time. The aliquots were centrifuged at max speed and supernatant was aspirated. All the pellets were frozen until use.

(d) Analysis of the expression results with SDS-PAGE and Western blot

I. SDS-PAGE analysis

The results of the expression experiment were tested with SDS- PAGE electrophoresis. Two gels with 18% resolving and 4% stacking parts were prepared according to Lab FAQs manual (Roche), for description see Table 15.

Table 15: *Composition of SDS-PAGE gels, volumes used are sufficient for 2 gels*

Components	Stacking 4% gel	Resolving 18% gel
40% Acrylamide/Bis (Amresco)	0.25 ml	4.5 ml
0.5 M Tris-HCl, pH 6.8	0.63 ml	_____
1.5 M Tris-HCl, pH 8.8	_____	2.5 ml
10% SDS	25 µl	0.1 ml
Distilled deionized water	1.59 ml	2.85 ml
TEMED (Sigma)	2.5 µl	5 µl
10% Ammonium persulphate (APS)	12.5 µl	50 µl

The collected cells from the pilot expression were resuspended in 150 µl of TE buffer. Fourteen µl of these solutions (per one well) were mixed with 1 µl of reducing agent and 5 µl of loading buffer (for composition see Table 16).

Table 16: *Composition of solutions for control of protein expression*

Solutions	Composition (Supplier)
TE buffer, pH 8.0	10 mM Tris-Cl, pH 8.0 (Sigma)
20x Reducing agent	2 M DDT (MBI Fermentas)
4x DualColor™ Protein Loading Buffer	(MBI Fermentas)

Samples were boiled for 10 minutes to denature, briefly centrifuged and loaded on the gel. The electrophoresis was run at 130 V for an hour and a half in 1x SDS-PAGE running buffer. For composition of the chemicals used see Table 17.

As a marker 5 µl of Spectra™ Multicolor Low Range Protein Ladder provided by Thermo Scientific were used.

Table 17: *Composition of chemicals used for SDS-PAGE*

Chemicals	Composition
SDS-PAGE running buffer (1x)	0.250 M Tris; 1.92M glycin; 1% SDS
PageBlue^{TM} Protein Staining Solution (Fermentas)	0.05 % Coomasie Brilliant Blue R-250; 50% methanol; 10% acetic acid

After electrophoresis was completed, both gels were washed twice with deionized water for 10 minutes and one of them was left overnight in Coomasie brilliant blue R250. After staining step the gel was washed three times for 10 minutes in deionized water.

The second gel was used for Western blot analysis.

II. Western blot analysis

Transfer of proteins from the gel to membrane:

The proteins separated on polyacrylamide gel were transferred to methanol activated polyvinylidene fluorid (PVDF) membrane with 0.2 μm pores (Millipore). This membrane is appropriate for transfer of proteins smaller than 20 kDa. The transfer was conducted using the Genie electrophoretic Blotter (IDEA Scientific Co, USA) according to manufacturer's recommendations.

The membrane was kept overnight blocking solution (in 2% milk in TBS-Tween). Composition of buffers is described in Table 18.

Table 18: *Composition of buffers for Western blot analysis*

Buffer	Composition
Blotting buffer	40 ml 99% methanol; 25mM Tris-Base; 150mM glycin, 0.1% SDS, H_2O up to 1l
TBS	10 mM Tris/HCl (Sigma), 150 mM NaCl
TBS- Tween (TBST)	20 mM Tris/HCl, 500mM NaCl, 0.05% Tween 20 (Loba Feinchemie)

Detection with Ni-NTA HRP Conjugates:

After proteins transfer, the membrane was washed three times for 10 minutes with TBS-buffer and incubated for 1 hour in 3% BSA in TBS at room temperature. The following washing step was repeated 3 times for 10 minutes with TBS-Tween.

The membrane was then incubated for 1 hour at RT in TBS-Tween buffer containing Ni-NTA Conjugate stock (dilution 1:1000) and again washed 3 times in TBS-Tween.

The signal was revealed with 24 ml of 1x Tris- saline, 6 ml of methanol, 18 ng of 4- chloro-1-naphtol and 60 µl 30% H_2O_2.

The expression profile was found out according to increasing intensity in the expected size range for the recombinant protein. The un-induced culture was used as a negative control. The pilot experiment was provided with the aim of determination of the optimal time for the induction of the recombinant protein.

(e) Purification of recombinant protein under native conditions

Purification of recombinant proteins was based on the interaction of histidine residues (His-Tag) and immobilized metal ions such as Zn^{2+}, Cu^{2+}, Co^{2+} or Ni^{2+} with a chelating part.

Five hundred ml of fresh LB/ampicillin complemented with 1% glucose (Lachema, CR) were inoculated with 10 ml of overnight culture and let to grow for two hours at 37˚C with shaking. Afterwards, the solution was centrifuged at 2500 rpm for 20 minutes and supernatant containing glucose was discarded. The pellet was resuspended in the fresh LB/ampicillin media and left for 1 hour at 37 ˚C. Then the induction with IPTG was performed.

After five hours of induction the cells were collected by centrifugation and frozen at - 20˚C until use.

I. Recombinant protein purification using Ni^{2+} ions

Ni-NTA resin was used for purification of recombinant protein (Qiagen). NTA occupies 4 of 6 ligand binding sites thus it prevents binding of impurities or metal-ion contaminations, it leaves two free sites for binding of 6x His-Tag (Figure 7).

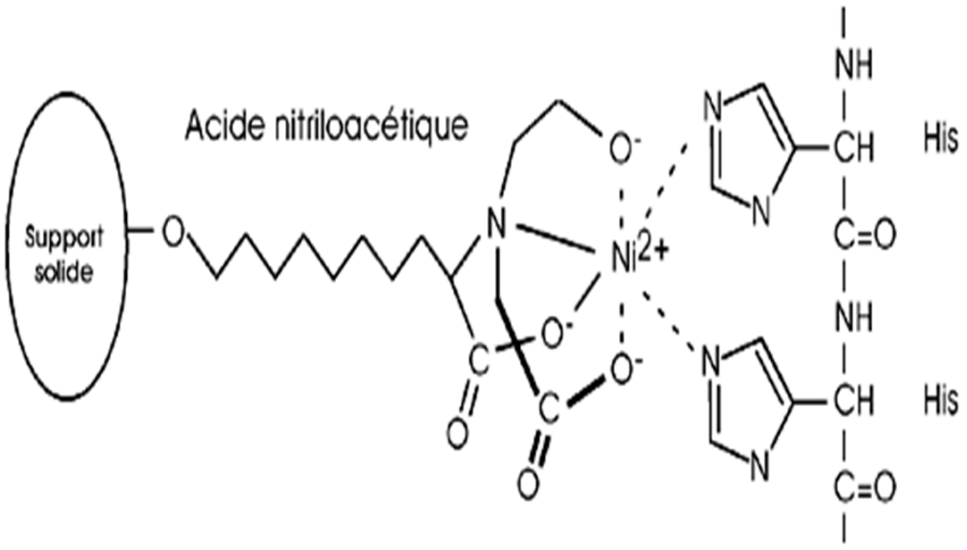


***Figure 7**: Interaction between two 6x His-Tag and Ni-NTA- matrix (source: http://pages.usherbrooke.ca/bcm-514-bl/5e1.html*

(i) Preparation of cleared E. coli lysates

The procedure was performed following the instructions by "The QIA*expressionist*™" protocol (Qiagen, USA).

Five hundred mg of the obtained cell pellet was thawed on ice for 15 minutes. The cells were resuspended in 3 ml of lysis buffer (Table 19).

Twenty five µl of Protease inhibitor cocktail (Sigma) was added to block out all enzymes which could degrade the protein. Lysozyme was added to final concentration of 1 mg/ml and the solution was incubated on ice for 30 minutes. Followed sonication was conducted on ice as: 10 s bursts with a 20 s cooling period between each burst. The lysate was centrifuged at 10000 x g for 20 min at 4˚C. Supernatant was transferred into clean tube and 40 µl were removed and stored at -20˚C.

***Table 19:** Composition of lysis buffer*

Buffer	Composition
Lysis buffer, pH 8.0 (adjusted using NaOH)	50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole

(ii) Preparation of Ni-NTA column and batch purification

Two ml of Ni-NTA resin were applied to column (Qiagen) according to "The QIAexpressionist™" manual. Settled resins were washed 5 times with distilled water. The column was equilibrated with lysis buffer.

Cleared cell lysate was applied to the column, capped and left at 4˚C for overnight on rotary platform with gentle shaking. The cap was removed and 50 µl of flow-through were collected and stored at -20˚C for further analysis. The resin was washed 5 times by 5 ml of washing buffer (Table 20). First three washing fractions (50 µl each) were collected and stored for further analysis.

Table 20: *Composition of washing buffer*

Buffer	Composition
Washing buffer, pH 8.0 (adjusted using NaOH)	50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole

Subsequently, the protein was eluted 5 times with 0.5 ml of elution buffer and all fractions were collected in five tubes for further SDS-PAGE analysis (Table 21).

Table 21: *Composition of elution buffer*

Buffer	Composition
Elution buffer, pH 8.0 (adjusted using NaOH)	50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole

(iii) Protein analysis by SDS-PAGE electrophoresis

SDS-PAGE was performed under the same conditions as described in section 4.1.6.

Recombinant protein purification under native conditions was performed with lowered concentration of imidazole both in lysis buffer (from 10 mM to 1 mM) and in washing buffer (from

20 mM to 10 mM) due to the presence of recombinant protein in fractions of flow-through and in washing fractions in the previous experiment ("The QIAexpressionist™", Qiagen, USA).

II. Recombinant protein purification using Co^{2+} ions

To optimize the procedure of the recombinant protein purification, different slurry (with different chelating metal ions) was checked.

(i) Preparation of cleared E. coli lysates

After overnight incubation at 37^{o}C the 10 ml of culture was transferred into 500 ml of fresh LB/ampicillin media, supplemented with 1 % glucose (Lachema, CR) and left to grow at the same temperature for additional 2 hours. Cell culture was than centrifuged at 2500 rpm for 20 minutes and supernatant containing glucose was discarded. The pellet was resuspended in the fresh LB/ampicillin media and left for 1 hour shaking at 37 ˚C. Induction with IPTG was performed.

After 5 hours the aliquots were centrifuged at max speed and supernatant was discarted. All the pellets were frozen at - 20^{o}C until use.

Whole amount of the obtained cells (2,459 g) was used for purification of recombinant protein.

(ii) Sample preparation- lysate preparation

Collected cells were resuspended in Buffer 1 (4ml/100ml of original culture)(Table 22). The suspension was sonicated 4x for 20 sec on ice and then spun down for 10 min at 4˚C at 13 000 rpm. Pellet was resuspended in Buffer 2 (3ml/100ml of original culture), again sonicated 4x 20 sec on ice, and centrifuged for 10 min at 4˚C at 13 000 rpm. This step was repeated untill the supernatant was colorless. The pellet was resuspended in Buffer 3 (5ml/100 ml of original culture) with permanent stiring at room temperature untill complete pellet dissolving. After centrifugation for 15 min at 4˚C at 13 000 rpm, the pellet was discarded and supernatant was filtrated through 0.22 μm filter.

Table 22: *Composition of buffers sample preparation in Co^{2+} protein purification procedure*

Buffer	Composition
Buffer 1	20 mM Tris-HCl, pH 8.0
Buffer 2	2M Urea, 20 mM Tris-HCl, 0.5M NaCl, 10mM imidazole, 1mM 2-mercaptoethanol, 2% Triton X-100, pH 8.0
Buffer 3	6M guanidine hydrochlorid, 20 mM Tris-HCl, 0.5M NaCl, 5 mM imidazole, 1mM 2-mercaptoethanol, pH 8.0

The clarified final solution was dialyzed against dH_2O to get rid of guanidine hydrochloride using cellulosic membrane (MEMBRA-CEL® dialysis tubing, SERVA; with cut off of 7 kDa).

(iii) Preparation of Co^{2+} column and batch purification

Five milliliters of the slurry (chelating colone, HiTrap™ 5 ml IMAC FF column, GE Healthcare) were loaded into the column, washed 2 times with H_2O, 3 times with 0.1 M EDTA and again with H_2O.

Subsequently, the column was washed with 10 ml of 0.1 M cobalt solution, rinsed with distilled water and equilibrated with 10 ml of buffer A (8 M urea, 0.5 M NaCl, 50 mM Tris, pH 8.5). The sample solution of 25 ml was applied to the column. The flow- through was collected and stored at 4˚C for further use.

Ten milliliters of buffer A was used as washing buffer and two washing fractions (5 ml each) were collected and stored at 4˚C. The protein was eluted using linear gradient (10 ml) of 0.5 M buffer B (8 M urea, 0.5 M imidazol, 0.5 M NaCl, 50 mM Tris/HCl, pH 8.5). Five 2 ml fractions were collected and analysed by SDS- PAGE electrophoresis as described before (section 4.1.6).

(iv) Purification of recombinant protein

Fractions (E1, E2) containing the desired recombinant protein were dialyzed against urea overnight, using cellulosic membrane-MEMBRA-CEL® dialysis tubing (SERVA; 7 kDa cut off).

Co^{2+} column was prepared as described above. Protein sample (9 ml) was applied to Co^{2+} column and flow-through was collected and stored at 4˚C. The column was washed twice with buffer A and the protein fractions were eluted by 5 ml of buffer B. Subsequently, 5 ml of buffer C (8 M urea, 10 mM imidazol, 0.5 M NaCl, 50 mM Tris/HCl, pH 8.5) were used. The results were controlled by SDS- PAGE electrophoresis (Section 4.1.6).

4.2. Native protein isolation and testing of antimicrobial potential

To confirm the presence of antimicrobial activity in tick, the tick hemolymph, salivary glands, gut and ovaries were collected.

4.2.1. Isolation of native protein from hemolymph of *I. ricinus*

The isolation of native protein from hemolymph was performed in Prague, at the Institute of Organic Chemistry and Biochemistry v.v.i. Academy of Science of the Czech Republic (IOCB AS CR) under supervision of Dr. Vaclav Cerovsky, PhD.

(a) Hemolymph collection

The hemolymph was collected from 400 partly fed (6 days fed) females of *I. ricinus* (section 4.1). To prevent hemolymph from degradation, it was mixed with acidic acetonitrile-trifluoroacetic acid solution in ratio 2: 1 and stored frozen at -20˚C (Table 23).

Table 23: *Storing solution for hemolymph*

Solution	Composition
Storing solution	50% acetonitrile, 1% trifluoroacetic acid

(b) Ultrafiltration

pH of hemolymph sample mixed with 50% acetonitrile (ACN) and 1% trifluoroacetic acid (TFA) was adjusted to value pH 2.0 by adding 500 µl of 50% ACN and 0.1% TFA.

The hemolymph solution was spinned for 5 minutes at 1360 rpm to remove insoluble

particles. The extraction was repeated with 1.2 ml of 50% ACN and 0.1% TFA. The resulting pellet was resuspended and spinned again for 5 min at 1360 rpm. The supernatants obtained from both centrifugations were transferred into ultrafiltration cell Amicon Ultra soaked for 3 hours in distilled water before use (Milipore; cut off 100 kDa).

Three ml of 5% acetonitrile (ACN) was added to the supernatant. Sample was centrifuged for 30 minutes at 4200 rpm. After centrifugation, the flow- through was transferred into pear- shaped flask and stored at 4˚C. This step was repeated 2 more times using 2 ml of 5% ACN. Sample was uniformly frozen using dry ice and left to lyophilize overnight. Next day, the lyophilized sample was weighted.

(b) High Performance Liquid Chromatography (HPLC)

HPLC was conducted at Agilent Technologies 1200 Series instrument with the parameters summarized in Table 24. Gradient of 5% ACN and of 70% ACN was applied (Table 25). HPLC instrument was washed with 5% CAN before use.

Table 24: *Parameters used during the measurement*

Parameters	Units
Column	L = 250 mm, I.D. = 4,6 mm
Stationary phase	Vydac® 218TP54, C_{18}
Mobile phase	5% ACN / 70% ACN, gradient
Flow	1 ml/ min
Injection volume	50 µl
Temperature	25 ˚C
Pressure	124 bar
Detection wavelength	220, 280 mAU

Table 25: *Gradient used in the experiment*

Time [min]	5% acetonitrile [%]	70% acetonitrile [%]
0	80	20
60	50	50

I. Sample preparation and sample measurement

Lyophilized sample was dissolved in 300 µl of 5% ACN. Injection volume for one measurement was 50 µl. As a blank measurement 5% ACN was used. With increasing absorbance , the 1 ml fractions were collected (section 5.2.1). The measurement was repeated 3 times, i.e. the total volume of 150 µl of the sample was used. Therefore, 3 sets of 24 fractions were obtained. The rest of the sample was stored at 4˚C.

(c) Antimicrobial activity tests- drop diffusion test

Acetonitrile was left to evaporate overnight from all the obtained fractions of one set. All samples were subsequently resuspended in 40 µl of MiliQ water.

I. Antimicrobial test preparation

Testing on Gram- positive bacteria

One hundred µl of suspension of Gram- positive bacteria *Micrococcus luteus*, supplemented with 2 ml of 0.5% LB media for uniform spreading was seeded on the LB/agar plate and left to grow for approximately 6 hours. Two microliters from 24 fractions were applied to the plate and left overnight at 37˚C (Figure 8).

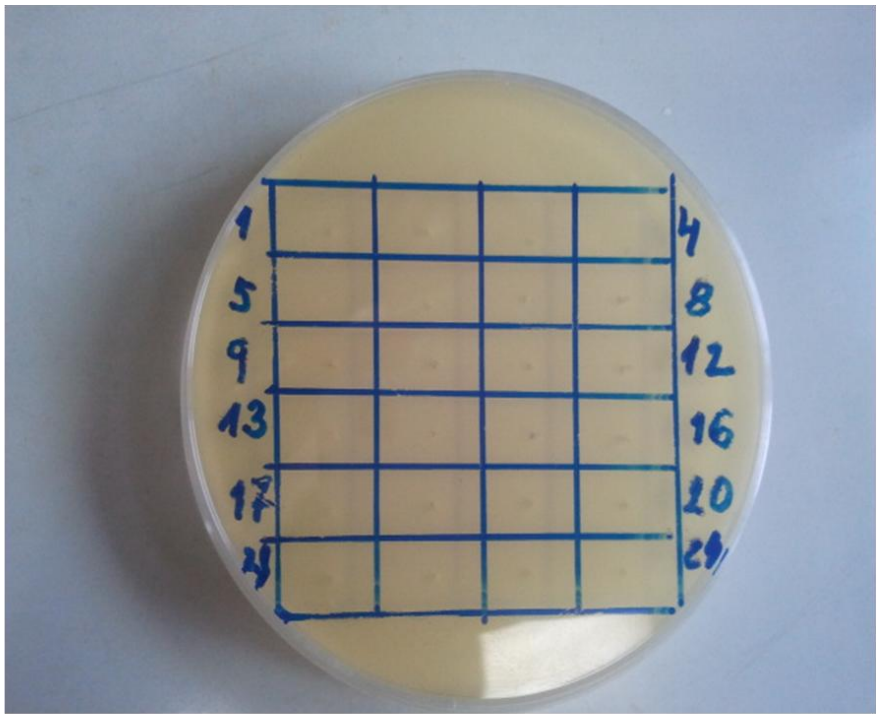


***Figure 8**: Antimicrobial activity testing - drop-diffusion assay*

Testing on Gram- negative bacteria

Some antimicrobial peptides are effective against Gram-negative bacteria. That is why the presence of antimicrobial activity in isolated fractions against Gram- negative *Escherichia coli* was tested with as described above. Remaining after antimicrobial testing samples was used for HPLC.

(d) Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) is a method in which molecules in solution are separated by their molecular weight. This technique is called "gel- filtration chromatography" when the samples are in aqueous solution and analysis is carried under non-denaturing conditions. The degree of retention depends on the size and shape of the solute molecule and the pore size of the packing. For SEC, HPLC Gel Filtration Column Bio-Sil® SEC-125 by Bio-RAD was used (Table 26).

Table 26: *Parameters used in SE chromatography*

Parameters	Units
Column	L= 300 mm, I.D. = 7.8 mm
Stationary phase	Bio-Sil® SEC 125
Mobile phase	0.1 M NH_4Ac, isocratic
Flow	1 ml/min
Injection volume	50 µ
Temperature	25 ˚C
Detection wavelength	220, 280 mAU

I. Sample preparation and measurement

The ACN, in collected RP- HPLC fractions where antimicrobial activity was proved, was let to evaporate overnight. Afterwards the fractions were combined together using 5% CAN. Two samples with final volume of 100 µl were obtained followed by acetonitrile evaporation step. Fifty microliters of 0.1M NH_4Ac (injection volume) were added to both vials. 0.1 M NH_4Ac was used as a blank sample. For identification of approximate retention time of studied peptide, peptide lucifensin was used as a standard. The fractions were collected following the shape of the curve in aim to collected whole peaks. The procedure was repeated twice.

(e) Measuring of antimicrobial activity of SEC fractions

Fractions obtained from SEC were dried overnight, than washed with 50 µl of H_2O to remove NH_4Ac salts completely and dried again. Tests were performed on Gram- positive *Micrococcus luteus* as described above.

4.3. Gene expression in organs of I. ricinus

To determine IRAMP tissue expression pattern in different ticks´ organs, real time PCR (qPCR) was performed. The expression in hemolymph was proved before (data not shown). Therefore salivary glands, gut and ovaries from thirty 6-days fed females were taken

and total RNA was isolated using NucleoSpin® RNA II kit by Machery-Nagel (Germany) strictly following producers instructions. One ng of RNA was used for cDNA synthesis using Transcriptor High Fidelity cDNA Synthesis Kit (Roche).

The qPCR with IRAMP-specific primers was performed. House-keeping gene actin was used as positive control (Table 27- 28).

Table 27: *Primers used in qPCR*

Primer	Sequence	Temp. of annealing [C°]
IRAMP R_{COR}	5´- TTA GCG GCA AGT GTT GTC GCC-3´	50
IRAMP $FORW_1$	5´- ATG CGT GCC GTA GCC ATT TTC ATC-3´	55
Actin F	5´-GATCATGTTCGAGACCTTCA-3´	60
Actin R	5´-CGATACCCGTGGTACGA-3´	60

Table 28: *Amplification conditions (qPCR)*

Steps	Temperature [°C]	Time [min]
1. Initial denaturation	95	5
2. Denaturation	95	0.1
3. Annealing	60	0.1
4. Extension	72	0.1
5. Final extension	72	5

The amplifications were performed in 96- wells plate in LightCycler 480 (Roche; Table 29).

Table 29: *Reagents per one qPCR reaction (25 microliters)*

Composition of reagents/ supplier	Used volume [µl]
Universal SYBR Green Master mix (FastStart Taq DNA Polymerase, Reaction Buffer, Nucleotides (dATP, dCTP, dGTP, dUTP), SYBR Green I, and ROX Reference Dye; Roche	12.5
Forward primer (10 µM); Generi Biotech, CZ	1
Reverse primer (10 µM); Generi Biotech, CZ	1
dH_2O	6.5
cDNA template*	4

* cDNA was prepared from 1 ng/ µl of RNA

4.4. Synthetic peptide testing

Synthetic linear peptide with the sequence identical to native and recombinant protein was prepared by company Bio-synthesis, USA. Its purity, as was declared by company, was higher than 99% and its molecular weight was 5.344 kDa. Initial peptide folding was necessary for analysis of the presence of antimicrobial activity in the sample.

4.4.1. Recognition of retention time

To find out the retention time of linear synthetic peptide, HPLC was performed. Less than one mg of peptide was dissolved in approximately 300 µl of 20% ACN. As mobile phase, gradient of 5% ACN and 70% ACN were used (section 4.2.1., Table 24, 25). The fraction with linear synthetic peptide was collected and left to dry overnight.

4.4.2. Peptide cyclization

Approximately 2 mg of synthetic peptide was dissolved in 7 ml of 0.1 M ammonium acetate (pH 7.8). Immediately after addition of the solvent, 50 µl of the sample was removed and used for HPLC to control the start of the reaction (Section 4.2.1). Solution was mixed at

room temperature and after 1, 2, 4, 6, 8 and 25 hours 50 µl was removed and used for HPLC to see the progress of cyclization. After 25 hours, the cyclization was completed.

4.4.3. HPLC separation of cyclized synhetic peptide

Lyophilized sample was dissolved in 200 µl 5% ACN and 20 µl were used for RP-HPLC to check the purity of cyclization after lyophilization. Several gradients were used (Table 30).

Table 30: *Gradient conditions used for RT- HPLC with synthetic cyclized peptide*

Time [min]	5% acetonitrile [%]	70% acetonitrile [%]
0	100	0
60	0	100

Several fractions were collected as multiple peaks were present after HPLC. After acetonitrile evaporation fractions were used for further analyses.

4.4.4. Time-of-flight mass spectrometry (TOF MS)

Peptide folding resulted in several peaks in HPLC spectra analysis. The mass spectrometry was performed to check for the presence of possible side products. Mass spectrometry was performed at the Institute of Organic Chemistry and Biochemistry v.v.i. Academy of Science of the Czech Republic (IOCB AS CR) using Micromass Q-Tof micro mass spectrometer (Waters). Samples were dissolved in 10 µl of 50% acetonitrile with 0.1% formic acid. Five microliters of samples were used for the first measurements to optimize the measurements conditions. The following experiments were conducted with 1 µl of samples.

4.4.5. Antimicrobial testing

(a) Drop- diffusion assay

I. Sample preparation

The samples used for MS were dried and dissolved in appropriate volume of water to make the concentration of protein in all samples equal.

II. Drop- diffusion assay

Antimicrobial drop- diffusion assay was conducted as described in section 4.2.1. with 2 different dilutions of the samples (10^{-1} and 10^{-2}) using Gram- positive *Micrococcus luteus* and *Staphylococcus aureus* and Gram- negative *Escherichia coli*.

(b) Antimicrobial assay- minimal inhibitory concentrations (MICs)

MICs, quantitative test, established by observing bacterial growth in the presence of synthetic linear IRAMP protein in multiwall plates were performed to confirm the results obtained by drop- diffusion assay. 2.3 mg of synthetic linear peptide was dissolved in 0.365 ml of physiological solution. The solution was diluted to LB media in two binary rows: 200, 100, 50, 25 μM. and 12, 6, 3, 1.5, 0.75, 0.375 μM respectively. One hundred μl of the sample were loaded into wells on microtitration desk and 0.1 ml of bacterial suspension was added (final volume of 200 μl).

Gram- positive *Bacillus subtilis, Micrococcus luteus, Staphylococcus aureus,*and Gram- negative *Escherichia coli, Pseudomonas aeruginosa* and yeast *Candida albicans* were used in the experiment with the final IRAMP concetrations of 0.375 – 50 μM for *B. Subtilis* and *M. luteus,* 3 - 100 μM for *S. aureus* and *C. albicans,* 1.5 - 100 μM for *E. coli* and 25 – 100 μM for *P. aeruginosa.*

The plates were left to incubate for 20 hours at 37 ˚C with constant shaking at Bioscreen C instrument; Oy Growth Curves AB Ltd., Finland. Every 15 minutes the absorbance at 540 nm was measured. Peptide antimicrobial potential was tested in duplicate.

5. Results

5.1. Characterization of antimicrobial peptide from Ixodes ricinus (IRAMP)

Using the primers designed on the sequence of AMP found in *I. scapularis*, ISAMP (104), the partial sequence of new gene similar to it was detected using *I. ricinus* cDNA as a template. The sequences of primers were corrected by RACE PCR and the I. ricinus-specific sequence was used for further work (Figure 9).

ATG	CGT	GCC	GTA	GCC	ATT	TTC	ATC	GTT	ACT	CTA	CTC
M	R	A	V	A	I	F	I	V	T	L	L
GTT	CTG	GAA	AAT	TGC	AAC	TTT	CTC	ATG	TCT	GAG	CCC
V	L	E	N	C	N	F	L	M	S	E	P
CTT	CCT	GGA	CAA	GCC	TGG	CAG	GTC	CCG	TCA	AAG	AGA
L	P	G	Q	A	W	Q	V	P	S	K	R
CCT	AAA	TGC	TAT	AGT	AAA	GAA	TGC	ACG	AAA	AAT	GAA
P	K	C	Y	S	K	E	C	T	K	N	E
GAC	TGC	AAG	TTT	GGG	TCG	TGC	ACA	TAT	TGC	AAT	AAT
D	C	K	F	G	S	C	T	Y	C	N	N
GGC	CTT	TGG	GGC	CTT	TGG	GGC	GAC	AAC	ACT	TGC	CGC
G	L	W	G	D	N	T	C	R	---	---	---
TAA											

***Figure 9**: The sequence of newly found antimicrobial peptide (IRAMP)*

Comparison of *I. scapularis* ISAMP and *I. ricinus* IRAMP gene sequences was performed using available public database (BLAST and FASTA). The BLAST search revealed 80 % of identity between two gene sequences of different tick species (Figure 10). http://fasta.bioch.virginia.edu/fasta_www2/).

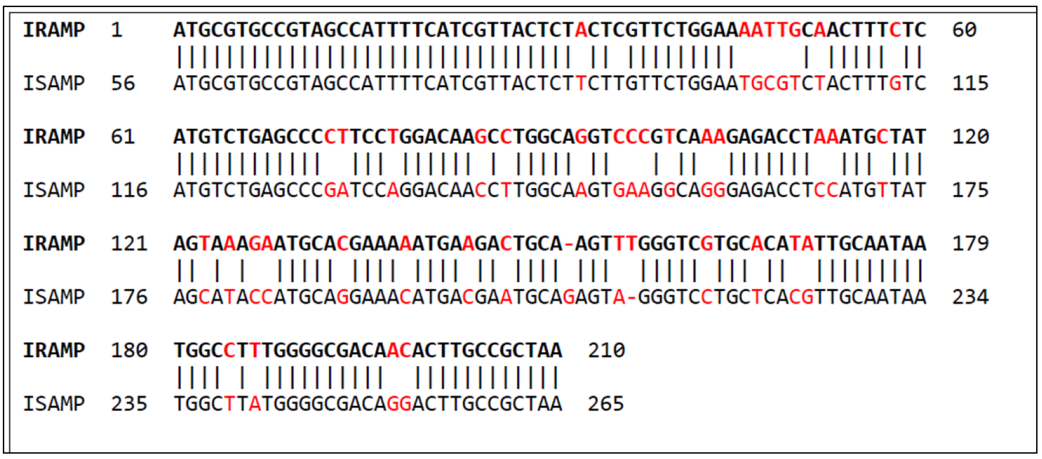


Figure 10: *Comparison of IRAMP and ISAMP sequence (BLAST; http://www.ncbi.nlm.nih.gov/nucleotide/22164301?report=genbank&logS=nuclalign&blast_rank=2&RID=C0ZHD5ZU014) ; identities 161/200 (80 %)*

The FASTA algorithm (BLOSUM50; http://fasta.bioch.virginia.edu/fasta_www2/) revealed 69.6 % of identity and 79.7 % of similarity on protein level (Figure 11).

ISAMP	MRAVAIFIVTLLVLECVY FVMSEPDPGQPWQVKAGRPPCYSIPCRKHDECRVGSCSRCNNGLWGDRTCR
	MRAVAIFIVTLLVLE F+MSEP PGQ WQV+ RP CYS C K+++C+ GSC+ CNNGLWGD TCR
IRAMP	MRAVAIFIVTLLVLENCNFLMSEP LPGQAWQVPSKRPKCYSKECTKNEDCKFGSCTY CNNGLWGDNTCR

Figure 11*: Comparison of ISAMP and IRAMP sequences (BLAST alignement), red letters sign difference in amino acid sequence*

In addition to homology with different secreted salivary proteins and ISAMP from *I. scapularis*, *I. ricinus* IRAMP reveals from 35 to 70 % of identity to secreted salivary protein from *I. pacificus*, *Drosophila melanogaster*, *Drosophila erecta*, *Drosophila yakuba*, *Janibacter sp.*, *Oryza sativa* (Asian rice) and *Hordeum vulgare L.* (barley).

The predicted prepropeptide sequence consists of 69 amino acids (aa) and has a molecular weight of 7.8 kDa. It contains a 22 aa signal peptide characteristic for secreted proteins, as it was determined by signal peptide database (www.cbs.dtu.dk/services/SignlP/).

The mature peptide has 47 aa and its molecular weight is 5.3 kDa. The presence of concerved cysteine motif was detected in protein sequence.

5.2. Recombinant protein production

Signal sequence was cut off with the help of newly designed forward primer (Figure 12, 13).

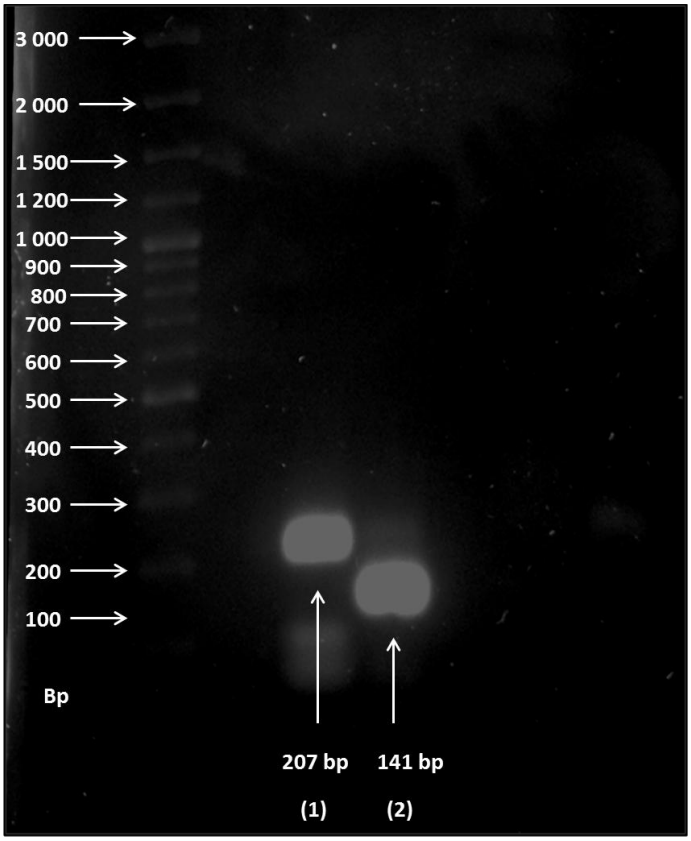


Figure 12: *PCR products: 1- PCR product with signal sequence, 2- PCR product without signal sequence*

ATG	CGT	GCC	GTA	GCC	ATT	TTC	ATC	GTT	ACT	CTA	CTC
M	R	A	V	A	I	F	I	V	T	L	L
GTT	CTG	GAA	AAT	TGC	AAC	TTT	CTC	ATG	TCT	GAG	CCC
V	L	E	N	C	N	F	L	M	S	E	P
CTT	CCT	GGA	CAA	GCC	TGG	CAG	GTC	CCG	TCA	AAG	AGA
L	P	G	Q	A	W	Q	V	P	S	K	R
CCT	AAA	TGC	TAT	AGT	AAA	GAA	TGC	ACG	AAA	AAT	GAA
P	K	C	Y	S	K	E	C	T	K	N	E
GAC	TGC	AAG	TTT	GGG	TCG	TGC	ACA	TAT	TGC	AAT	AAT
D	C	K	F	G	S	C	T	Y	C	N	N
GGC	CTT	TGG	GGC	CTT	TGG	GGC	GAC	AAC	ACT	TGC	CGC
G	L	W	G	D	N	T	C	R	---	---	---
TAA											

Figure 13: *The IRAMP sequence with and without signal peptide*

PCR product was cloned into Champion pET100 Directional TOPO vector and the expression of the fusion protein was performed. The N-terminal fusion tag increased the size of the target protein up to 8.3 kDa.

The pilot expression experiment showed that the optimal time for the induction of the recombinant IRAMP is 5 hours. The induction was initiated by 1mM IPTG. The results of the pilot experiment, analyzed by SDS- PAGE as well as Western blotting, are shown in Figure 14.

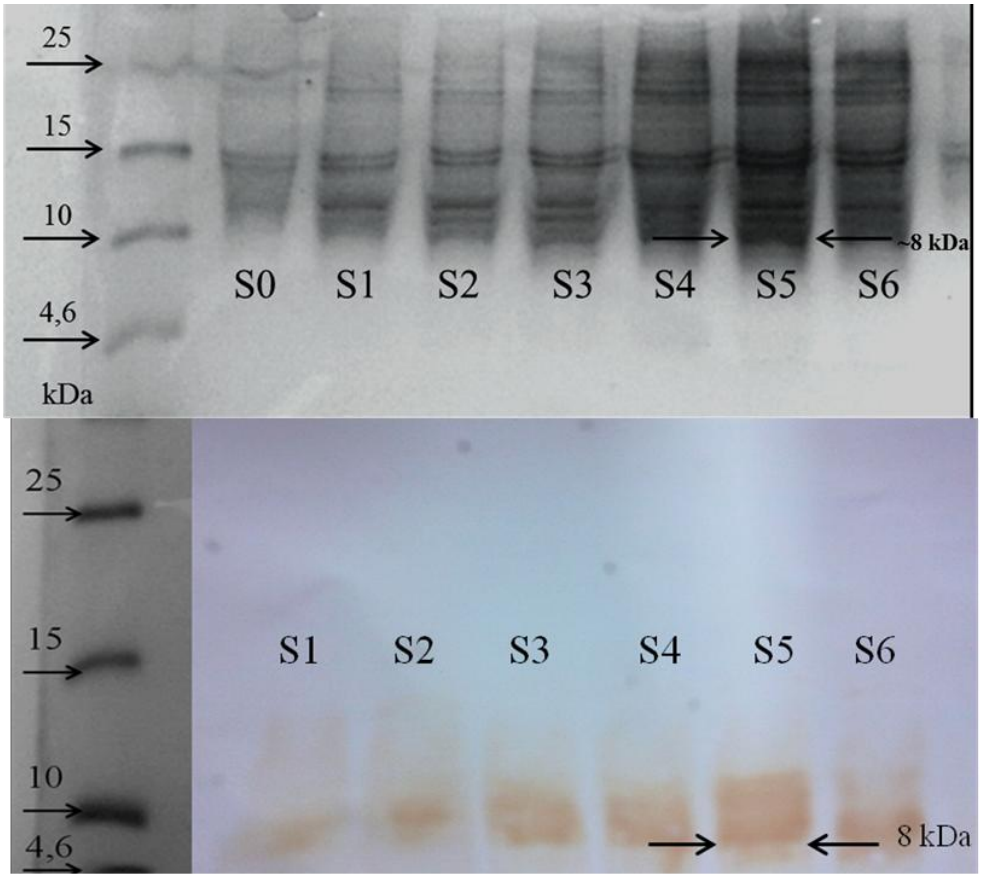


Figure 14: *Pilot expression: Development of expression in time; before induction: S0; after induction: S1- S6 (nb. of sample corresponds to hours after induction), arrows indicate the protein of our interest.* **(A)- SDS-PAGE; (B)- Western blotting**

The first experiment of fusion protein purification revealed the presence of IRAMP in flow through and washing fractions (Figure 15). No bands were visible in the elution fractions. This could be possibly due to the hidden His-tag in the structure which prevents the protein to bind to resins. To overcome this problem, Co^{2+} resin was used and recombinant protein of predicted size was obtained in two elution fractions, E1, E2 (Figure 16). However, except the target purified protein some nonspecific proteins were present. Additional attempts to increase the purity of recombinant IRAMP failed.

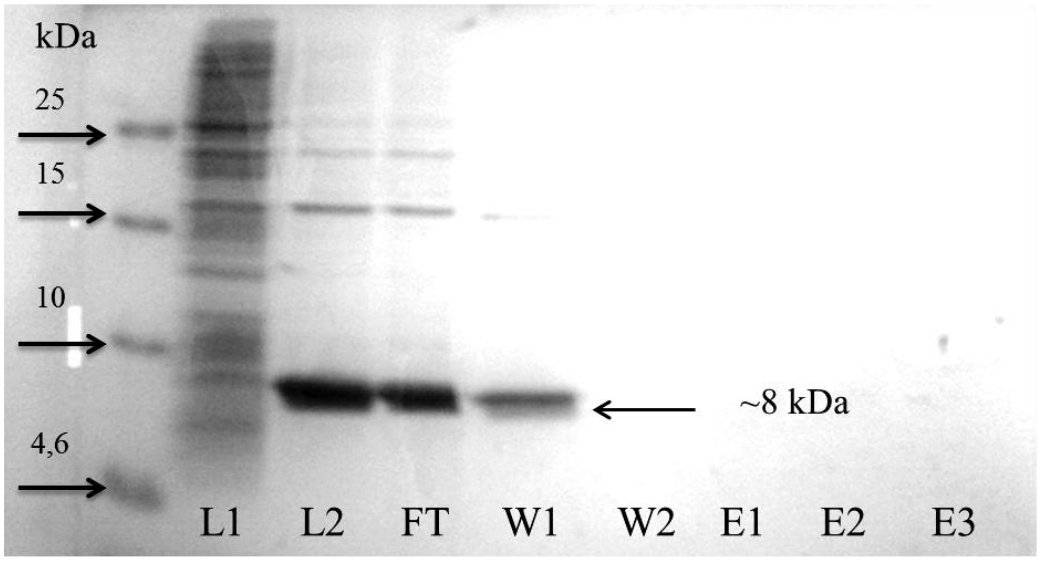


***Figure 15**: Recombinant protein purification with Ni2+ resins; with arrow is indicated desired recombinant protein of size 8 kDa*

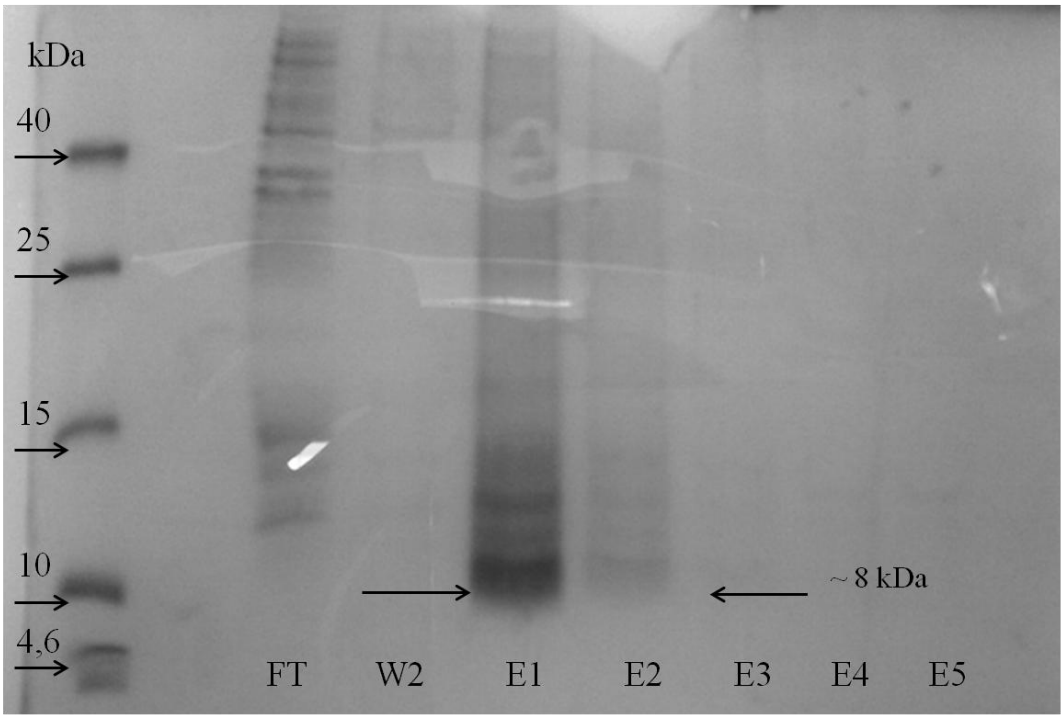


***Figure 16:** Recombinant protein purification with Co^{2+} resins, desired recombinant protein of corresponding size is indicated by arrows*

In order to receive the pure recombinant IRAMP, the composition of elution buffer for purification under native conditions was changed. Additional purification under denatured conditions was performed. Applied improvement steps, either it was the change of the elution buffer or IRAMP purification under denatured conditions (data not shown), did not result in obtaining of the preparative amount of the recombinant protein. Taking into consideration the difficulties related to recombinant protein production, that were, most probably, caused by the small protein size, inappropriate protein folding, and the presence of high amount of nonspecific histidine-rich bacterial proteins in *E.coli* expression system, the native protein from *I. ricinus* tick (Section 5.2) as well as synthetic linear peptide of IRAMP were prepared (Section 5.3.) with purpose to analyze the properties and possible antimicrobial activity of *I. ricinus* IRAMP.

5.2. Native protein isolation

Hemolymph from 400 ticks of *I. ricinus* was collected and further analyses were performed at the Institute of Biochemistry and Organic Chemistry (IBOC) in Prague.

5.2.1. High performance liquid chromatogramy (HPLC)

HPLC was performed from lyophilized sample of hemolymph and all appeared peaks were collected as separate fractions to vials (Table 31).

***Table 31**: HPLC- collected fractions, fractions with antimicrobial activity are highlighted*

Fraction	Flow through [ml]	Fraction	Flow through [ml]
0	2.9- 4.2	12	23.5- 24.0
1	14.9- 15.3	13	24.0- 24.5
2	16.1- 16.6	14	24.5- 25.0
3	17.3- 18.0	15	25.0- 26.0
4	18.0- 19.0	16	26.0- 27.0
5	19.0- 20.0	17	27.0- 28.0
6	**20.0- 21.0**	18	28.0- 29.0
7	**21.0- 21.5**	19	29.0- 30.0
8	21.5- 22.0	20	30.0- 31.0
9	22.0- 22.5	21	31.0- 32.0
10	22.5- 23.0	22	32.0- 33.0
11	23.0- 23.5	23	51.2- 52.2

5.2.2. Drop- diffusion assay

Drop- diffusion assay that demonstrates suppression of bacterial growth in the area of applied solution was performed using dry HPLC fractions diluted in 40 µl of water.

Antimicrobial effect was slightly detected in fractions 6 and 7 (Figure 17). It corresponds to retention time of 20- 21.5 min. Unfortunately, it was not clearly visible on the picture (Figure 18). The fractions with antimicrobial activity are highlighted in Table 31 in red.

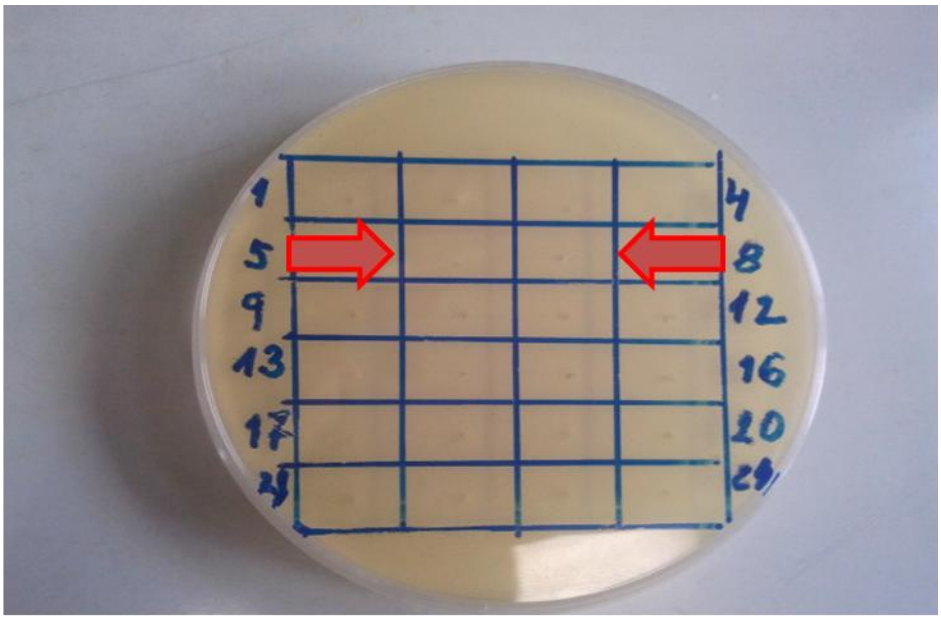


Figure 17: *Drop- diffusion assay with M. luteus, dilution 40 µl*

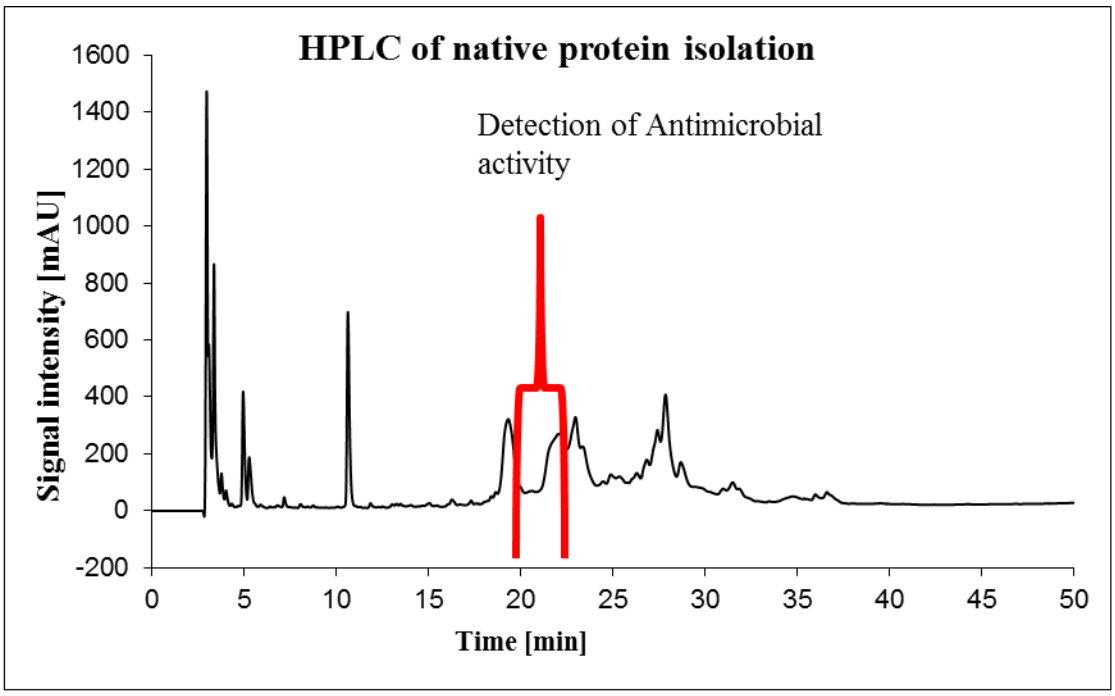


Figure 18: *HPLC chromatogram of native protein isolation*

As antimicrobial activity was not very strong, the HPLCs with the rest of the sample were performed and 1 ml fractions were collected just in the area of proved antimicrobial activity (11 fractions).

Drop- diffusion assay with *M. luteus* was repeated with the increased concentration of IRAMP and resulted in higher effect on bacteria in antimicrobial assay in fraction 3 (Figure 19). The fraction (volume of 21. 0- 22. 0) corresponds to the fractions (6 and 7) with antimicrobial activity detected in previous assay.

Antimicrobial potential against Gram- negative bacteria E. *coli* revealed no suppression of bacteria growth (Figure 20). As it was detected in above discussed tests native IRAMP protein from *I. ricinus* revealed noticeable, but not strong antimicrobial activity against Gram- positive bacteria. No suppression of Gram- negative bacteria growth was detected with the use of mentioned concentration of native tick IRAMP.

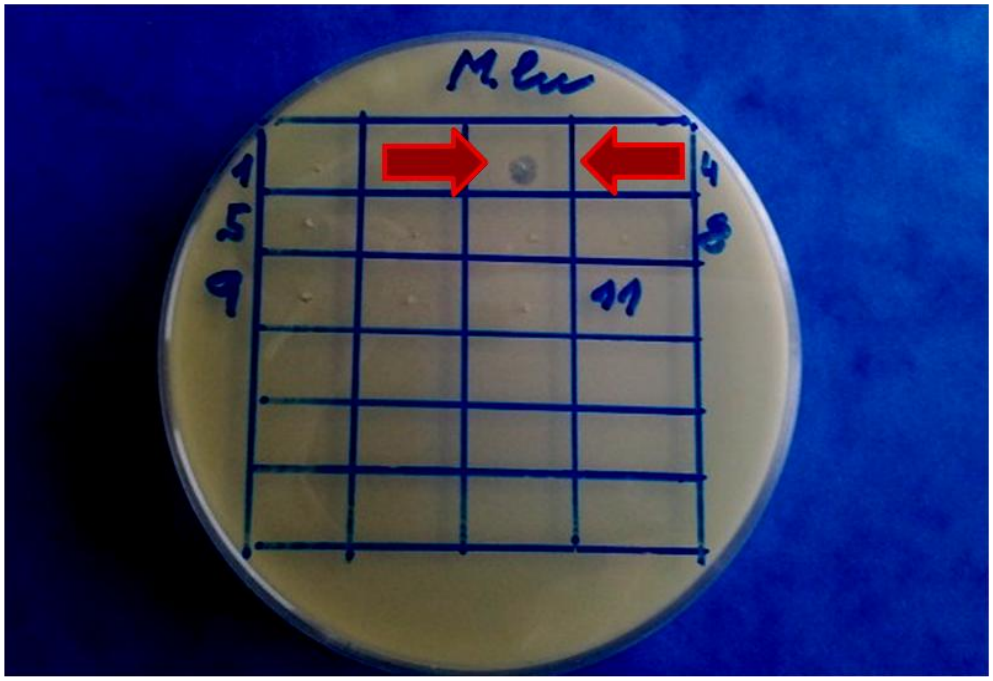


***Figure 19**: Drop- diffusion assay with M. luteus*

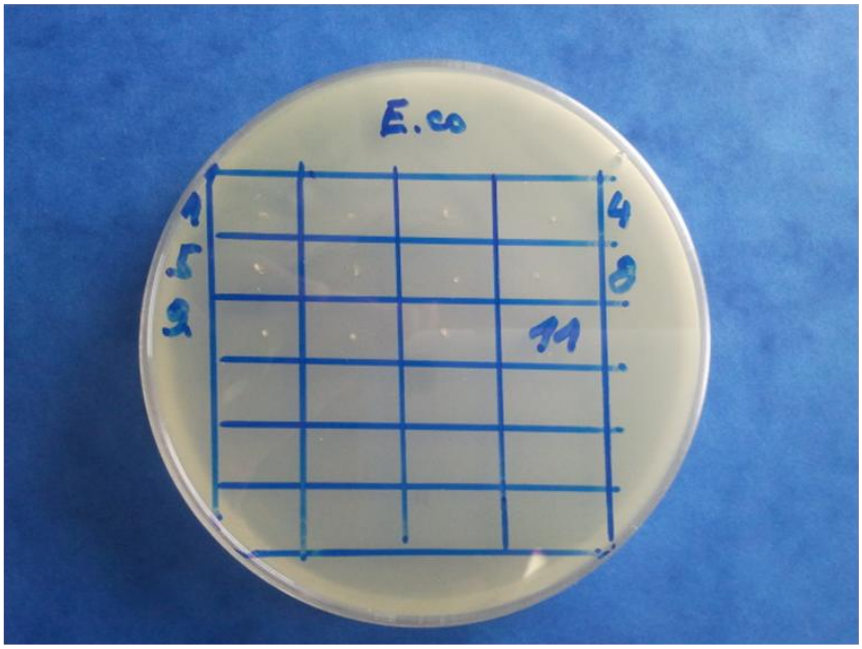


***Figure 20**: Drop- diffusion assay with E. coli*

5.2.3. Size exclusion chromatography (SEC) and antimicrobial assay

After further separation of the compounds in antimicrobial active fractions using SEC the antimicrobial activity was not detected (Figures 21 - 22).

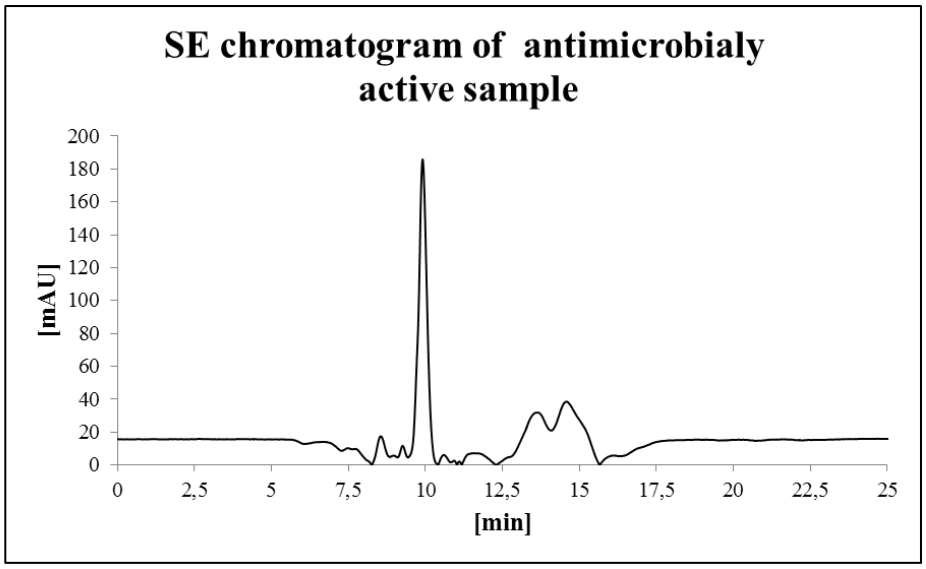


***Figure 21**: Size exclusion chromatogram of sample where was detected antimicrobial activity*

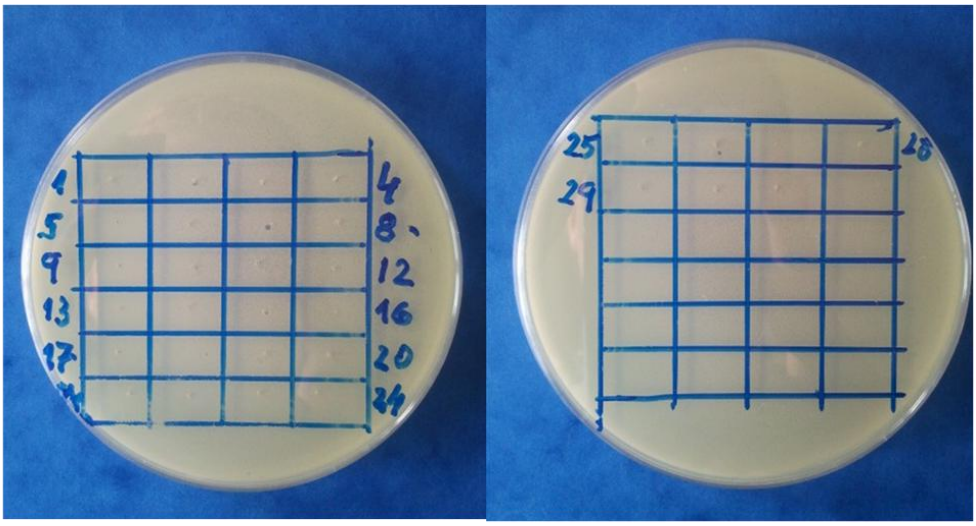


Figure 22: *Drop- diffusion assay on M. luteus after sample separation by SEC*

5.3. Differential expression of detected gene in tick tissues

The Real time PCR performed on the different tick tissues (salivary glands, midgut, ovaria) revealed the expression of IRAMP protein in all of them in different levels. However, the highest expression was observed in salivary glands following by the expression in midgut and ovaria (see Figure 23 for the results). Expression in hemolymph was performed and proved at the beginning of this work (data shown).

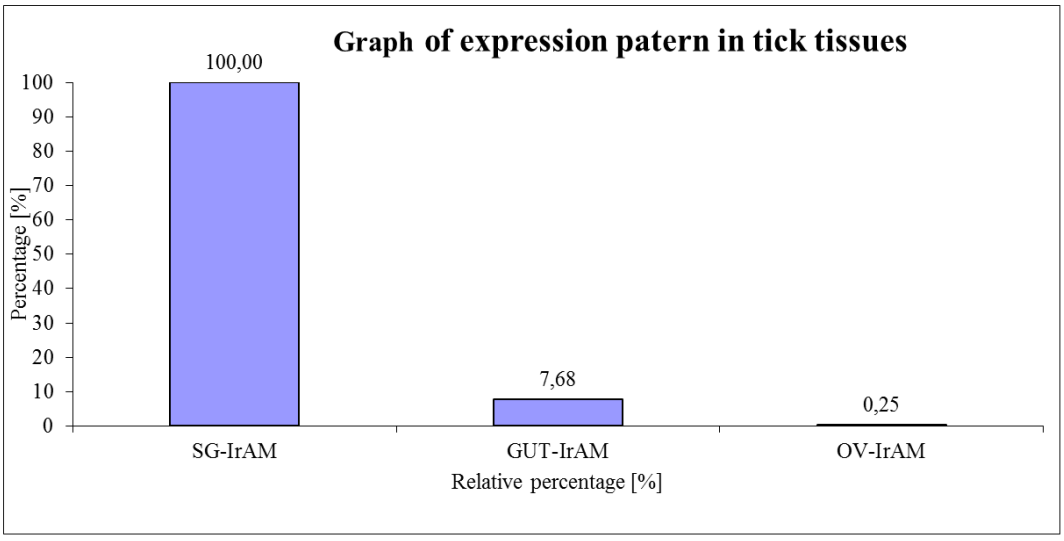


Figure 23: *Tissue expression pattern of IRAMP*

5.4. Synthetic peptide manufacturing

5.4.1. Synthetic peptide cyclization

RP- HPLC with linear synthetic peptide revealed the retention time of 23 minutes, as it was expected based on the previous experience of work with natural antimicrobial peptides from tick hemolymph (Figure 24).

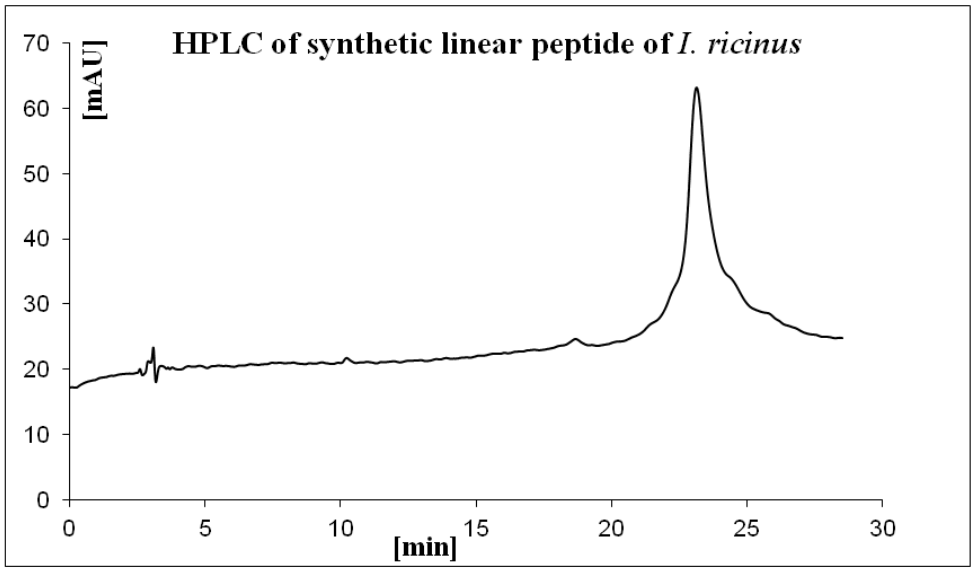


***Figure 24**: Retention time of unfolded linear synthetic peptide*

Cyclization of linear synthetic peptide was performed. For development of cyclization see Figure 25. In Figure 26, cyclized peptide is presented.

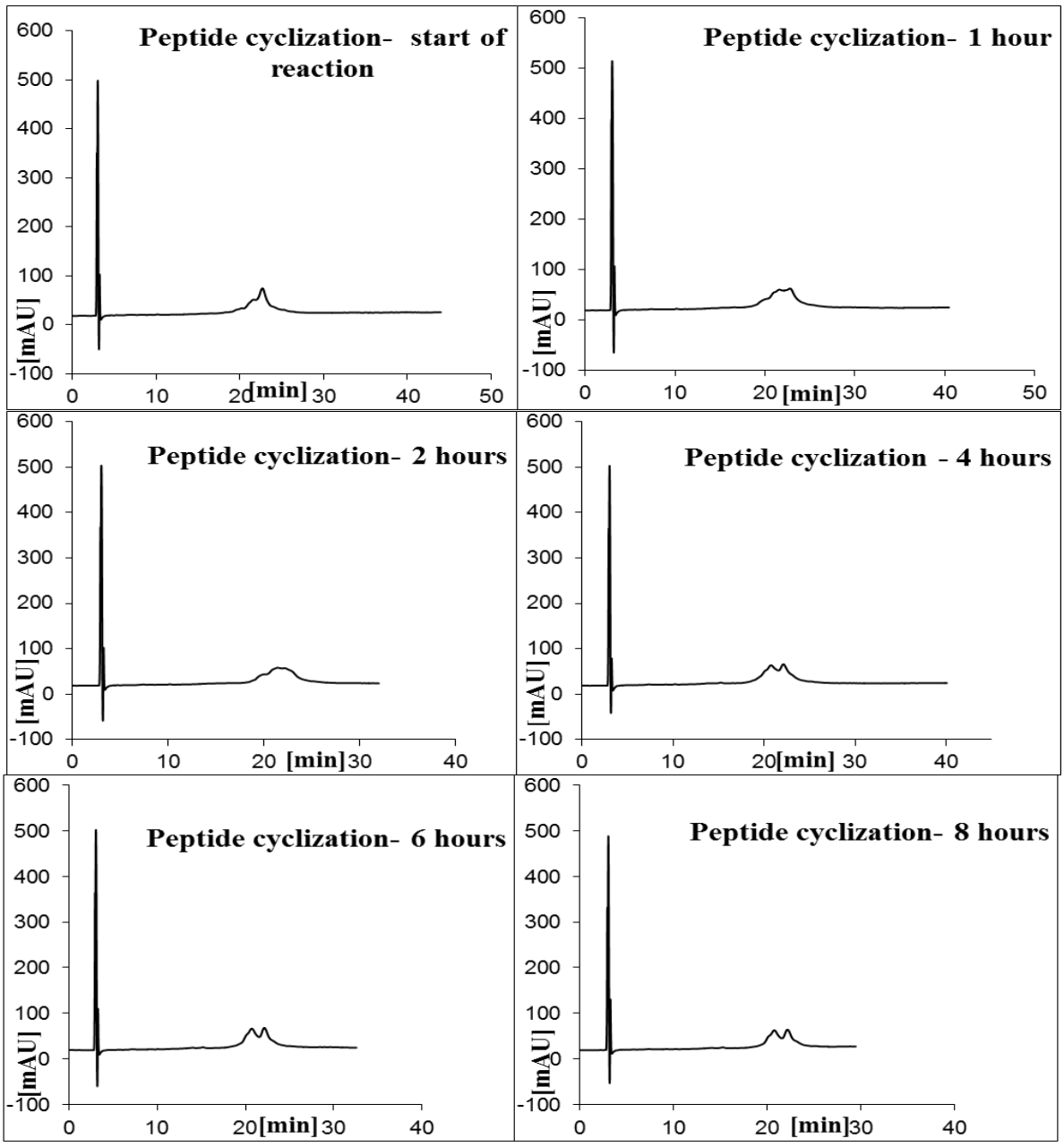


Figure 25: *Development of cyclization in time; first peak in the chromatogram corresponds to the solvent, ammonium acetate*

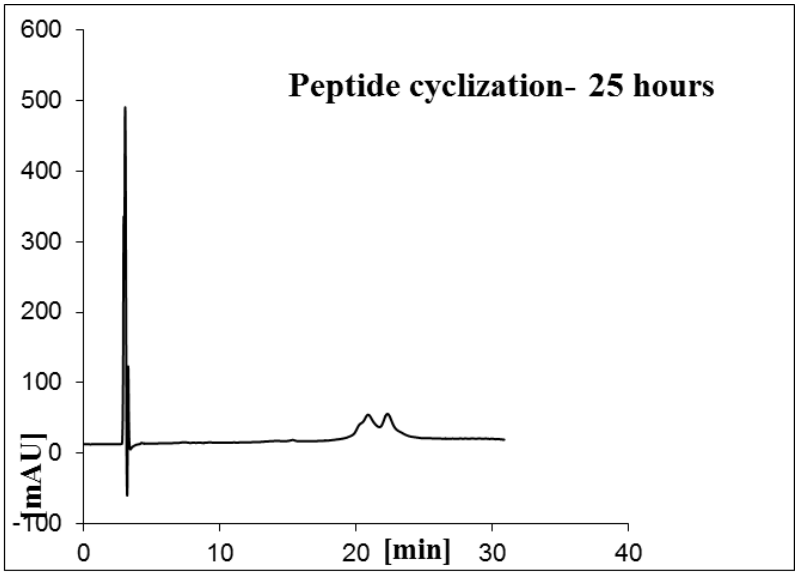


Figure 26: *HPLC chromatogram of the end of cyclization (after 25 hours)*

After cyclization the synthetic peptide was separated into fractions using HPLC analysis. Fractions were divided according the peaks showed below (Figure 27).

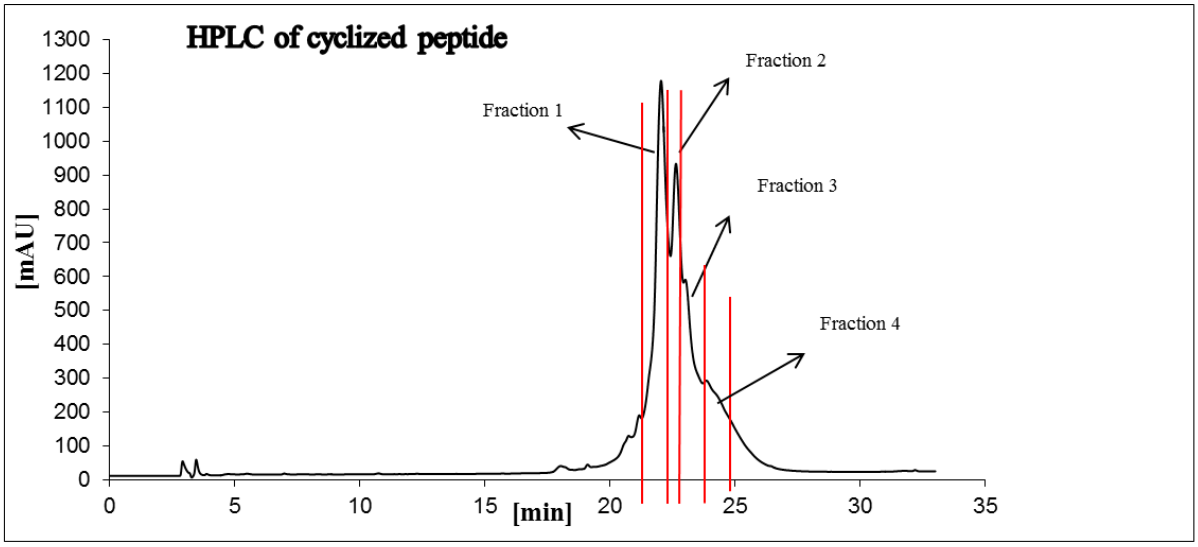


Figure 27: *HPLC chromatogram of cyclized synthetic IRAMP and separation into 4 fractions*

Further analyses by TOF Mass Spectrometry technique revealed the same composition of compounds present in each fraction. Therefore only one mass spectrum is added in Figure 28.

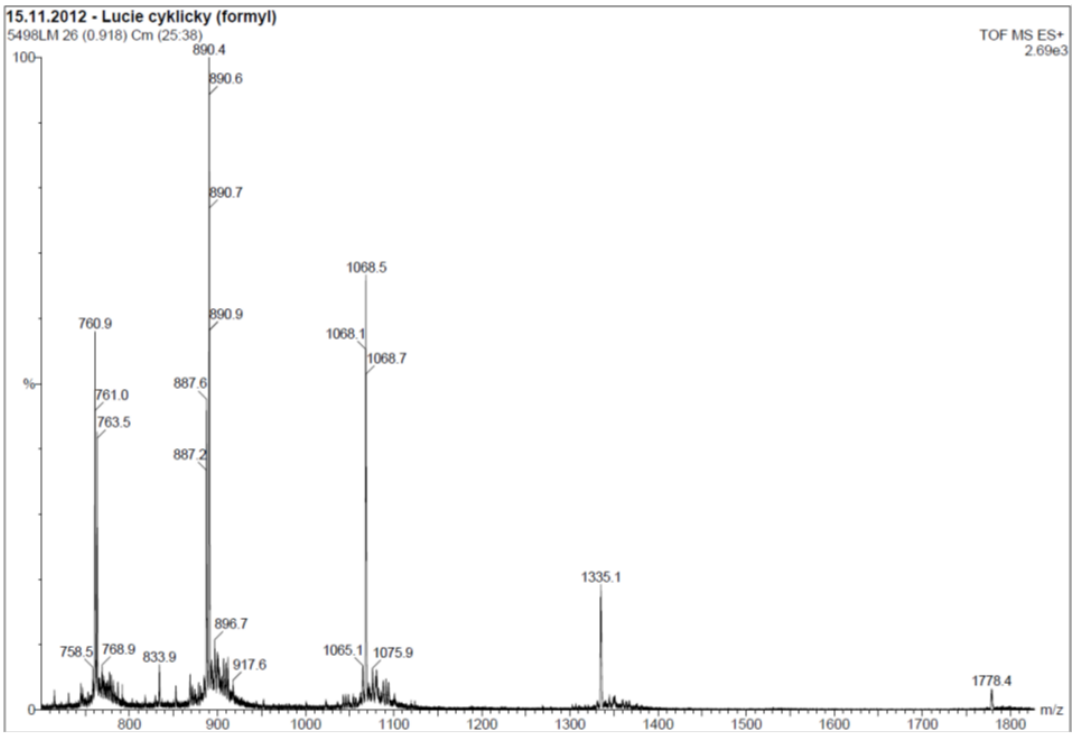


***Figure 28**: MS spectra of cyclized synthetic peptide- first peak has a molecular weight of 4995 Da, second one of 5316 and third and fourth one of 5334 Da*

The MS analysis of the three compounds of different molecular weight revealed the following: the first compound with the MW of 4995 Da corresponds to IRAMP peptide without first two amino acids, glutamic acid and proline, which could be lost during synthetic peptide manufacturing. The second compound with MW of 5316 Da also corresponds to IRAMP peptide. However, some of the amino acids were dehydrated and it resulted in a compound 16 Da smaller than the desired peptide. Last two peaks with MW of 5334 Da represent cyclized synthetic IRAMP peptide. The obtained peptides were used in antimicrobial testing.

Antimicrobial testing of cyclized synthetic peptides was performed with Gram-positive *M. luteus.* Moreover, dilution factors of 10^{-1} and 10^{-2} were used. Results are summarized in Figure 29.

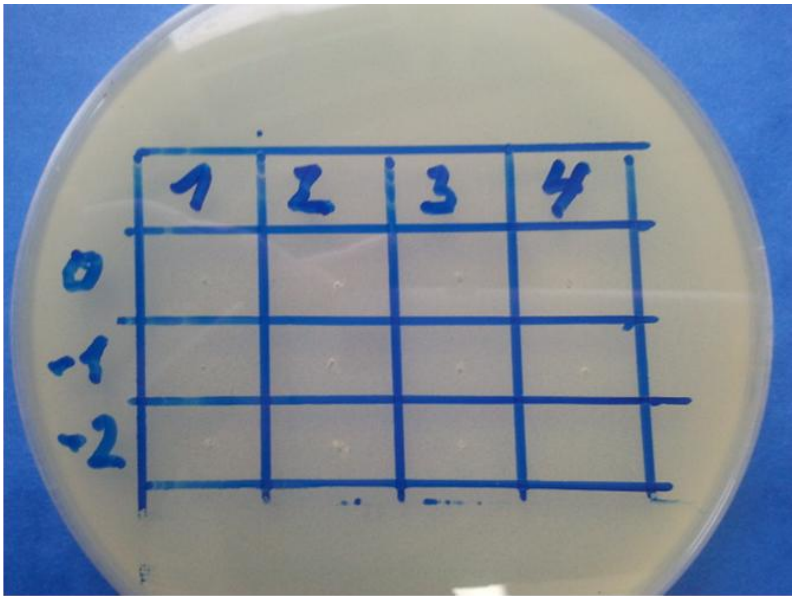


Figure 29: *Drop- diffusion assay on M. luteus with HPLC fractions (1-4) of cyclized synthetic peptide, dilution of 20 µl, with no dilution (signed as 0), dilution factors of 10^{-1} (row -1) and 10^{-2} (row -2)*

To increase the test specificity and accuracy in order to be sure that formic acid did not influence the affectivity of the peptide, the samples which were not used for MS were included in the experiment. To eliminate the possibility of incorrect cyclization conditions with effect on antimicrobial activity, one milligram of linear synthetic peptide was dissolved in physiological solution and let to fold oxidatively (107).

Testing was performed on *M. luteus* as well as on *S. aureus* and *E. coli* (Figure 30). However, none of the tested fractions was activity against tested bacteria.

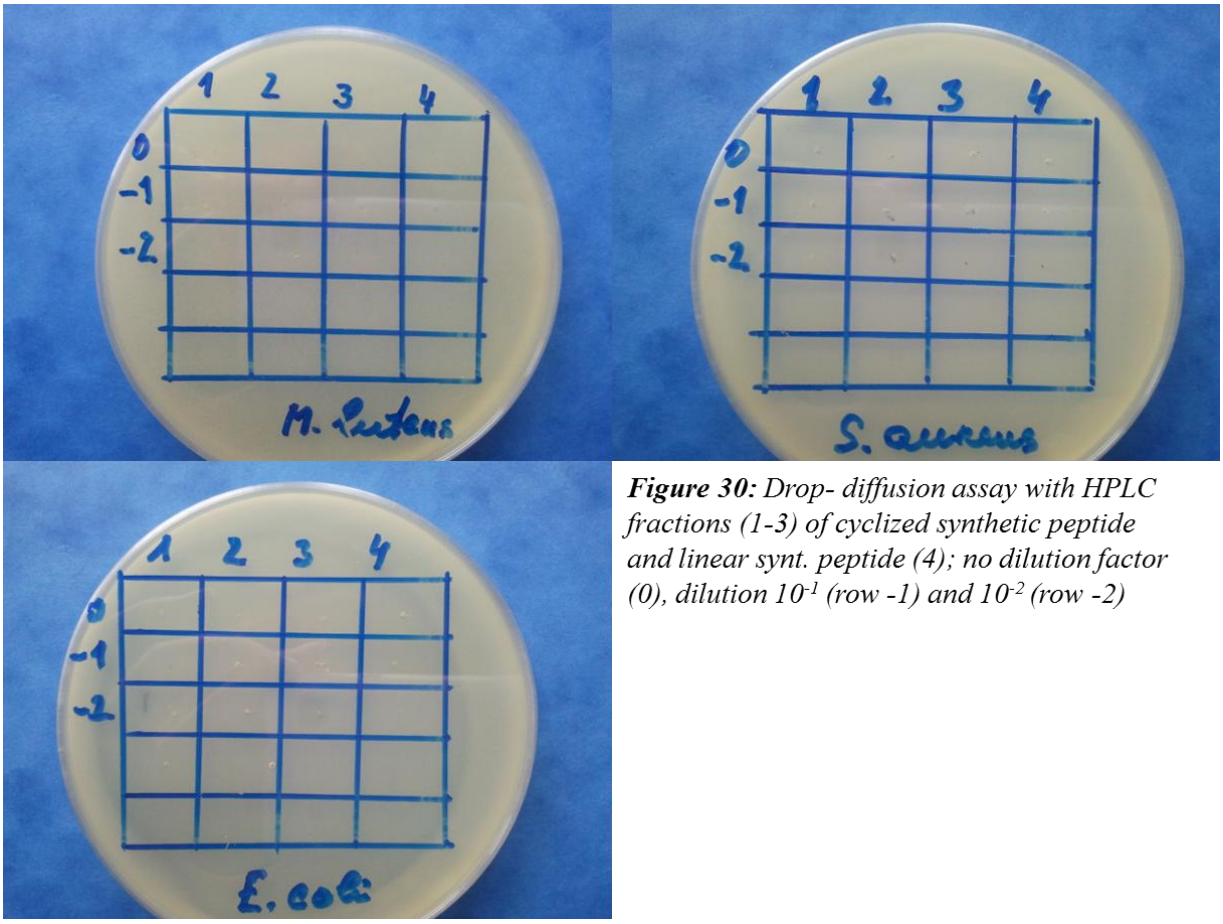
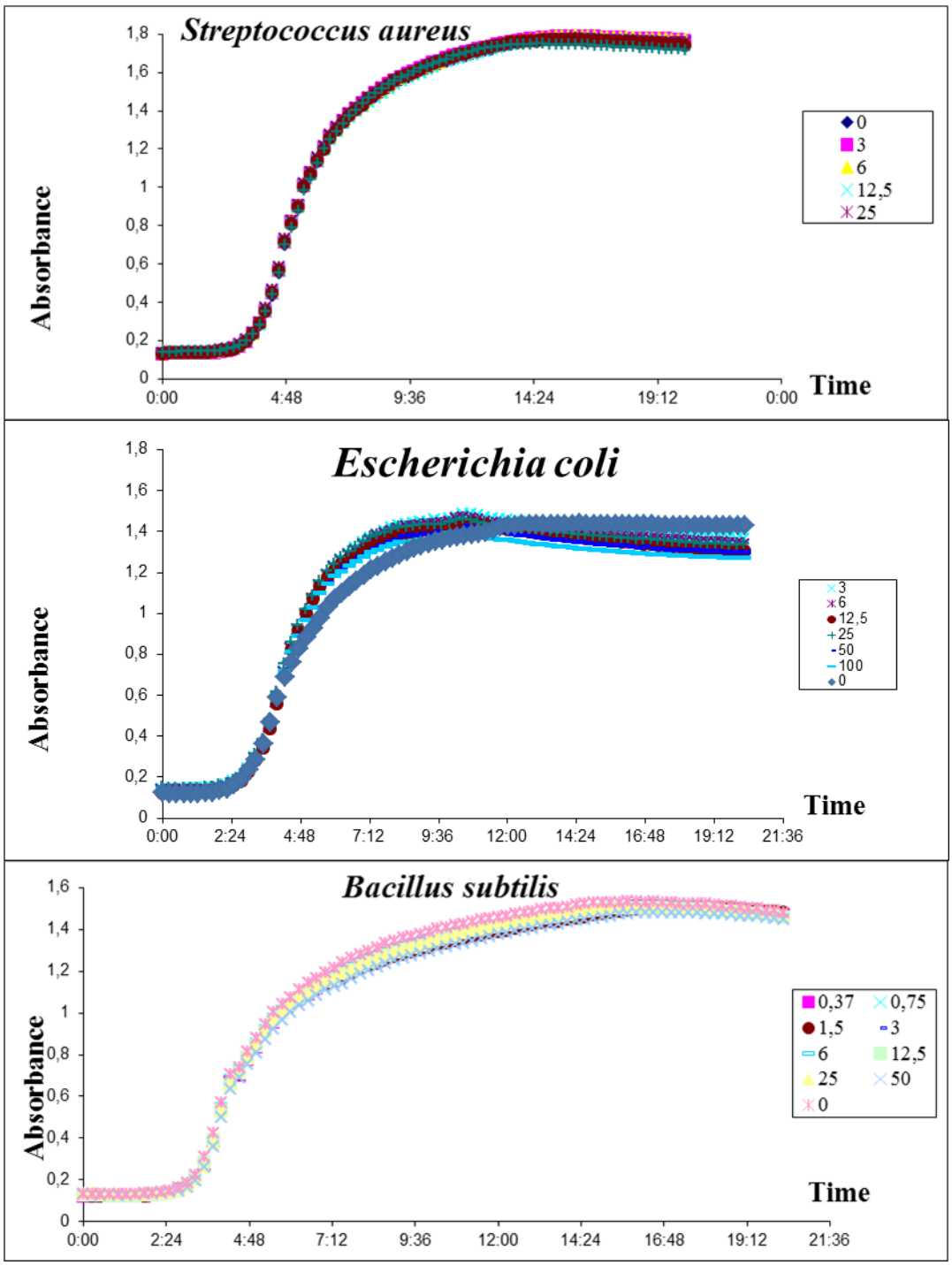


Figure 30: *Drop- diffusion assay with HPLC fractions (1-3) of cyclized synthetic peptide and linear synt. peptide (4); no dilution factor (0), dilution 10^{-1} (row -1) and 10^{-2} (row -2)*

The use of the different concentrations of the linear peptide (0.375 µM-200 µM) in minimum inhibition concentrations (MICs) did not revealed any antimicrobial activity (see Figure 31).


Streptococcus aureus
Absorbance
Time
0
3
6
12,5
25
Escherichia coli
Absorbance
Time
3
6
12,5
25
50
100
0
Bacillus subtilis
Absorbance
Time
0,37
0,75
1,5
3
6
12,5
25
50
0

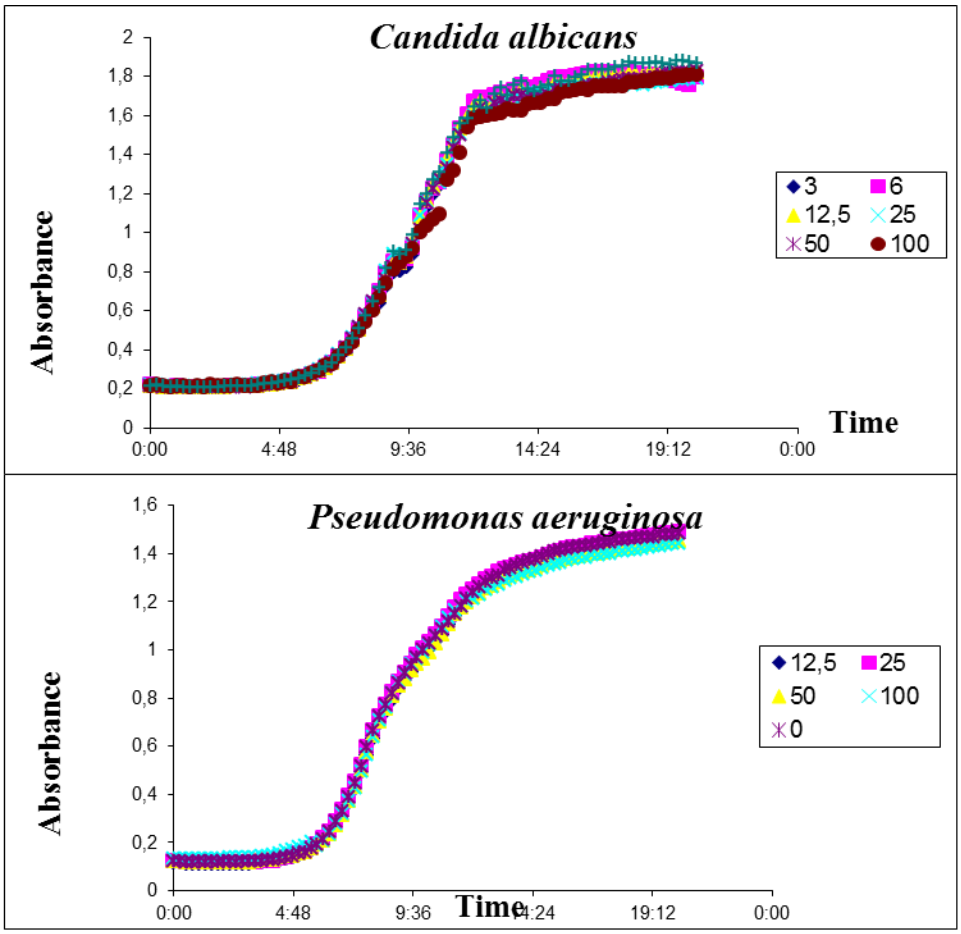


Figure 31: *Determination of antimicrobial activity by MICs assay: Linerar synthetic IRAMP peptide was tested against G+ (B. subtilis, M. luteus, S. aureus), Gram-negative (E. coli and S. aureus) and yeast (Candida albicans). The activity was meausered using the peptide concentrations from 0.375 to 200* μM*. Only values from one measurement are presented. It is obvious that peptide was not active in any of tested concetration under used conditions.*

The synthetic peptide in concentrations varied from 0.365 to 200 μM was tested against Gram-positive, Gram- negative bacteria and against yeast. No antimicrobial activity against tested organisms was revealed in these tests.

6. Discussion and conclusion

While taking a blood meal ticks encounter a large diversity of pathogens from hosts. The immune system provides ticks with efficient defense from pathogens in case of their penetration into the body. One of the facets of the tick defense is a rapid and transient synthesis of a set of potent antibacterial peptides (AMPs) following infection or trauma. The naturally occurring AMPs form the first line of tick defense. Due to extreme diversity they are active against wide variety of pathogens. With the growing problem of pathogens that resist conventional antibiotics, ticks are becoming fruitful sources to find novel pharmaceutical substances to treat infections.

In general, *I. ricinus* survives systematic pathogen infection without any significant mobilization of defense reactions of its innate immunity. It seems that neither one of the pathogens that *I. ricinus* transmitt threaten the tick life during any of its development stage. In case of the causative agent of Lyme disease spirochetes the situation is different as, in non competent tick vectors, tick hemocytes eliminate the majority of spirochetes by phagocytosis in hemocoel before the pathogen will reach the salivary glands. It may be assumed that the absence of tick defense reactions against certain pathogens (like TBEV) and, on the other hand, the presence of the defense response against some others (like LD spirochetes) reflect the period of pathogen co-existence with molecular factors in the inner environment of ticks during the evolution.

Arthropod antimicrobial peptides in general and, ticks defense proteins, in particular, have been studied very intensively within the last decade. Research on this topic could bring the answer why some tick species are being such successful vectors of multiple pathogens while the other tick species are eliminating invaders fast. Many tick antimicrobial peptides (AMPs) have been already described. It was proved that the majority of ticks AMPs are active against Gram- positive bacteria (111). However, some of them are active against Gram-negative bacteria as well (112). Certain tick AMPs reveal significant antimicrobial activity against wider spectrum of pathogens. AMP called ixosin, from hard tick *I. sinensis*, is active against Gram-negative, Gram- positive bacteria and yeast as well (113). Another example,

defensin peptide 1 and 2 from *Amblyomma americanum* are also active against Gram-negative and Gram- positive bacteria (114).

In 2009, a team of Dr. Pichu (106) introduced a new cationic AMP from hard tick *I. scapularis* and called it "ISAMP" (***I****xodes* ***s****capularis* **a**nti**m**icrobial **p**eptide). ISAMP revealed strong antimicrobial activity against Gram- positive and Gram- negative bacteria. Taking into consideration the similarity between North American *I. scapularis*, and in Old continent widespread *I.ricinus*, we started to look for homolog of ISAMP in European tick *I.ricinus*. Results of our search of gene that encodes a novel antimicrobial protein in *I.* ricinus, that we named "IRAMP" (***I****xodes* ***r****icinus* **a**nti**m**icrobial **p**eptide) are present in this study. The tissue expression pattern of IRAMP was defined and the possible presence of antimicrobial activity was analyzed in native and recombinant AMP as well as in synthetic IRAMP analog.

Comparison of genes encoding discussed antimicrobial peptide in *I. scapularis* and *I. ricinus* showed their relatedness with high level of identity and shared specific features, like the presence of the same conserved cysteine motif.

The basic idea of our project was to produce the recombinant IRAMP protein in sufficient amount to perform analysis of its antimicrobial potential (115), keeping in mind that, for example, the modes of action of AMPs are not always accepted by the metabolic system of the host and in some situations it results in cellular stress response (116). Production of preparative amounts of recombinant IRAMP in bacterial expression system failed. Multiple reasons might be responsible for unsuccess and one of them could be the small size of mature IRAMP protein (5.3 kDa). The His-tag selected for the production of recombinant IRAMP, is approximately the same size as the mature peptide itself that makes the expression of the target protein in the *E. coli* expression system and it purification very problematical. It was shown as well that short recombinant peptides are frequently degraded by *E. coli* proteases (117). Moreover, IRAMP is short protein with 6 conserved cysteine residues that form disulfide bridges, using the same pairing Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6. Disulfide bridges formation is vital for functional defensin activities. Unfortunately, proteins which require disulfide crosslinks are often unstable in cell compartment (118). Moreover, it is well known that the presence of 8- 15 hydrophobic amino acids stretched in a protein sequence makes its expression extremely complex (119). According to the predicted protein sequence of

IRAMP it has 13 hydrophobic amino acids in a chain. The failure of bacterial expression system to produce reasonable amounts of active recombinant peptide requires involvement of other more advanced systems for this purpose.

Tick defensins have been shown to be expressed in multiple tick tissues such as midguts, salivary glands, hemocytes and fat body. It was already discussed that the site of expression of AMPs in vector body reflects the certain functional properties in arthropod antimicrobial peptides (106, 114, 120-124). Tissue- specific expression pattern of IRAMP showed the highest level of its expression in salivary glands that is in agreement to the expression profile of ISAMP. However, contrary to ISAMP, the expression of IRAMP transcripts was detected in midgut. These results suggest that IRAMP has an impact in protection of ticks while it is present in tissues with high possibility to be exposed to infectious agents, i.e. in the first line of defense.

As it was already published, AMPs in insects are mainly synthesized in the fat body and their gene transcription is strongly induced after an injury and/or infection (104, 125). The synthesis of AMPs in chelicerates seems to be constitutive and occurs mostly in the hemocytes where they remain stored in granules, before being released into the hemolymph after microbial stimulation (126). This stimulation of tick innate immunity, i.e. activation and induction of expression of defensins including IRAMP, is started during feeding when ticks can be exposed to many pathogens (127).

As recombinant approach used in our study did not bring the extected results, the native IRAMP protein was isolated from hemolymph of 6 days fed *I. ricinus* females. Antimicrobial activity was detected in several fractions (128) in assay against Gram- positive *M. luteus*, as expected. However, the tested fractions did not affect growth of tested Gram- negative bacteria *E. coli*. As it was already published, none of the hemolymph AMPs showed activity against Gram- negative bacteria, *E. coli* (122). Our results are in agreement with a previous observation that the total hemolymph and the gut contents of tick *B. microplus* did not exhibit any activity against Gram-negative bacteria using *in vitro* assays (129). The reason why Gram-negative bacteria are more resistant to cationic antimicrobial peptides could be due to the effectively permeable barrier in their outer membrane of cell wall (130). Thus, some of the peptides are not able to interact with the cell membrane.

As the tick hemolymph contains a wide spectrum of different compounds it was hard to

confirm on the basis of HPLC separation only, that antimicrobial activity was caused by IRAMP protein alone. Several other antimicrobial peptides were already described in *I. ricinus* (128). We applied exclusion chromatography approach to analyze the antimicrobial potential of native *I. ricinus* IRAMP. Collected fractions did not reveal any significant antimicrobial activity. The lack of activity might be explained by the low concentration of protein in the sample, or by the site of protein expression in the tick tissues, that does not define such activity for IRAMP, or by the possibility that our protein has evolved to target different types of pathogens, or simply by the fact, that the immune responses are very complex and affectivity of the protein is dependent on presence of other compounds. This fact was already observed in small recombinant peptides (88). To confirm or reject the above mentioned reasons which possibly affect antimicrobial activity of the protein, further studies are required.

Antimicrobial activity was also tested with the synthetic IRAMP peptide both in linear form and after cyclization. It was believed that disulfide bridges are important for ticks defensin activity in order to preserve its three-dimensional structure. From the other side, three-dimensional structure is not so important for some mammalian as well as insect defensins, like lucifensin (110). These defensins exhibit antimicrobial activity even in a linear form. To check all possibilities, antimicrobial test of IRAMP MICs was performed with non-folded synthetic linear peptide as well. IRAMP was left to fold oxidatively and the test was performed with Gram- positive bacteria (*M. lutes, S. aureus, B. subtilis*), Gram- negative *(Pseudomonas aeruginosa*) and with yeast (*Candida albicans*). Both forms of synthetic analog of IRAMP, linear and refolded, failed to show suppression of bacterial or yeast growth.

The presence of significant antimicrobial activity of tested native, recombinant and synthetic IRAMP against selected species of Gram-positive, Gram-negative bacteria and yeasts, was not confirmed in this study, except of weak but rather noticeable antimicrobial activity against *M. luteus* that was found in fraction 3 of HPLC separated *I. ricunus* hemolymph. However, our results cannot give the direct answer to the question if IRAMP possesses the studied antimicrobial activities in general or not. Most probably, the spectrum of microorganisms, selected for our project, was incomplete and inappropriate and might need further re-evaluation. Such conclusions are strongly supported by the very recent discovery, published by E. Fikrigs group on North American relative of European tick, *I. scapularis*.

I. scapularis transmits the agent of human granulocytic anaplasmosis, among other pathogens. The mechanisms used by the tick to control *Anaplasma phagocytophilum* are not known. A salivary gland gene family encoding 5.3-kDa antimicrobial peptides (to which *I. ricinus* 5.3 kDa IRAMP revealed significant identity) is highly induced upon *A. phagocytophilum* infection of tick salivary glands. Gene expression and electrophoretic mobility shift assays showed that the 5.3-kDa antimicrobial peptide–encoding genes are regulated by tick STAT. Silencing of these genes increased *A. phagocytophilum* infection of tick salivary glands and transmission to mammalian host.

The results of this study did not fully explain the property of newly found protein and its role as an antimicrobial peptide remains unclear. However, the antimicrobial activity of IRAMP needs to be clarified in order to determine whether or not this peptide can be effective against certain causative agents of vector borne diseases. The priority for the future extencion of our project will definitely involve the wider spectrum of pathogens that have a great impact in human health.

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