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Master thesis

Identification and characterization of histidine-rich peptides from hard ticks *Ixodes ricinus* and *Ixodes scapularis*.

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ANNOTATION:

Antimicrobial (cationic) proteins play an important role in innate imunity. Such proteins can possess antibacterial, antiendotoxic or fungicidal abilities. The rising resistence of microbes to common antibiotics evokes acute need of studying more endogenous proteins to reveal new potential antibiotics. Ticks, the blood-feeding ectoparasites with effectual defense system, present an endless source of newly described and unknown antimicrobial peptides/proteins with significant theurapeutic potential.

This study represents identification of histidine-rich proteins detected in *Ixodes ricinus* and *Ixodes scapularis*, that are related to recently described new family of proteins isolated from *Rhipicephalus microplus* (protein microplusin) and *Amblyomma hebraeum* (protein heraein). Analysis and characterization of newly identified histidine rich proteins, study of their antimicrobial and protease inhibitory effect are the main goals of this study.

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DECLARATION:

Prohlašuji, že svoji diplomovou práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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České Budějovice, 3. 1. 2011

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Veronika Dorňáková

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1. INTRODUCTION:

The early years after Fleming's great discovery of penicillin in 1929, and Florey's subsequent demonstration of the chemotherapeutic activity, were followed by a golden era of discovery. However, the influence of new antibiotics was soon replaced by the spectra of resistance. This has led to a constant race between researchers developing new compounds, and bacteria developing resistance.

However, the heyday of antibiotic discovery in the last century has given way to the present situation where we have a plethora of derivatives of the old classes, but few new leads in the biopharmaceutical pipeline.

The new classes of compounds are really needed. A potentially rich source of peptide therapeutics that is being investigated by researchers is the innate immune response, the effectual weapon against microbial attack - antimicrobial proteins (AMP).

2. STATE OF KNOWLEDGE:

2.1. Antimicrobial proteins (AMPs)

Living organisms are exposed daily to different pathogens (bacteria, viruses, protozoa, fungi) that sometime might cause severe infections. In order to defend themselves against the abrasive environment, each organism have developed potent defensive mechanisms that are part of innate and adaptive immunity. One mechanism of innate immunity is the production of anti-microbial substances – antimicrobial proteins (AMPs).

Antimicrobial proteins (AMPs) are ancient weapon with the fundamental impact on successful evolution of complex multi-cellular organisms. These innate immune effectors are widely distributed throughout the animal and plant kingdoms and demonstrate a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria (Pachon *et al.,* 2009; Linde *et al.,* 2008; Ramanathan *et al.,* 2002), fungi (Aerts *et al.,* 2008; Thevissen *et al.,* 2007), parasites (Lofgren *et al.,* 2008) and enveloped viruses (Harder *et al.,* 2007; Slocinska *et al.,* 2008). Some have been reported to possess antitumor properties as well (Lu *et al.,* 2008; Hoskin *et al.,* 2008; Dennison *et al.,* 2006; Slocinska *et al.,* 2008).

2.1.1. Characterization of AMPs:

Since the emergence of the field more than 30 years ago, the antimicrobial peptides have been characterized as small amphipathic and cationic molecules, comprised of 12–60 amino acids with molecular weights of less than 10 kDa. Although this is the case for the majority of antimicrobial peptides, many recent studies have identified exceptions to this classic definition. Recently, polypeptides and proteins considerably larger than defined antimicrobial peptides have been shown to have unambiguous antimicrobial activity (e.g. lactoferrins, kinocidins). The antimicrobial effect of anionic peptides was also proved (LaForce et al., 1981; LaForce et al., 1984; Lai et al., 2004; Harris et al., 2009). As a result, the definition of host-defense peptides has been expanded to include the larger molecules with not only cationic charge.

(i) The diversity of AMPs:

The broad variety of antimicrobial peptides has been isolated. The brief introduction of existing AMPs databases is shown in the table 1.

The diversity of antimicrobial peptides is so wide that it is difficult to categorize them except broadly on the basis of their secondary structure. There are four prominent structural classes of AMPs: the α-helical (linear) peptides, the disulfide-bonded β-sheet peptides, the loop-structured peptides and the extended peptides (Jenssen et al., 2006). Other classification criteria could be based upon source (e.g., neutrophils or other leukocytes), enrichment in specific amino acids (e.g., proline–arginine or tryptophan rich), precursor structure (e.g., cathelicidin-derivatives of cathelin), extent of intramolecular bonds (e.g., cysteine array or cyclization), or other parameters (Yount *et al,* 2006).

The **α -helical peptides** are linear, noncysteine-containing molecules with no rigid structure in aqueous media that form amphipathic helices in proximity to hydrophobic membranes, e.g. magainins (Zasloff, 1987) and melittins (Steiner *et al*., 1981). In some cases these peptides contain a central hinge region (e.g. dermaseptins (Lequin *et al*., 2003), caerin1.1 (Pukala *et al*., 2004)).

The **β-sheet peptides** represent a highly diverse group of molecules at the level of primary structure. These peptides are frequently comprised of several antiparallel β-strands that are stabilized by a series up to six disulfide bonds, e.g. defensins (Ganz & Lehrer, 1994) and protegrins (Steinberg *et al*., 1997). Recently, a multidimensional proteomic analysis discovered a common motif that integrates all of the cysteine-stabilized antimicrobial peptides. This motif, termed the γ-core, is composed of two antiparallel β-sheets, with basic residues

Table 1.: The brief overview of existing database of AMPs:

features / databases	link	citation	nature	number of AMPs presented
APD	http://aps.unmc.edu/AP/main.html	Wang *et al.* 2004	general	1518
RAPD	http://faculty.ist.unomaha.edu/chen/rapd/index.php	Li *et al.* 2008	specific recombinant AMPs	149
PhytAMP	http://phytamp.pfba-lab.org	Hammami *et al.* 2009	specific plant AMPs only	273
BACTIBASE	http://bactibase.pfba-lab.org	Hammami *et al.* 2007	specific bacterial AMPs only	177
Defensins knowledgebase	http://defensins.bii.a-star.edu.sg	Seebah *et al.* 2007	specific defensin family only	457
PenBase	http://penbase.immunaqua.com	Gueguen *et al.* 2007	specific penaeidin family only	29
Peptaibol Database	http://www.penbase.immunaqua.com	Whitmore *et al.* 2004	specific peptaibols only	317
AMSDb	http://www.bbcm.units.it/~tossi/pag1.htm	no citation	specific eukaryotic AMPs only	895
SAPD	http://oma.terkko.helsinki.fi:8080/~SAPD	Wade *et al.* 2002	specific synthetic AMPs only	247
CAMP	http://www.bicnirrh.res.in/antimicrobial	Thomas *et al.* 2010	general	3782

polarized along its axis (Yount and Yeaman, 2004). Remarkably, conservation of the γ-core motif across all biological kingdoms suggests it is an antimicrobial peptide archetype.

In contrast, the **loop-structured** and **extended peptides** are relatively unstructured, with the exception of the loop structure itself.

There are also so called specific residue-rich peptides in which one or more amino acid residues predominate, e.g. indolicidin (intensified amounts of tryptophan) (Selstev *et al.,* 1992) and histatins (histidine-rich) (Oppenheim *et al.,* 1988). The structure of peptides with such composition is different from prototypic α-helical or β-sheet structures. For example, tryptophan-rich indolicidin is thought to adopt a wedge-shaped structure in membrane-mimetic environments (Rozek *et al.,* 2000).

(ii) Mode of action

Antimicrobial peptides target a previously under-appreciated "microbial Achilles heel", a design feature of the microbial cellular membrane that distinguishes broad species of microbes from multi-cellular plants and animals (Zasloff, 2002). Bacterial membranes are organized in such a way that the outermost leaflet of the bilayer, the surface exposed to the outer world, is heavily populated by lipids with negatively charged phospholipid headgroups. In contrast, the outer leaflet of the membranes of plants and animals is composed principally of lipids with no net-charge; most of the lipids with negatively charged headgroups are segregated into the inner leaflet, facing the cytoplasm (Matsuzaki, 1999). Numerous studies indicate that many antimicrobial peptides, in contrast to conventional antibiotics, act directly on the membrane of the target microorganism, and are not receptor mediated. However, consequently the exception was shown. The antibacterial peptide nisin Z uses the membrane-anchored cell wall precursor Lipid II as a receptor (Breuking *et al.,* 1999).

The antimicrobial peptides can permeabilize the membrane and/or translocate across the membrane into the cytoplasm without causing major membrane disruption. Hence, the modes of action of antibacterial peptides can be broadly categorized as either membrane acting (I.) or non-membrane acting (II.).

I. Membrane-permeabilizing peptides:

The killing mechanism, for both gram-positive and gram-negative bacteria, is the formation of channels in the cytoplasmic membrane that causes leaking of important ions out of the cell (Hancock *et al.,* 1995; Falla *et al.,* 1996). Three predominant models have traditionally been proposed for antimicrobial peptide membrane permeabilisation and/or poration: torroid-pore model, barrel-stave model and carpet model.

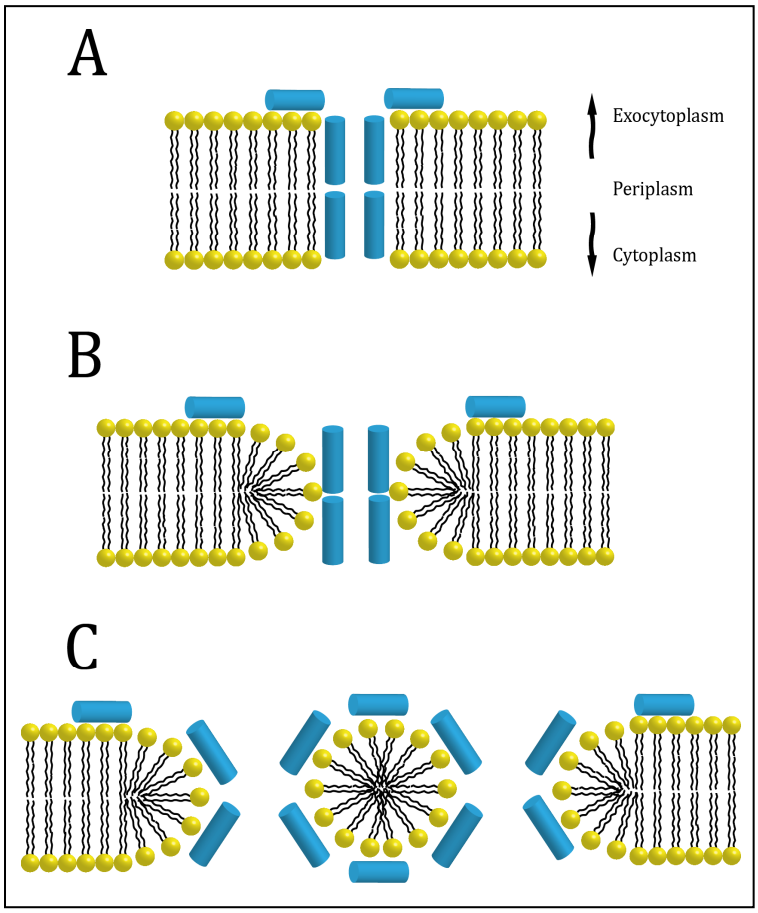


Fig.1.: Three predominant models of antimicrobial peptide membrane permeabilisation – toroidal model (A), *barrel-stave model* (B) *and carpet model* (C).

The torroidal model *(Fig.1A)* is the complex where the phospholipids bend from the membrane interface towards the hydrophobic interior and are intercalated between the long axis of the peptide molecules, which are oriented perpendicular to the bilayer plane. The example of AMP with such mode of action is magainin 2 (Matsuzaki *et al.*, 1996).

For the antimicrobial peptide alamethicin a pore of barrel-stave type *(Fig.1B)* was described (He *et al.*, 1996). Theoretically such pores can be formed from as few as 3 molecules. To allow pore formation the inserted molecules should have distinct structures, such as, an amphipathic α-helix, hydrophobic α-helix, β-sheet or both α-helix and β-sheet structures.

The carpet model *(Fig.1C)* is characteristic for peptides that bind to the phospholipid membrane surface until a certain threshold concentration is reached.

Subsequent insertion of the peptides into the hydrophobic core results in permeation/disruption of the membrane. The detailed mechanism of membrane permeation or disruption is less defined. It could be disintegration in a detergent-like manner (Shai, 2002; Bechinger and Lohner, 2006) or formation of pores (Matsuzaki *et al.,* 1995; Ludtke *et al.,* 1996) and channel like aggregates (Hancock and Rozek, 2002).

II. Peptides that do not act by membrane permeabilization:

A growing number of peptides have been shown to translocate across the membrane and accumulate intracellularly, where they interfere with a variety of essential cellular processes to mediate cell killing. Novel modes of action that have recently been demonstrated include inhibition of nucleic acid synthesis, protein synthesis, enzymatic activity, and cell wall synthesis (Brogden, 2005).

Inhibition of nucleic acid synthesis has also been demonstrated for antimicrobial peptides from different structural classes, such as the β-sheet human defensin, HNP-1 (Lehrer *et al*., 1989), and the extended-structure bovine peptide indolicidin (Subbalakshmi and Sitaram, 1998).

Pyrrhocidin, the proline-rich insect antimicrobial peptide, is the example of inhibitor of cellular enzymatic activity. After the penetration of membrane it binds to DNaK, a heat shock protein that is involved in chaperone-assisted protein folding, leading to accumulation of misfolded proteins and cell death (Kragol *et al*., 2001).

The inhibitors of cell wall synthesis could be presented by bacterially produced lantibiotics – mersacidin and nisin. These peptides interfere with transglycosylation of lipid II, a necessary step in the synthesis of peptidoglycans (Brotz *et al.,* 1997).

2.1.2. Resistance:

In the past 50 years, resistance to every new antibiotic has appeared in microbial population within a few years of its introduction (Palumbi, 2001). Unlike conventional antibiotics such as penicillin, which microbes readily circumvent, acquisition of resistance by a sensitive microbial strain against antimicrobial peptides is surprisingly improbable (Zasloff, 2002). The great diversity of AMPs, the presence of several different kinds at the infection site and their different mode of

action might have impeded the evolution of resistance in natural bacterial population (Perron *et al.,* 2006).

Resistance of different microorganisms to AMPs has been identified. Resistant species of genera such as *Morganella* and *Serratia* express an outer membrane that lacks the appropriate density of acidic lipids to provide peptide-binding sites for AMPs. Other resistant species, such as *Porphyromonas gingivalis*, secrete digestive proteases that destroy AMPs (Zasloff, 2002).

Evolutionary biologists have argued about the therapeutic use of particular AMPs, the way thay might alter the natural environments by creating a source of specific and continued selection leading to the evolution of resistance (Bell and Gouyon, 2003). This theory was support by Perron and colleagues (2006). Their results show that experimental lines of *Pseudomonas fluerescens* and *Escherichia coli* evolved high resistance to an antimicrobial peptide. The laboratory selection experiment is inconsistent with the conditions bacteria experience in nature. There are many factors such as stress, chemical agents, etc. that might influence the rise and establishment of resistance in microorganisms. In spite to this fact the study shows that resistance can be expected to evolve rapidly whenever bacterial populations are consistently exposed to elevated levels of AMPs (Perron *et al.,* 2006). Thereby the use of AMPs as therapeutics should be carefully and appropriately regulated to minimize emergence of resistant organisms from treated individuals and from environments such as hospitals and stockyards (O'Brien, 2002).

2.1.3. The importance of AMPs:

Because of the ability of AMPs to kill rapidly broad spectrum of infectious agents and modulate both innate and adaptive immunity, significant efforts have been made to exploid their therapeutic potential.

One of the examples is nisin, bacterial AMP. It is used as a safe alternative for food preservation chemical reagents in approximately 50 countries for over 40 years. Nisin is in the preclinical stages of medical application in clinical and veterinary therapies (Ryan *et al.*, 2002; Breukink and de Kruijff, 2006). It is effective against bacterial mastitis, oral decay and enterococcal infections and is effective in peptic ulcer treatment. The inhibition of methicillin-resistant *Staphylococcus epidermidis*

should not be left out (Ghiselli *et al.,* 2004). Another potential application of nisin is as a contraceptive agent, because nisin inhibits sperm motility (Aranha *et al.,* 2004).

LL-37, an important multifunctional human cathelicidin, is currently being tested in the treatment of skin diseases (Zasloff, 2006). The effectiveness LL-37 to counterattack infectious or inflammatory gastrointestinal diseases is widely documented in animal models (Yang *et al.,* 2006; Chromek *et al.,* 2006). Another study also demonstrated the role of LL-37 in enhancing the innate immunity in atherosclerosis (Edfeldt *et al.,* 2006).

The anticancer action of cecropins, insect AMPs, was reported by Moore and collegues (1994). Their study reported that Cecropin B significantly increases the median survival time of mice with colon adenocarcinoma when injected intraperitonally at a dose 80 mg/kg. Consequently, expression constructs carrying cecropin were introduced into a human bladder carcinoma-derived cell line and the resultant cell clones were analysed for tumourigenicity in nude mice. The result was the reduction or even a complete loss of tumour induction (Winder *et al.,* 1998). But futher studies need to be done to get into the preclinical stages of medical application.

But AMPs are important not only in medicine, but in agriculture as well. Thionins, plant AMPs, have already been utilized to obtain transgenic plants resistant to phytopathogenic fungi. For instance, the α-hordothionin gene expression in tobacco that increase the resistance against *Pseudomonas syringae* (Carmona *et al.,* 1993), viscotoxins expression in *Arabidopsis thaliana* that confer resistance to *Plamodiophora brassicae* (Holtorf *et al.,* 1998) and expression of an oat thionin in transgenic rice seedlings lead to protection against the phytopathogenic bacteria, *Burkholderia plantarii* and *B. glumae* (Iwai *et al*., 2002).

2.2. Ticks

2.2.1. Importance of the tick:

Ticks are blood-feeding ectoparasites and vectors of various pathogens, affecting humans and animals worldwide. The wide variety of pathogenic organisms transmitted by ticks include viruses, bacteria, fungi and protozoa, and significantly

exceeds the number of pathogens transmitted by other hematophagous arthropods (Jongejan and Uilenberg, 2004; Pagel Van Zee *et al.,* 2007).

Ixodes ricinus and *Ixodes scapularis* belong to the family of hard ticks (*Ixodidae*). While *I. ricinus* is a main hard tick in Europe, *I. scapularis* is typical for the territory of the eastern, northern and midwestern United States. A number of tick-borne diseases (e.g. Lyme borreliosis) are transmitted by these vectors.

Ticks from the family *Ixodidae* feed on three different hosts during their life cycle, developing from larvae, through nymph, into the adult. The most important for the progress from one development stage to the other is the blood uptake.

The blood-feeding means for ticks modulation of host haemostatic, inflammatory and immune responses mediated by molecules present in tick saliva. Simultaneously, the tick defense system must eliminate microbial infection, or at least reduce it to a level accaptable for the vector. The major role in mentioned processes is played by antimicrobial peptides.

2.2.2. Antimicrobial peptides of the tick:

Ticks lack an adaptive immune system. Their only weapon against invading pathogens is the innate immunity. The nature of the innate immune response involves two major components: cellular defenses, represented by phagocytosis, encapsulation and nodule formation, and humoral responses, involving antimicrobial proteins. While the great number of antimicrobial peptides was already studied and described from different organisms, the knowledge of AMPs from ticks is very limited. Brief overview of tick AMPs present five identified protein groups: lysozymes, defensins, hemoglobin fragments, protease inhibitors and 'other AMPs' group of peptides that do not belong to any group mentioned above.

(i) Lysozymes

The bactericidal effect of tick lysozymes was proved by Podboronov mainly against Gram-positive bacteria (*Microccoci, Staphylococci*) (Podboronov, 1982 and 1990).

Lysozyme antimicrobial activity was reported from the hemocytes of *I. ricinus*, cell lines of *D. andersoni* and *I. scapularis,* and from *I. persulcatus* (Kuhn and Haug,

1994; Podbornov, 1990; Mattila *et al*., 2007). Mattila and colleagues (2007) confirmed that lysozyme expression was not up-regulated in the response to challenge by endosymbiont *R. peacock*; however, expression was up-regulated in the cells challenged with *E. coli* and *M. luteus*.

(ii) Defensins

Defensisns are the most studied group of tick antimicrobial peptides. To date, ticks defensins (with several isoforms) were identified from nearly twenty hard and soft tick species (Sonenshine and Hynes, 2008; Saito *et al.* 2009; Rudenko *et al*., 2005; Rudenko *et al*., 2007; Chrudimská *et al.* 2010).

The antimicrobial activity of defensins is primarily directed against Gram-positive bacteria, but some isoforms are effective also against Gram-negative bacteria, yeasts and protozoa (Nakajima *et al*., 2003; Tsuji *et al*., 2008; Isogai *et al*., 2009; Saito *et al*., 2009).

Varisin, purified from the hemolymph of hard tick *Dermacentor variabilis* challenged with *Borrelia burgdorferi* (Johns *et al.,* 2001), showed antimicrobial activity against Gram-positive bacteria *Bacillus subtilis* (Johns *et al.,* 2001). Varisin revealed low activity against cultured spirochetes, however, when it was combined with lysozymes, 65 % of cultured *B. burgdortferi* were killed within 1 hour (Johns *et al.,* 2001). Thus, significantly increased borreliacidal activity indicates a possible synergism between varisin and lyzozyme. The role of varisin in the innate immunity of *D. variabilis* was also proven by RNAi. The activity of varisin-knock-down tick hemolymph against Gram-positive bacteria *Microccocus luteus* was reduced only by 50%. This fact indicates that varisin is important but not vital for the antimicrobial activity of *D. variabilis* hemolymph (Hynes *et al.,* 2008). Interesting findings were presented in the study of Kocan and colleagues (Kocan *et al.,* 2008) where the silencing expression of varisin resulted in reduced infection of tick by *Anaplasma marginale*. A second defensin isoform from hard tick *D. variabilis,* defensins 2, was recently isolated from the midgut (Ceraul *et al.,* 2007). Its role in tick innate immunity was not defined yet.

Two defensin isoforms, def1 and def2, were identified from another hard tick *Ixodes ricinus* (Rudenko *et al.,* 2005 and 2007; Chrudimska *et al*., 2010). Def1 and def2 inhibits the growth of Gram-positive bacteria (*M. luteus, B. subtilis, S. aureus*) in

very low concentrations (MIC 0.37 – 50 μM). While the defensin isoforms differs by only one amino acid in their mature peptide sequence the def2 isoform is much more effective than def1 (Chrudimská, personal communications).

The defensin from *I. persulcatus* (Saito *et al.,* 2009) markedly inhibited the growth of Gram-positive bacteria including *Staphylococcus aureus*, *Bacillus subtilis* and *Corynebacterium renale*, but not Gram-negative bacteria except *Escherichia coli*.

Results of Isogai and collegues (2009, 2010) showed that bacteria naturally co-existing with tick (e.g. pathogenic spirochete *Borrelia garinii*, bacteria *Bacillus* sp.) possess the natural resistance to tick defensins.

Tsuji and colleagues (2007) described another defensin-related molecule, longicin, from babesial vector *H. longicornis.* Recombinant peptide was active against wide range of fungi, Gram-positive and Gram-negative bacteria, including multi-drug resistant strains. But what is most interesting is the fact that recombinant protein was also active against *Babesia.*

Defensins were identified also in soft ticks. The antimicrobial activity of successfully purified defensin A from soft tick *Ornithodoros moubata* was shown against bacteria *Staphylococcus aureus* (Nakajima, 2002). Functional analysis of synthesized defensin A determined that it was active against panel of Gram-positive bacteria but did not reveal any activity against Gram-negative. The mode of action of this defensin is permeabilization of the bacterial membrane (Nakajima *et al.,* 2003).

(iii) Hemoglobin fragments

The hemoglobin fragments of the host were shown to be an important part in antimicrobial control. A. Fogaca and colleagues (Fogaca *et al.,* 1999) demonstrated that the 3.2 Da fragment of the bovine α-hemoglobin from the tick *R. microplus* showed bacteriacidal activity against Gram-positive bacteria and fungi. Also hemoglobin fragments from soft tick, *O. moubata* (Nakajima *et al.,* 2003), and hard tick *D. variabilis* (Sonenshine *et al.,* 2005), were active against Gram-positive bacteria *S. aureus* and *M. luteus*. These data indicate that both hard and soft ticks use host hemoglobin in their defence mechanism. It was determined that α-hemoglobin fragment functions via disrupting the bacterial membrane (Sforca *et al.,* 2005), but more studies are needed to explain the whole mechanisms of action of hemoglobin fragments.

(iv) Protease inhibitor

Proteases are important factors of virulence used by pathogens. There are few reports on cysteine (cystatines) and serine (serpins) protease inhibitors from ticks.

Cystatins were reported to suppress the host's adaptive immune response, e.g. Sialostatin L, cystatin of *I. scapularis,* inhibits T lymphocytes proliferation and have an antiinflammatory effect (Kotsyfakis *et al.,* 2006); OmC2, the cystatin from *O. moubata*, reduces the production of the pro-inflammatory cytokines tumour necrosis factor alpha and interleukin-12, and proliferation of antigen-specific CD4+ T-cells of the host (Salat *et al.,* 2010).

It was reported that cystatines affect not only the immune system of the host, but also invading pathogens. For example, cystatin from the tick *Haemaphysalis longicornis*, Hlcyst-2, showed a significant growth-inhibitory effect on *Babesia bovis* cultured *in vitro*.

Serine protease inhibitor were also identified in several hard tick species (e.g. *A. americanum* (Mulenga *et al.*, 2007), *Rhipicephalus appendiculatus* (Mulenga *et al.,* 2003), *R. microplus* (AAP75707), *I. ricinus* (Mulenga *et al*., 2009), *I. scapularis* (Ribeiro *et al*., 2006)). Their potential role in tick control was showed in several studies that present serpins as promising candidates for anti-tick vaccine (Sugino *et al*., 2003; Imamura *et al*., 2006; Prevot *et al*., 2007; Chmelar *et al.,* 2010).

(v) Other AMPs

There are a large number of AMPs that does not belong to any group mentioned above.

Longicornsin (Lu *et al.,* 2010), peptide isolated from salivary glands of the hard tick *H. longicornis*, is similar to defensin-like peptides mainly with conserved cysteines, but it differs by a C-terminal extension. The antimicrobial tests showed that longicornsin is active against bacteria and fungi, and it revealed strong antimicrobial activity against drug-resistant microorganisms and *Helicobacter pylori* as well.

Two defensin-like peptides (ADP1 and ADP2) were identified in *Amblyomma hebraeum* tick (Lai *et al.,* 2004). Having all conserved cysteines as defensins, they lack the typical furin cleavage motif. The other difference is that these two peptides

are anionic. Analysis of their antimicrobial activity showed that ADP2 was active against Gram-positive and Gram-negative bacteria, but showed no effect against fungi.

Another antimicrobial peptides, microplusin and hebraein, belong to a new type of antimicrobial protein family with multiple histidines and a novel secondary structure composed of four to six α-helices. Hebraein, a 102 amino acids long anionic peptide was identified in *A. hebraeum* tick (Lai *et al.,* 2004) and revealed strong antimicrobial activity against Gram-positive, Gram-negative bacteria and the yeast *Candida glabrato,* but not against *Candida albicans*.

Microplusin was isolated from *R. microplus* (Fogaca *et al.,* 2004). The recent functional analysis showed (Silva *et al.,* 2009) that microplusin chelates copper ions by binding them most probably to histidines (H2, H74). Microplusin was active against Gram-positive bacteria, some filamentous fungi like *Aspergillus niger* and against yeast *Candida neoformans,* but not *Candida albicans.* The bacteriostatic effect of microplusin on *Microccocus luteus* was probably due to its ability to take the copper ions from bacteria that are vital for bacterial respiration. Supplementing of medium with copper can restore the growth of *M. luteus.*

Another cystein-rich antimicrobial peptide, named ixodidin, was identified in *R. microplus* (Fogaca *et al.,* 2006). Ixodidin contains ten cysteins forming five disulfide bridges. Suppressing the growth of *E. coli* and *M. luteus,* ixodidin possesses inhibitory activity against two exogenous serine proteinases, elastase and chymotrypsin.

In contrast to the peptides mentioned above, antimicrobial peptides from the hard tick *Ixodes sinensis,* Ixosin (Yu *et al.,* 2006) and Ixosin-B (Liu *et al.,* 2008), do not contain any cysteine-residue, presenting in this way the linear antimicrobial peptides. Both peptides were isolated from the salivary gland and their activity against *E. coli, S. aureus*, and *C. albic*ans was shown.

ISAMP antimicrobial peptide isolated from saliva of *I. scapularis*, (Pichu *et al.,* 2009) was reported to possess antimicrobial activity against both Gram-positive and Gram-negative bacteria.

2.3. Projectives

The great variety of antimicrobial peptides from different sources was described till the date, but there is still need to continue the detection and characterization of new antimicrobial molecules as new tools against resistant bacteria. Ticks represent a rich source of AMPs. Very little is known about ticks antimicrobial peptides, even less is known about their functional characteristics. The understanding of tick immune system provides us the possibility to discover the new pharmaceutically important molecules that represent a new source of antibiotics of new generation with significant pharmacological potential.

3. MAIN GOALS:

The master thesis is focused on analysis of the new type of antimicrobial peptides with multiple histidines from the hard ticks *I. ricinus* and *I. scapularis.* The main goals of this study were to detect homologues of previously described histidin-rich antimicrobial peptides from *Amblyomma hebraeum* (hebraein) and *Rhipicephalus (Boophilus) microplus* (microplusin) (HM) in *I. ricinus* and *I. scapularis* and to compare all sequences from this unique group of antimicrobial peptides. An essential part of our study was devoted to expression and purification of recombinant HM protein for further analysis of it antimicrobial activity and analysis of tissue-specific differential expression pattern in *I. ricinus* and I*. scapularis*. One of the goals was to express and analyse a recombinant protein, ricinusin, a novel member of histidine-rich protein family that was identified in *I. ricinus* and characterized in bachelor thesis (Dornakova, 2008).

- To detect hisitidine rich sequences in *I. ricinus* and *I. scapularis.*
- To express and purify recombinant ricinusin and newly revealed recombinant his-rich proteins.
- To test the antimicrobial activity of acquired recombinant proteins.

4. MATERIALS AND METHODS:

Tab.2. Primers used in this study:

primer	sequence	T_A(°C)
RicinusRev	5´- CGC ATC ATG GTG GGC CGC TTC AG -3´	52
Ricinus mature	5´- CAC CGA GGA AGC CCA TGG AGC C -3´	55
SCA-Forw	5' - ATG AAG TCG CTY CTG GTT TGY C - 3'	48
SCA-Rev	5' - TTA RTG GTG TCC GTG TCC GTG - 3'	49
H/Msca_matureF	5' - CAC CCA TCA TGT AGA GCT GTG C - 3'	50
H/Msca_matureR	5' - TTA GTG GTG TCC GTG TCC GTG TTC GTG - 3'	55
HMsca_FWD_Ndel	5' - GGA CAT ATG CAT CAC GTA GAG CTG TGC AAA AAG - 3'	55
HMsca_Rev_Xhol	5' - ATT CTC GAG TTA GTG GTG TCC GTG TCC - 3'	54
ACTINgamaF	5' - CAC CTT CCA GCA GAT GTG GAT CTC - 3'	50
ACTINgamaR	5' - CAA ACC ATC GTT AGG GCA TCG G - 3'	50
T7-Forw	5´- TAA TAC GAC TCA CTA TAG GG -3´	50
T7-Rev	5´- GCT AGT TAT TGC TCA GCG G -3´	50
M13-Forw	5´- GTA AAA CGA CGG CCA -3´	50
M13-Rev	5´- CAG GAA ACA GCT ATG AC -3´	50

4.1. Ticks:

Ixodes ricinus ticks were provided by the internal tick facility of the Biological Centre, Institute of Parasitology, Academy of Science of the Czech Republic, and *Ixodes scapularis* ticks were obtained from the Department of Microbiology and Immunology, University of Maryland School of Medicine, USA. Uninfected ticks were fed on adult guinea pigs so that three unfed females and one male (per animal) were placed into the cell, attached to the shaved area on the guinea pig back and left there until they had been fully engorged (Rudenko *et al*., 2005). Laboratory animals were treated in accordance with the Animal Protection Law of the Czech Republic no. 246/1992 Sb.

4.2. Primers design, PCR and sequence analysis:

The degenerate primers for amplification of hebraein/microplusin like ('HM like') genes (see tab.2. Sca-forw and Sca-Rev) were designed according to hebraein and

microplusin homologues sequences found in genome of *I. scapularis* (http://iscapularis.vectorbase.org). Homologous genes were amplified by PCR using cDNAs (see 4.3.) from blood-fed *I. scapularis* and *I.* ricinus females as templates. The amplification was performed in 0.2 ml thin-wall PCR tubes using Mastercycler (Eppendorf, Germany) thermal cycler.

PCR reaction:
2x GoTaq Colorless Master Mix (Promega, USA) . 10µl
Forward primer (10 µM) 1µl
Reverse primer (10 µM) 1µl
DNA template* 0.2-1µl
dH_2O ..... up to 20 µl
*(120 – 150ng/ml)

The results of the PCR were analysed by agarose gel-electrophoresis.

Tab.3. used chemicals:

buffer	**composition/supplier**
Agarose (SERVA)	1.5 % agarose dissolved in 1x TAE buffer
50x TAE buffer	2M Tris-acetate; 50mM EDTA
5x loading dye	20% Ficoll; 10mM Tris-HCl (pH 8.0); 1mg/ml 'Orange G'
SYBR Green	500x 'SYBR Green'(Sigma) in 5x loading buffer

The PCR products were cut off and eluted from the agarose gel (QIAquick® Gel Extraction Kit', Qiagen, USA), cloned into pCR®4-TOPO® (Invitrogen, USA) vector for sequencing according to manufacturer's protocol and the construct was transformed in One Shot® TOP10 competent cells. Isolated plasmid DNA (QIAprep® Miniprep Handbook, Qiagen, USA) was consequently sequenced with M13 forward/reverse primers (see tab.2.) in both directions at the laboratory of FS SBU (Faculty of Science, University of South Bohemia) and BC ASCR (Biology Centre of the Academy of Sciences of the Czech Republic) in České Budějovice (ABI 3130, Applied Biosystems, USA). Acquired nucleotide and predicted amino acid sequences were compared to related sequences available from public databases.

4.3. Semi-quantitative reverse transcriptase-polymerase chain reaction analysis (RT-PCR):

To analyze the expression of the homologous genes in *I. ricinus* and *I. scapularis* the semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. The total RNA was isolated from fed and unfed larvae, nymph and adult females and different organs (salivary glands, malphigian tubes, midgut and ovary) from blood fed female of *I. ricinus* and *I. scapularis*. The RNA was extracted from all tick samplesusing TRI reagent (Sigma, USA) according to the recommendations of the manufacturer. The integrity of total RNA was checked on 1.2% agarose gel and the concentration of the acquired RNA was determined by measuring the absorbance at 260 nm (A_{260}) on Nanodrop (Thermo Scientific, USA).

Consequently, the cDNAs were synthesized from isolated RNAs by two steps process with anchored oligo $(dT)_{23}$ primers using the 'Enhanced Avian HS RT-PCR kit' (Sigma, USA) according to manufacturer's recommendations. Synthesized cDNAs were controlled by measuring the concentration at 260nm on Nanodrop (Thermo Scientific, USA).

As a positive control in RT-PCR the gen-specific primers for amplification of housekeeping gene actin (AY333957) (see tab.2.) were used.

Tab.4. used chemicals:

buffer	**composition**
Agarose (SERVA)	1.2% agarose dissolved in 1x TBE buffer
1 x TBE buffer	89mM Tris base; 89mM Boric acid; 2 mM EDTA
SYBR Green	500x 'SYBR Green'(Sigma) in 6x loading buffer
6x loading dye	0.25% (w/v) bromphenol blue; 0.25% (w/v) xylene cyanol; 30% glycerol;1.2% lauryl suphate (SDS); 60mM sodium phosphate; pH 6.8

4.4. Preparation of recombinant protein:

4.4.1. The ligation of fresh PCR products into the vector:

Two different vectors were used to prepare recombinant proteins with and without his-tag, pET 100/D-TOPO® (Invitrogen, USA) and pET-17b (Novagen, USA). Gene-specific primers for amplification and further cloning of insert into vectors were synthesized according to kit requirement.

pET 100/D-TOPO® vector allows the expression of fusion protein with N-terminal histidine-tag (6xHis) for detection and purification. The TOPO® cloning reaction was done according to recommendation of manufacturer.

The specially designed primers containing Ndel and Xhol restriction sites were used for amplification and further cloning of PCR products into pET-17b vector. The PCR products were digested with restriction enzymes Ndel and Xhol, gel – purified (QIAquick® Gel Extraction Kit', Qiagen, USA) and cloned into pET-17b vector using T4 DNA ligase under standard conditions.

Both recombinant constructs were transformed into competent cells OneShot®TOP10 (Invitrogen, USA). The construction was verified by sequencing.

4.4.2. Expression of recombinant protein:

The prepared constructs (see 4.4.1.) were used to transform *E. coli* BL21 (DE3) (Invitrogen, USA) and BL21 (DE3) pLysS (Novagen, USA) competent cells according to manufacturer recommendation. The expression of recombinant proteins was induced in two ways, using glucose or isopropyl-β-d-thiogalactopyranoside (IPTG) protocols.

In the first case, the transformed bacteria were grown in LB broth with ampicillin (50 μg/ml), chloramfenicol (34 μg/ml, in the case of BL21 (DE3) pLysS cells) and glucose (20mM) at 37°C to an approximately OD_{600} =1. Then, the glucose was washed out and the culture was grown in fresh LB medium overnight at 37°C.

The induction with IPTG was done in similar way, but the bacterial culture was prepared in LB broth with ampicillin (50 μg/ml), chloramfenicol (34 μg/ml, in the case of BL21 (DE3) pLysS cells) but without glucose. 1M IPTG (final concentration

0.5mM) was added to grown bacterial culture (OD_{600}=1) and incubated overnight at 37°C.

4.4.3. Purification of recombinant protein:

Expressed protein was purified in different ways depending on the presence or absence of his-tag.

Recombinant protein with his-tag:

The recombinant protein containing his-tag was purified using cobalt (Co^{2+}) HiTrap IMAC FF (5ml, GE Healthcare) column under native and denaturating conditions. Resuspended and lyzed bacterial pellet was loaded into the pre-equilibrated resin. After washing, the bound proteins were eluted from the resin by rising concentration of imidazol in used buffer.

Tab.5. used buffers:

buffer	composition	pH
Purification under native conditions:		
lysis	50mM NaH_2PO_4, 300mM NaCl, 10mM imidazol	8.0
washing	50mM NaH_2PO_4, 300mM NaCl, 20mM imidazol	8.0
elution	50mM NaH_2PO_4, 300mM NaCl, 250mM imidazol	8.0
Purification under denaturating conditions:		
washing	8M urea, 50mM Tris, 500mM NaCl, 10mM imidazol	8.0
washing II	8M urea, 50mM Tris, 500mM NaCl, 50mM imidazol	8.0
washing III	8M urea, 50mM Tris, 500mM NaCl, 70mM imidazol	8.0
elution	8M urea, 50mM Tris, 500mM NaCl, 250mM imidazol	8.0
elution II	8M urea, 50mM Tris, 500mM NaCl, 1M imidazol	8.0

Recombinant protein without his-tag:

Total proteins were isolated from cytoplasm, membrane and inclusive bodies of the obtained bacterial pellet (see 4.4.3). Recombinant protein was detected in one of these fractions. Protein purification was done on MONO Q anion exchange column. Proteins were eluted with rising concentration of salt.

Tab.6. used buffers:

buffer	composition	pH
resuspend (cytoplasm protein)	20mM Tris-HCl	8.0
isolation (membrane protein)	2M urea; 20mM Tris-HCl; 0.5M NaCl; 10mM imidazol; 1mM 2-mercaptoethanol; 2% Triton X-100	8.0
solubilisation (inclusive bodies proteins)	6M guanidin hydrochlorid; 20mM Tris-HCl; 0.5M NaCl; 5mM imidazol; 1mM 2-merkaptoethanol	8.0
buffer for purification through MONO Q		
20mM Tris-HCl		8.0
20mM Tris-HCl, 1M NaCl		8.0

4.4.4. Refolding of recombinant protein:

Purification under denaturing conditions (see 4.4.3) destroyes the protein tertiary structure that is vitally important for the function of the protein so the refolding procedure of the purified protein is necessary to restore its functional abilities.

The eluted recombinant protein was dialyzed against different buffers (see tab.6.). Dialysis buffer I contained 8M urea that was subsequently gradually replaced by glycerol (dialysis buffer II). Finally, glycerol was also slowly washed out by dialysis buffer III

Tab.7. used buffers:

buffer	composition	pH
dialysis buffer I	50mM Tris; 150mM NaCl; 8M urea	9.0
dialysis buffer II	50mM Tris; 150mM NaCl; 20% glycerol	8.5
dialysis buffer III	50mM Tris; 150mM NaCl	8.5

4.5. The analysis of recombinant protein:

4.5.1. SDS-PAGE:

The presence and the purity of the proteins were tested using the SDS-PAGE. The 15% gels for the electrophoretic analysis were prepared according to manual (Lab FAQs, Roche). The samples were mixed with 2 x sample buffers and denaturated at 97°C for 5 minutes before loading on the gel. The electrophoresis was run through stacking gel at 100 V then the voltage was increased to 200 V (for protein separation in running gel). After the end of electrophoresis the gel was stained by Comasie brialliant blue R250 and unstained by unstaining solution or distilled water. The samples containing guanidine hydrochlorid were dialyzed against distilled water for 1 hour at 4°C.

Tab.8. used buffers:

buffer	composition
5x sample	250mM Tris-HCl (pH 6.8); 500mM dithiotreitol; 10% SDS; 0.2% bromophenol blue; 50% glycerol
10x running	0.250 M Tris; 1.92M glycin; 1% SDS
staining	0.05 % Coomasie Brilliant Blue R-250; 50% methanol; 10% acetic acid
unstaining	25% methanol; 10% acetic acid

4.5.2. Preparation of specific antibodies:

Specific antibodies from mouse:

The purified protein was used for the preparation of specific antibodies in mice. The protein (50-100 μg/ml) was mixed with Freund's adjuvant (SERVA) in the ratio 1:1. BALB/c mice were immunized with this solution 3x in 7 days interval. Mice were bled 7 days after the last injection. The blood was left to coagulate and consequently centrifuged (2 000 rpm, 4°C, 20min). The obtained serum was mixed with glycerol (1:1). The prepared antibodies were stored at -20°C.

Specific antibodies from rabbit:

The rabbit was immunized with purified protein (100-200 µg/ml) mixed with Freund's adjuvant (SERVA) 5x in 7 days. After the coagulation and centrifugation the acquired serum was precipitated with caprylic acid (25 µl/ml). After the centrifugation and filtration the supernatant was dialysed against sodium phosphate (5 nM). The prepared antibodies were stored at -20°C.

4.5.3. Western- blot analysis:

Transfer of proteins from the gel to the membrane:

The samples separated on the acryl-amid gel were transferred to PVDF (polyvinylidene fluorid) membrane (0.2 µm, Millipore) using the western-blot method (Towbin *et al.*, 1979).

Tab.9. used buffers:

buffer	composition
blotting	20% methanol; 25mM Tris-Base; 150mM glycin

Detection of the protein with Ni-NTA conjugates:

The recombinant protein containing his-tag (see 4.2.2.) was detected using Ni-NTA conjugates. The hybridization was done according to manufacturer's manual (QIAexpress® Detection and Assay Handbook; Qiagen; protocol 9: Detection with Ni-NTA Conjugates). After blocking and incubation with antibodies the signal was revealed with HRP (horseradish peroxidase) solution. HRP was prepared immediately before staining according to manufacturer's recommendation.

Detection of the protein with specific antibodies:

The specific antibodies obtained as described above (see 4.5.2.) were used in this hybridization. During the hybridization the membrane was incubated in blocking buffer followed by incubation with primary antibodies diluted in blocking buffer and final incubation with secondary antibodies SwAR/Px (1: 1000, 5% milk). The signal was revealed with HRP as described above (see 4.2.2. '*Detection of the protein with Ni-NTA conjugates*').

4.6. Antimicrobial assays:

4.6.1. Pilot antimicrobial test – double-layer technique:

This experiment was originally developed for bacteriophage titration. It was used as a quick qualitative determination of the antimicrobial properties according to Čeřovský *et al.* (2008).

LB agar in Petri dishes was overlaid with "soft" agar (0.5% agar in LB without ampicillin) containing grown bacterial culture of *Micrococcus luteus* (about 10^7 colony forming units (CFU)). After the solidification of the upper layer the tested protein solutions (2 μl) were dropped on it. The plates were incubates at 37°C for few hours.

4.6.2. Determination of the minimal inhibitory concentrations (MICs):

This experiment was performed at the Institute of Organic Chemistry and Biochemistry in Prague. The minimal inhibitory concentrations (MICs) were established by observing bacteria growth in the presence of the tested protein. The MIC was measured in multi-well plates. The bacteria in mid-exponential phase were added to individual wells containing LB broth with different concentrations of the tested peptides (ranged from 0.5 to 100 μM, final volume 0.2 ml). The plates were incubated with continuous shaking at 37°C for 20 hrs in a Bioscreen C instrument (Helsinki, Finland). The ability to inhibit the growth of bacteria was measured at A_{540} nm every 15 min. The tested bacteria were *Bacillus subtilis, Micrococcus luteus*, *Staphylococcus aureus* and *Escherichia coli*.

4.6.3. Determination of the minimal bactericidal concentration (MBCs):

The minimal bactericidal concentration was established according to Tran *et al.* (2008). The overnight bacterial culture of *M. luteus* and *B. subtilis* was subcultured with fresh LB without ampicillin and incubated with shaking at 37°C until the bacterial reached the log-phase. After the dilution with 1 x PBS (pH 7.4) (1-2 x 10^6 cells/ ml) the cells were mixed with the tested protein (final concentration 100μM).

Subsequently, the mixture was incubated for 1 hour at 37°C without shaking. The reaction was finally diluted 100 x or 1000 x, spread on LB agar plates and incubated at 37°C overnight. Grown colonies were counted and compared with number of grown colonies on the control plates (bacteria without protein).

4.7. Serine protease inhibition assays:

The analysis was perfomed by Michalis Kotsyfakis, PhD (The Institute of Parasitology AS CR in České Budějovice).

All assays were performed at 30°C. Recombinant protein (2 μM) was pre-incubated with selected set of serine proteases for 10 min before addition of the corresponding substrate. All used enzymes were of human origin, purified or recombinant. Alpha (α)-chymotrypsin (0.025 nM) was purchased from Sigma; elastase (0.18 nM) was purchased from Elastin Products; cathepsin G (4.4 nM) was from Molecular Innovations; proteinase 3 (5.5 nM) was from Merck; and sequencing-grade trypsin (0.1 nM) was purchased from Roche.

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

Table 10: used chemicals

buffers for	**composition**
elastase & protease 3	50 mM Hepes buffer, pH 7.4, 100 mM NaCl, 0.01% Triton X-100
trypsin & α-chymotrypsin	50 mM Tris-HCl, pH 8, 150 mM NaCl, 20 mM $CaCl_2$, 0.01% Triton X-100
cathepsin G	50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.01% Triton X-100
used substrates	
elastase & proteinase 3	Suc-Ala-Ala-Pro-Val-AMC
trypsin	Boc-Gln-Ala-Arg-AMC
α-chymotrypsin & cathepsin G	Suc-Ala-Ala-Pro-Val-AMC

ANOVA analysis was used for statistical analysis of the observed inhibition in the presence of the protein, and statistical significance was considered when $p < 0.05$ when comparing the enzymatic activity in the presence or absence of the inhibitor.

4.8. Synthesis of C-terminal his-rich part of the protein:

The peptide was prepared by solid-phase peptide synthesis (SPPS). The synthesis runs in the opposite way from biosynthesis, i.e. from C-terminal end of the peptide to N-terminus. Due to the possibility of unwanted interactions, the amino acids used were protected with 9-fluorenylmethyloxycarbonyl (Fmoc). The Fmoc method utilizes a base, usually piperidine (20%) in dimethylformamide (DMF) to remove protected group and exposes the α-amino group for the reaction with another activated amino acid. The general principle of SPPS consists in repetition of coupling-wash-deprotection steps. (see fig.2.)

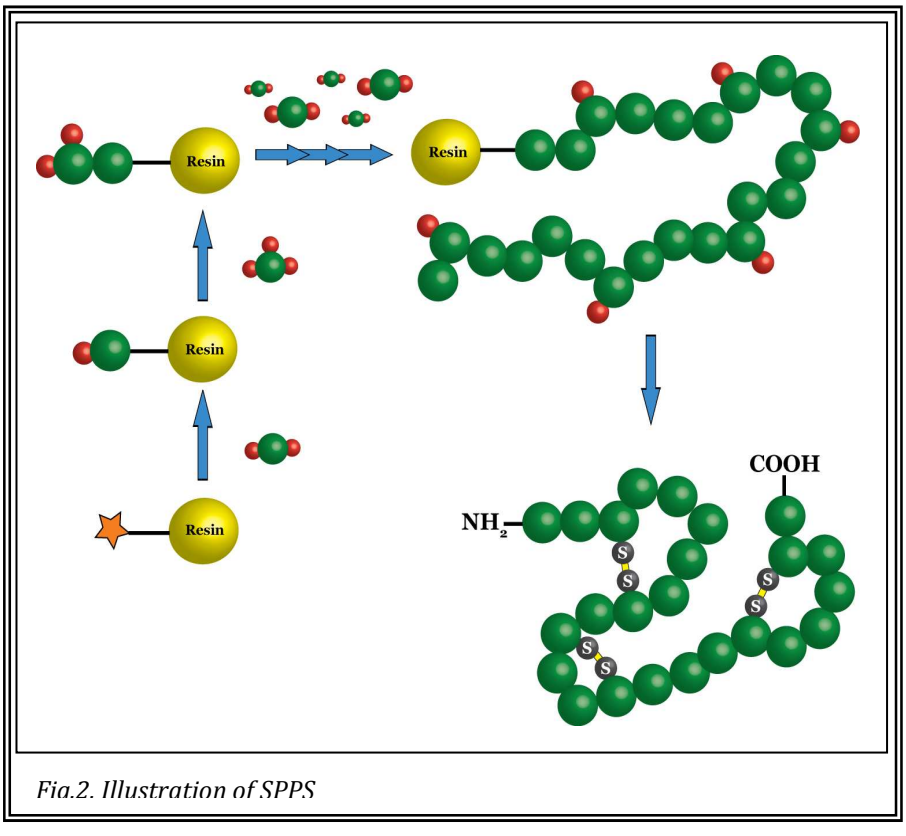


Fig.2. Illustration of SPPS

4.8.1. Preparation of synthetic peptide:

The Fmoc- protected peptide was synthesized on the preloaded Fmoc-Rink-Amide-MBHA resin (0.11g) in 10 ml propylene syringe. Fmoc deprotection was achieved with 20% piperidine in dimethylformamid (DMF)(1x 4ml, 10 min; 1x 4ml, 20 min). After each coupling and deprotection step, the resin-peptide was washed with DMF (3x 3ml), isopropanol with DMP (3x 3ml) and again with DMP alone (3x 3ml). In the coupling step, Fmoc-amino acid derivates, 1-hydroxy-benzotrazole (HOBt) and bromphenol blue indicator (BB) (1 µl) in DMF (600 µl) was mixed with resin-petide. The resin-petide was then soaked with a 2M solution of diisopropylcarbodiimid (DIC) in DMF and gently agitated.

Thus, the peptide sequence was elongated by changing the coupling, deprotection and washing steps. With the growing peptide chain, the reaction time was prolonged from 2 to 15 hours.

4.8.2. Monitoring of the synthesis:

After the eighth and the fourteenth cycles, ≈ 10 mg of dry protected resin-peptide was treated with the mixture of trifluoroacetic acid (TFA)/TIS/H_2O in the ratio 95:2.5 : 2.5 (200 µl) (3 hours, room temperature) for cleaving the peptide out of the resin. The resin was filtered and TFA was removed from the solution by "blowing" with CO_2. The acquired peptide was precipitated with anhydrous tri-butyl methyl ester (TBME). The fully deprotected peptide was analyzed for mass spectrometry (MS) and HPCL.

4.8.3. Cleavage of the synthesized protein:

After the coupling of the last Fmoc-protected amino acid followed by deprotection, the resin-peptide was washed by isopropanol (10x 3ml) and methanol (10x 3ml). The washed resin-peptide was dried in vacuum and then cleaved with the mixture of TFA/TIS/H_20 in a ratio 95: 2.5 : 2.5. The resin was filtered, TFA was "blown away" with CO_2 and the peptide was precipitated with TBME. The crude peptide was purified by preparative HPLC and its identity was verified by mass spectrometry (MS).

4.9. Bioinformatics:

Amino acid sequences were aligned in BioEdit 7.0.5 (Hall, 1999) using Clustal W algorithm with default setting. Alignment was then manually edited in BioEdit (Hall, 1999), unaligned and ambiguously aligned sites were deleted from the data set. Evolution model was estimated using Prottest 2.4 (Abascal *et. al.*, 2005). According to the Akaike's information criterion (AIC), the Dayhoff + Γ (with gamma distribution in 4 categories) model was selected. Maximum likelihood (ML) tree was constructed using PhyML 3.0 (Guindon and Gascuel, 2003)

5. RESULTS:

5.1. Characterization of hebraein/microplusin homologues:

Using the degenerate primers designed on the basis of microplusin (AY233212.1) and hebraein (AY437139.1) sequences new genes homologous to above mentioned AMPs were detected in *I. ricinus* and *I. scapularis.*

Six isoforms were identified in engorged females of *I. ricinus* (HM_ric_1-6) and three (HM_sca_1-3) in *I. scapularis* female. Predicted amino acid sequences for acquired homologues have the same cysteine motif and also comprise a histidine rich C-terminus as hebraein and microplusin with the similarity of about 45% and 40%, respectively. The number of histidine residues near their C terminus varies. While his-rich C-terminus of microplusin and hebraein consist of 4 and 9 histidines, respectively, the acquired homologues have 6 (HM_sca_2), 9 (HM_ric_4), 10 (HM_sca_1 & 3 and HM_ric_5,) and 12 (HM_ric_1-3 & 6) histidines on their ends.

The similarity between acquired sequences is shown on the table 11.

Table 11: Identity/similarity matrix of detected HM protein - the numbers represents the percentage identity and divergence found between the predicted amino acid (AA) sequences:

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9		
1		90.7	88.4	91.4	86.0	89.1	89.1	84.5	91.5	**1**	HM_ric_1
2	10.4		97.7	87.5	93.0	98.4	89.1	84.5	87.6	**2**	HM_ric_2
3	13.2	2.5		85.2	90.7	99.2	86.8	82.2	85.3	**3**	HM_ric_3
4	4.4	9.1	12.1		93.0	85.9	96.1	90.6	100.0	**4**	HM_ric_4
5	11.0	2.6	5.3	8.1		91.5	94.6	89.1	93.0	**5**	HM_ric_5
6	12.3	1.6	0.8	11.1	4.4		87.6	82.9	86.0	**6**	HM_ric_6
7	7.1	7.1	10.0	4.4	6.2	9.0		94.6	96.1	**7**	HM_sca_1
8	7.5	7.5	10.6	5.6	7.5	9.5	0.9		90.7	**8**	HM_sca_2
9	4.4	9.0	11.9	0.0	8.1	11.0	4.4	5.6		**9**	HM_sca_3
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**		

All sequences encoding *Ixodes* HM genes were less than 400 bp long and deduced protein sequences were approximately 120 amino acids (aa) including signal sequence of 19 aa (Signal P; http://www.cbs.dtu.dk/services/SignalP/). No

glycosylation was identified by NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc) and NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/). For details see table 12.

HM_sca_1 recombinant was chosen for all further analysis.

Table 12: genes identified in this study

gene	tick species	nucleotide sequence (bp)	AA no.	MW (kDa)	pI	predicted glycosylation	signal sequence
HM_ric_1	*I.ricinus*	375	125	13.65	6.36	no	19 aa
HM_ric_2	*I.ricinus*	375	125	13.61	6.73	no	19 aa
HM_ric_3	*I.ricinus*	375	125	13.66	6.85	no	19 aa
HM_ric_4	*I.ricinus*	357	119	13.05	6.43	no	19 aa
HM_ric_5	*I.ricinus*	357	119	13.01	6.67	no	19 aa
HM_ric_6	*I.ricinus*	375	125	13.63	6.85	no	19 aa
HM_sca_1	*I. scapularis*	357	119	12.99	6.55	no	19 aa
HM_sca_2	*I. scapularis*	339	113	12.32	6.65	no	19 aa
HM_sca_3	*I. scapularis*	357	119	13.05	6.43	no	19 aa

5.2. Phylogenetic analysis:

The amino acid sequences used for phylogenetic analysis are shown in table 13. Neutrophil elastase inhibitor from *R. microplus* was used as an outgroup.

Table 13: Sequences used for phylogenetic analysis

name used in phylogenetic analysis	tick species	GenBank accession no.
hebraein	*Amblyomma heraeum*	AAR97292
microplusin 1	*Rhipicephalus microplus*	Q86LE5
microplusin 2	*Rhipicephalus microplus*	AAO48492
AMM	*Argas monolakensis*	ABI52699
ISM1	*Ixodes scapularis*	XP_002409778
ISM2	*Ixodes scapularis*	AAY66495
ISH1	*Ixodes scapularis*	XP_002410155
ricinusin	*Ixodes ricinus*	ABB79785
OCM	*Ornithodoros coriaceus*	ACB70336
OCH1	*Ornithodoros coriaceus*	ACB70335
OCH2	*Ornithodoros coriaceus*	ACB70337
OPH	*Ornithodoros parkeri*	ABR23424
neutrophil elastase	*Rhipicephalus microplus*	ABH10604

The phylogenetic tree represents two main groups (see fig. 3). The first group comprises ricinusin and microplusin-like protein from *I. scapularis* detected in GenBank. Results separated ricinusin from hebraein and microplusin, indicating that it might belong to other protein group.

The second group consists of hebraein/microplusin-like family and contains the sequences identified in this study. It is obvious that identified HM sequences are related to hebraein and microplusin and to their homologues published in the GenBank. On the other hand, despite the low bootstrap support this group is divided into two subgroups: one group containing hebraein and microplusin and their homologues detected in soft ticks (*Argas* and *Ornithodoros*) and the second

comprising hebraein/microplusin homologues from hard tick species (*I. ricinus* and *I. scapularis*) identified in this study.

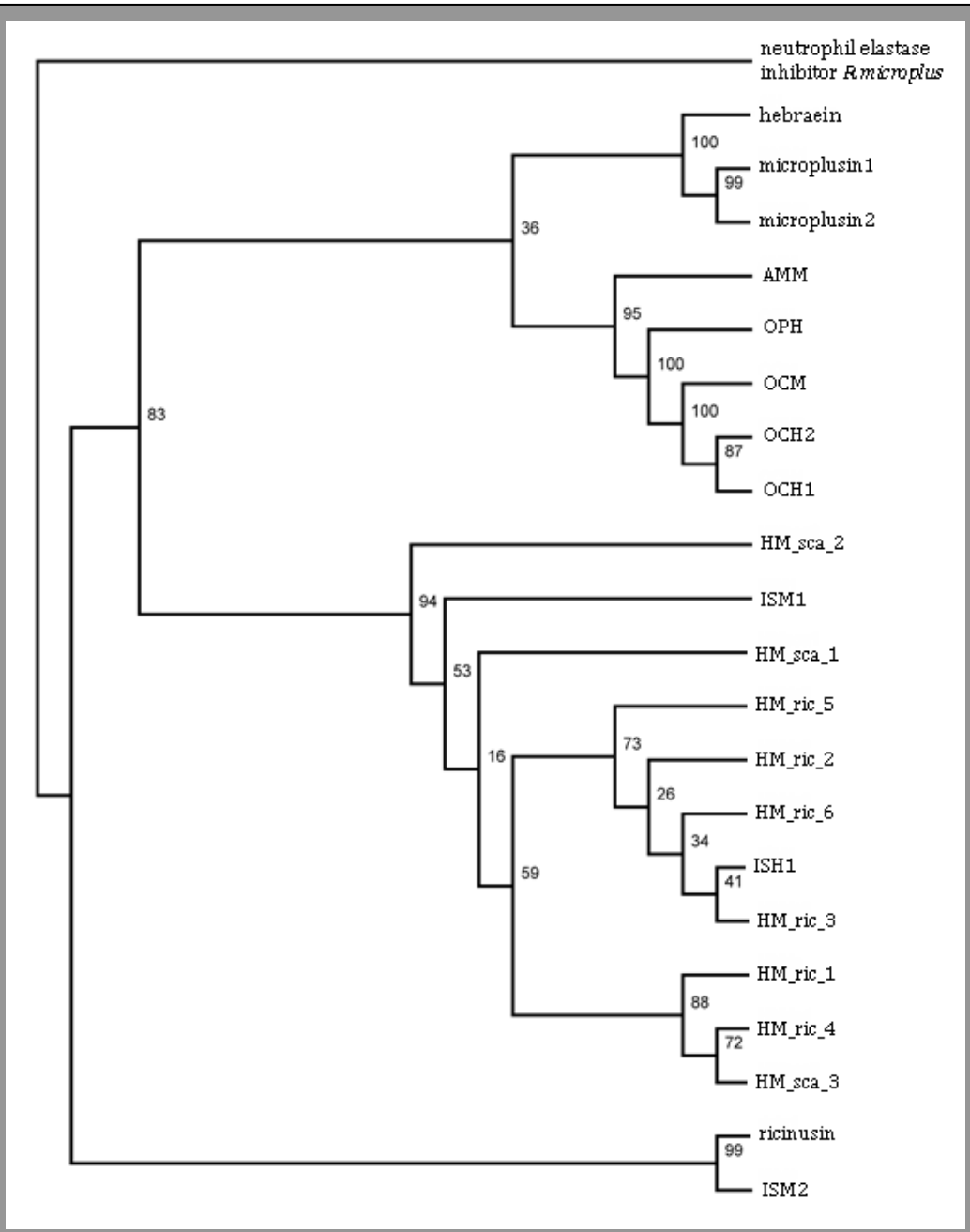


Figure 3: *The tree is showing the phylogenetic relationship based of amino acid sequences of protein identified in this study and hebraein/microplusin like proteins published on GenBank. The numbers represent the percentage of 100 replications (bootstrap support) for which the same branching patterns were obtained.*

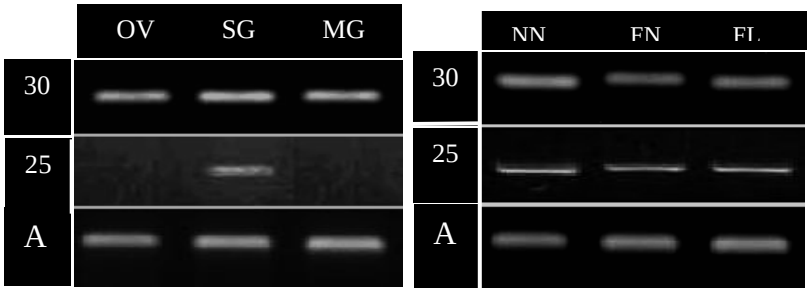
5.3. Differential expression of detected genes:

Synthesized cDNAs from various tissues of *I. ricinus* and *I. scapularis* were analysed by PCR (30 and 25 cycles) for the tissue-specific pattern of expression of HM genes. Although, the results revealed that HM genes are transcribed in all tested tissues (see fig.4.A.), the stronger expression of HM_sca_1 – representative of genes identified from *I. scapularis* – was confirmed in salivary glands. Salivary glands and midgut were defined as a primarily sites of expression of homologues detected in *I. ricinus* (see fig.4.B)

We studied the differential expression of detected genes in different tick developmental stages as well. While the level of expression of HM_sca_1 is the same in *I. scapularis* larvae, nymph and adult, the genes from *I. ricinus* showed lower expression in larvae (unfed and fed) than in other analysed stages. The induction of expression with blood feeding was not proved.

These data indicate that homologues genes might be a part of defence against invading pathogens received during blood feeding taking in concideration their sites of expression (salivary glands and midgut). However the induction of expression was not proved.

A) Differential expression of HM_sca_1 gene (I. scapularis)



B) Differential expression of genes detected from I. ricinus

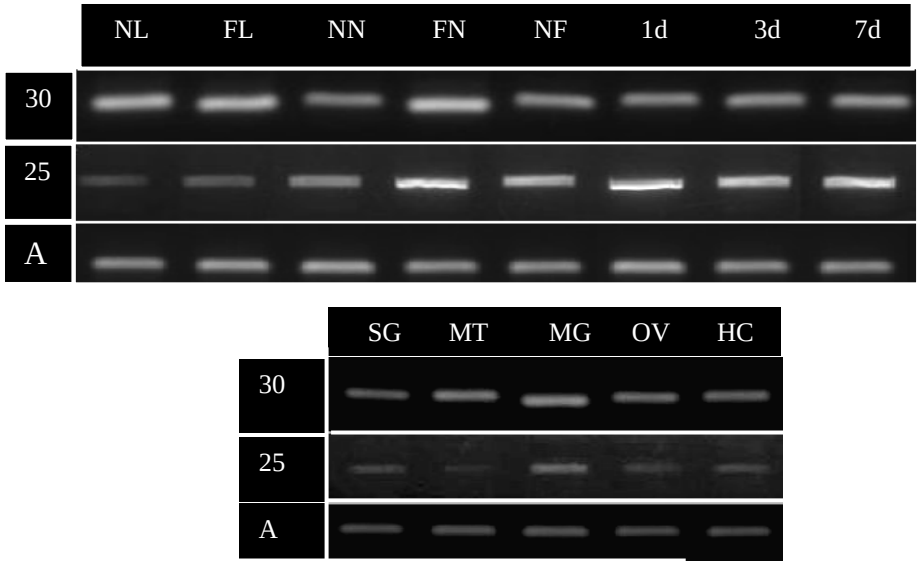


Figure 4: Differential expression of genes identified in this study *was determined using the RT-PCR with synthesized cDNA from different organs and develop. stages from I. ricinus and I. scapularis. The PCR was done for 30 and 25 cycles to see that in some cases the level of expression differs. As a control the gene-specific primers for housekeeping gene actin were used.*

30 – 30 cycles
25 – 25 cycles
A – actin

OV – ovary
SG – salivary gland
MT – malphigian tubes
MG – midgut
HC – hemocytes

NL/FL – non-fed/fed larvae
NN/FN – non-fed/fed nymph
NF – non-fed female
1d/3d/7d – female fed for 1/3/7 days

5.4. Functional analysis of recombinant ricinusin:

His-tag fusion recombinant ricinusin was expressed in *E. coli* BL21 (DE3). The expression was induced by washing out the glucose from the cultivation medium. The expressed recombinant was purified on Co^{2+} resin (see fig.5., E1-E5) and concentrated. Recombinant ricinusin with predicted size of 16.68 kDa was identified using the western-blot with Ni-NTA conjugates and mass spectrometer analysis that was conducted by Peter Koník (University of South Bohemia in České Budějovice).

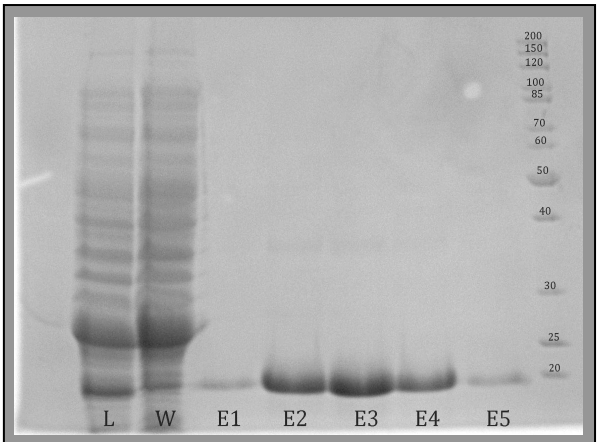
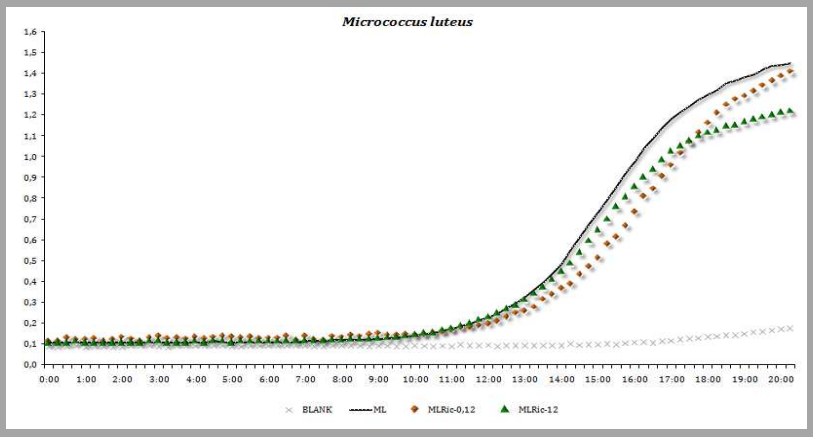


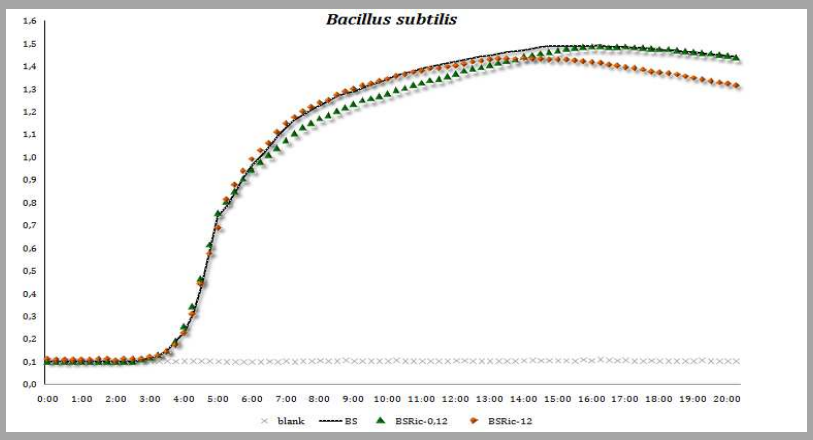
Figure 5: Purification of recombinant ricinusin through CO^{2+} resin.
L – lysate
W – wash
E1-E5 – fractions with eluted protein

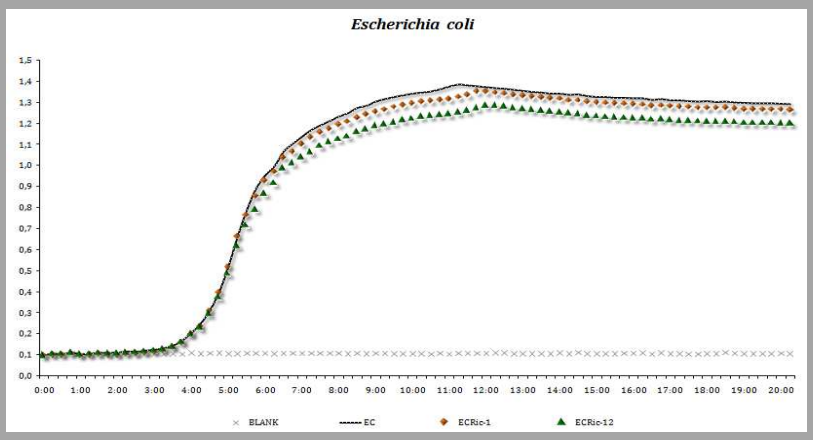
Purified ricinusin was used for preparation of specific antibodies in mice. Unfortunately, mice antibodies were not able to detect recombinant protein. Because of this inefficiency the specific antibodies in rabbit were prepared. The results were the same as described in the previous case.

Prepared recombinant ricinusin was used in antimicrobial assays. The activity was measured against four bacteria: *M. luteus, B. subtilis*, *E. coli* and *S. aureus.* The recombinant protein was used in diverse concentration: 2 µg/ml (0.12 µM), 4 µg/ml (0.25 µM), 8 µg/ml (0.5 µM), 16µg/ml (1 µM), 33 µg/ml (2µM), 67µg/ml (4 µM), 100 µg/ml (6 µM), 133 µg/ml (8µM) and 200µg/ml (12 µM). No antimicrobial activity was detected under used conditions (see fig. 6.).

Because of predicted glycosylations the recombinant ricinusin was prepared in baculoviruses (Dr. Rudenko N; work was conducted in Center for Ecology and Hydrology, Oxford, UK). Analysis of baculovirus-originated recombinant ricinusin in antimicrobial tests in concentration of 100 µg/ml (6 µM) didn't reveal any activities against mentioned bacteria under used conditions.







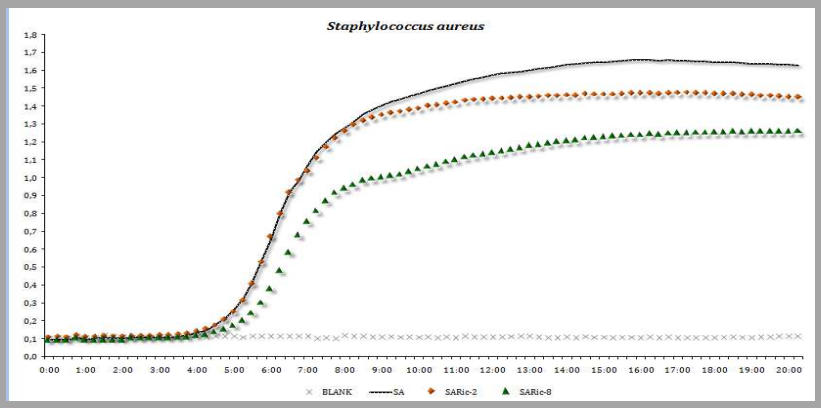


Figure 6: Determination of antimicrobial activity: *Recombinant ricinusin was tested against Gram⁺ (M. luteus and B. subtilis) and Gram⁻ bacteria (E. coli and S. aureus). The activity was measured using the protein concentrations from 0.12 to 12 μM. Only results of the lowest and highest used protein concentration are presented. LB-broth was used as the blank.*
It is obvious that recombinant ricinusin was not active in any of tested concentrated under used conditions.
The small decrease of absorbance in the case of the highest used concentration is caused by lower dilution of bacteria because the concentration of the protein was not high.

5.5. Recombinant HM-like peptides:

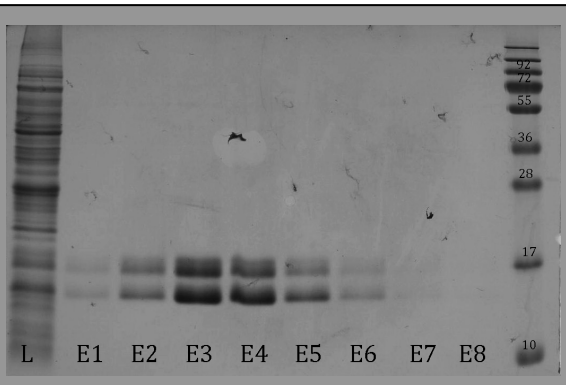


Figure 7: SDS-PAGE with the results of purification.
L-lysate E1-8 – taken fractions.
The protein was purified in fractions E1-E7. The highest concentration of eluated protein is in the fractions E3 and E4.

Recombinant his-tag fussion HM protein (14.09 kDa, including 3 kDa his-tag) expressed in *E. coli* BL21 (DE3) cells (induction with glucose) was purified (see fig. 7.; E1-E7). The purified protein was presented as a double-band on the SDS gel(fractions E1-E7). Western-blot analysis with Ni-NTA conjugates confirmed that both bands are his-tag-fusion protein of interest. These results might indicate that the protein forms multimers under conditions used for its purification. Protein identity was proved by mass spectrometer analysis, conducted by Peter Koník (University of South Bohemia in České Budějovice).

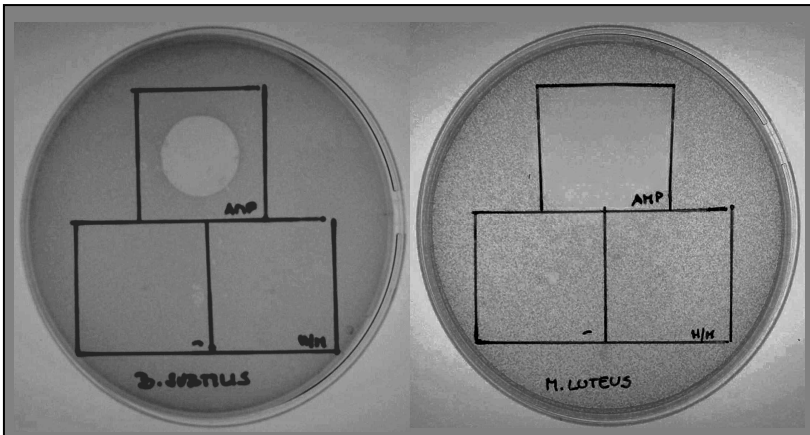


Figure 8: Tested antimicrobial activity of recombinant protein by double-layer technique
AMP – ampicillin (50mM)-positive control
- – negative control – buffer in which the protein is diluted
H/M – tested protein

Purified and refolded recombinant protein was tested against Gram-positive bacteria *M. luteus* and *B. subtilis* by double-layer technique (see fig. 8.). The tested concentration (400 μg/ml ≈ 30 μM) did not prove any antimicrobial activity.

Table 14: Measured concentrations

sample	concentration (μg/ml)	sample	concentration (μg/ml)
A2/3	70	A13/14	210
A7	70	A15	90
A8	300	B14	90
A9	130	B13	100
A10	280	B12	170
A11	370	B5	40
A12	430		

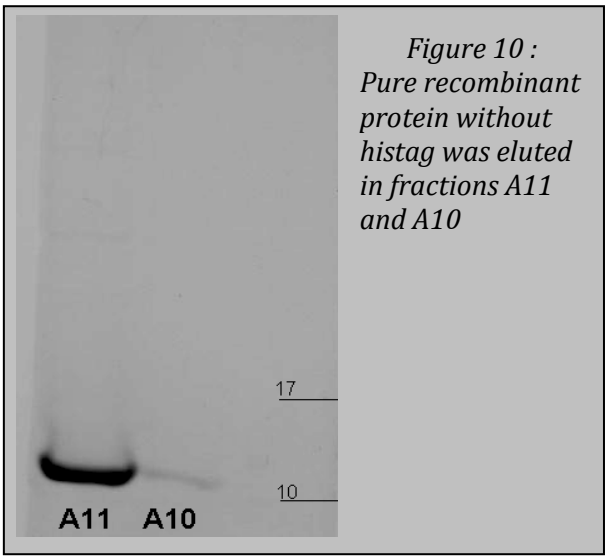


Figure 10 : Pure recombinant protein without histag was eluted in fractions A11 and A10

To be sure that the inefficiency of the protein in antimicrobial assays is not caused by his-tag, recombinant HM protein without his-tag was prepared in *E. coli* BL21 (DE3) pLysS cells. It was shown that after IPTG induction of HM protein expression it is produced mainly in cytoplasm. The results of purification are shown on fig.9. Fractions A2-3, A7-15, B14-12 and B5 were analyzed by SDS-PAGE. The most pure recombinant protein was eluted in fraction A11 and A10 (see fig.10.). Concentration of acquired protein measured on Nanodrop (Thermo Scientific, USA) was 370 μg/ml.

Selected fractions were tested by double-layer technique against Gram-positive bacteria *M. luteus* and *B. subtilis* with the highest possible protein concentration used (see tab.14.). The presence of antimicrobial activity was not revealed under used conditions (see fig. 11.).

Obtained results indicate that tested HM-like protein from *I. scapularis* does not possess antimicrobial activity under used conditions.

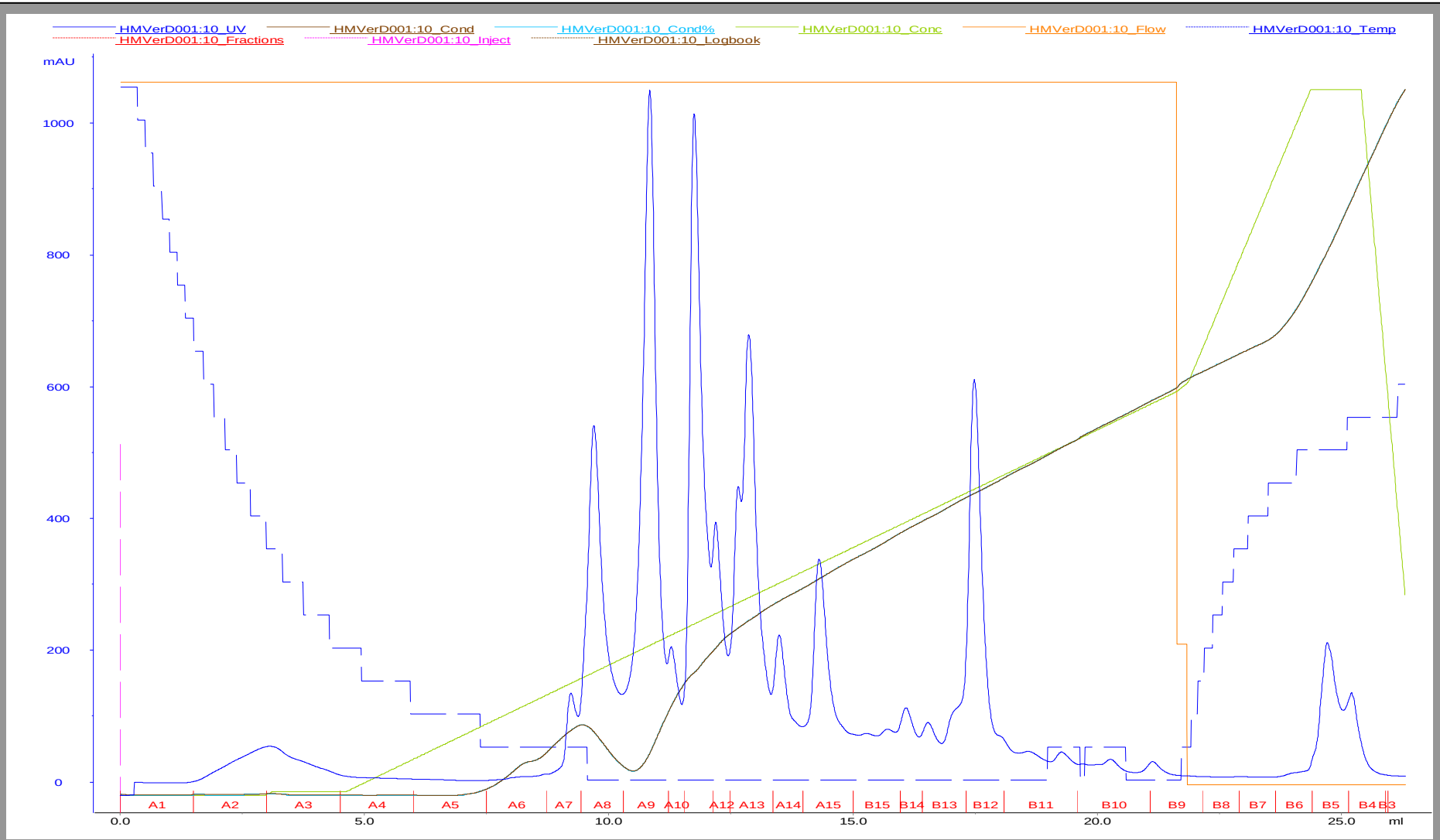


Figure 9: Process of purification through MONO Q.
The protein was eluted in fractions A2-3, A7-15, B14-12. These fractions were chosen for other analysis

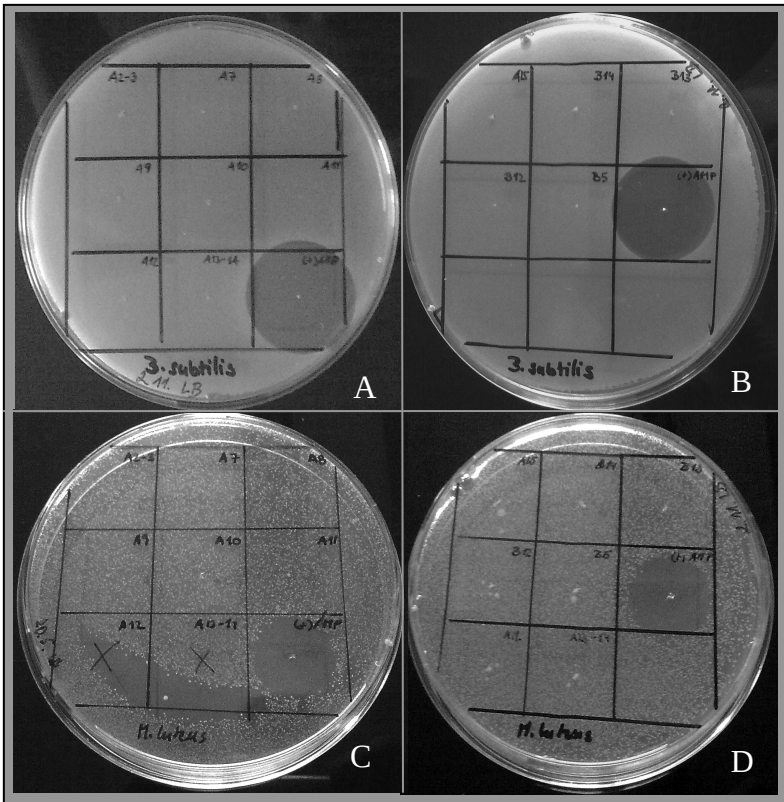


Figure 11: Double-layer assay with eluted proteins from MONO Q purification.

The fractions A2-3, A7-A15, B14-12 and B5 were tested against Gram-possitive bacteria B. subtilis (A & B) M. luteus (C & D). Ampicillin (50mM) was used as positive control. The proteins were used in the highest possible concentration (tab.13.) Non of the tested proteins was active against tested bacteria.

Subsequenctly, the fraction A11 containg eluted recombinant protein was used for serine protease inhibition assays. The protein was tested for inhibitor activity against the two 'archetypes' of serine proteases the enzymes chymotrypsin and trypsin and the elastase-like enzymes cathepsin G, proteinase 3 as well as pure elastase. All results were negative (see tab. 15 and fig. 12.).

Table 15: measured date

enzyme	Percentage of remaining enzymatic activity in the presence of 2.5 uM B11 fraction
elastase	105.9 ± 5.9
a-chymotrypsin	100.5 ± 0.8
trypsin	102.6 ± 2.4
cathepsin G	96.2 ± 0.4
proteinase 3	98.3 ± 6.8

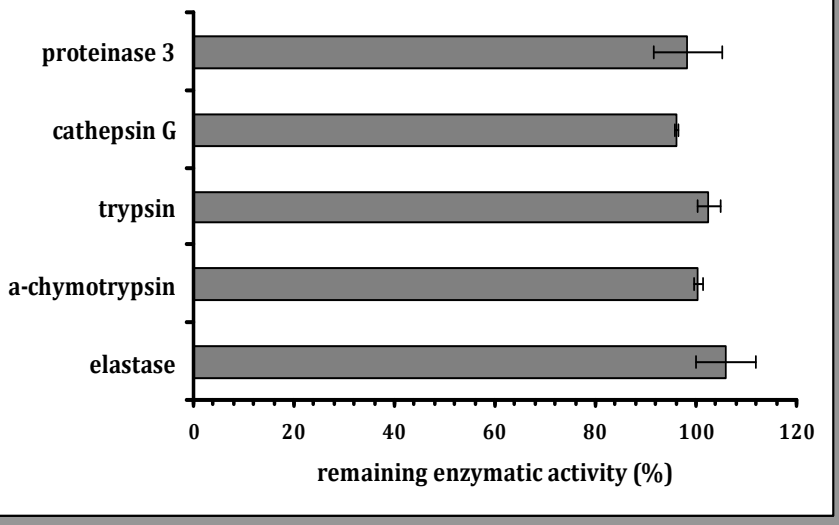


Figure 12: Graph representing measured data

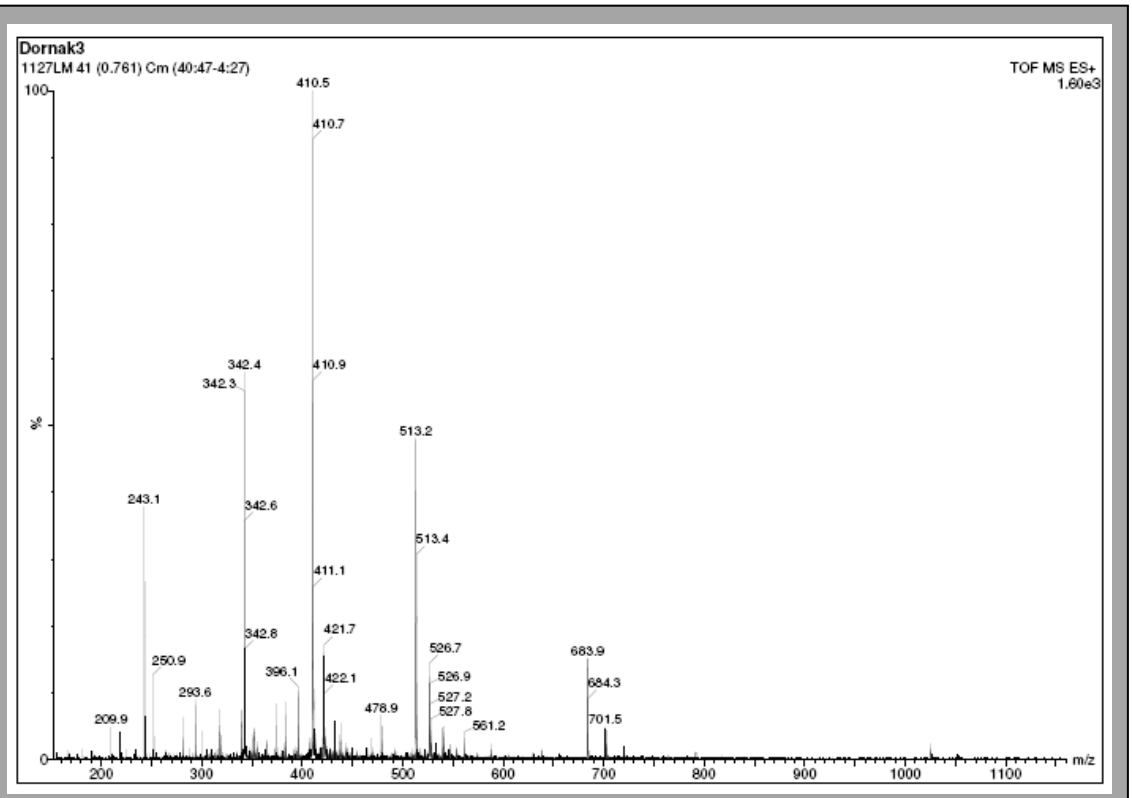
5.6. Histidine-rich C-terminal part of HM-like peptide:

The 20 amino acid peptide shown on fig. 14 was synthesized using solid phase peptide synthesis. Synthesized histidine rich protein was purified by preparative HPLC (see fig.15.) and was analyzed by mass spectrometer technique (see fig.15.).

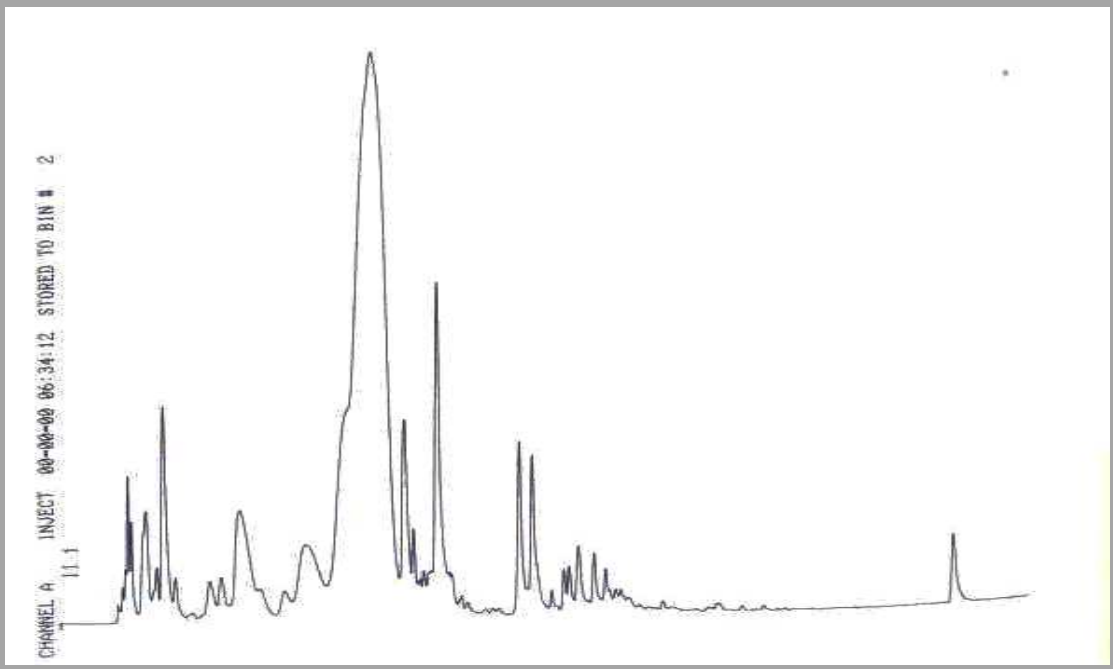
H-His-Ser-His-Glv-His-Glv-His-Glu-His-Ser-His-Glv-His-Glu-His-Glv-His-Glv-His-His-NH_2

Figure 15: The sequence of synthesized histidine rich peptide

The peptide was tested against Gram-positive bacteria *M. luteus* and *B. subtilis* in concentrations from 100 to 0.75 μM. It was proved that synthetised histidine-rich C-terminus of HM protein doesn't reveal any antimicrobial activity against tested bacteria.



A



B

Figure 16: Results of MS analysis (A) and preparative HPLC (B)

6. DISCUSSION:

Antimicrobial peptides/proteins are an essential part of tick innate immune system that is employed in protection of organism from invasion of pathogenic microorganisms. In terms of co-evolution, ticks have developed multiple antimicrobial factors because they encounter a large diversity of pathogenic organisms during their life cycle. More diversity of antimicrobial protein types makes understanding the mechanisms of antimicrobial action more complex. At the same time this diversity provides more opportunities for establishment of novel and effective antimicrobial agents.

Recently, novel family of antimicrobial proteins with multiple histidines and unique secondary structure was identified in ticks. To date, only two proteins from hard ticks, hebraein (AAR97292) from *Amblyomma hebraeum* and microplusin (AY233212) from *Rhipicephalus microplus*, belonging to this family were characterized (Lai *et al.,* 2004; Fogaca *et al.,* 2004). In this study we present the results of our research of hebraein- and microplusin-like genes in *Ixodes ricinus* and *Ixodes scapularis*. Predicted amino acid sequences of detected homologous genes contain the same cystein motif and his-rich elongation of C-terminus as above mentioned AMPs.

Lai and collegues (2004) suggested that histidine residues of play an important role in antimicrobial activity of the protein. Therefore the chemical synthesis of histidine rich C-terminus of HM protein from *I. scapularis* was performed to test antimicrobial activities. Negative results obtained in our study demonstrated that histidine-rich C-terminus is not responsible for antimicrobial activity of the protein and were supported by study of Silva and collegues (2009) where the structure and mode of action of microplusin were described. It was proven that bacteriostatic effect of microplusin is caused by binding copper II which is essential for proper assembly and functioning of terminal oxidases of the electron transport chain (Horn and Barrientos, 2008). The copper II binding regions were mapped and, as it appeared, none of the C-terminal histidines was involved in the copper-protein complex.

While the gene expression of microplusin was observed in the ovaries, fat body, and hemocytes (Fogaca *et al.,* 2004) and the up-regulated expression of gene for hebraein was observed in response to blood-feeding (Lai *et al.,* 2004), the tissue-specific expression pattern of HM genes showed higher level of gene expression only in salivary glands and midgut. These results indicate the possible involvement of HM proteins in protection of tick from invading pathogens during the blood-feeding. Therefore, the recombinant HM_sca_1 protein was prepared to be tested for antimicrobial activity.

The second prepared recombinant protein was ricinusin. Ricinusin was previously identified by Rudenko and collegues (2005) in *I. ricinus* cDNA substriction library and was characterized in bachelor thesis of Dornakova (2008). In spite of the fact that ricinusin possess only two histidines on its C-terminus, we expected it to function in the similar way as hebraein and microplusin due to the presence of unique HEAHEA repeat near the N-terminus. The recombinant ricinusin was prepared both in prokaryotic and baculovirus expression system.

It was obvious from our results that the produced recombinant ricinusin as well as HM protein did not possess antimicrobial activities under our experimental conditions as it was shown in case of microplusin and hebraein studies. Microplusin was active against *M. luteus*, *E. coli* and *C. albicans* (Fogaca *et al.,* 2004) and hebraein revealed activity against *S. aureus, E. coli* and *C. glabato* (Lai *et al.,* 2004). The reasons for ineffectiveness of the tested recombinant HM protein and ricinusin can be incorrect refolding, innapropriate conditions for exposing of antimicrobial activities or that mention proteins revealed activities other than antimicrobial. In case of HM recombinant the probability of incorrect refolding is very low as tested recombinant protein was obtained from cytoplasm. To confirm or reject the validity of other mentioned reasons affecting antibacterial activities of HM protein and ricinusin further studies are required.

The antimicrobial activity of tested recombinant proteins was not proved in this study. This might be a preliminary confirmation of the fact that hebraein- and microplusin-like homogues from different tick species might not possess the same antimicrobial activity as in original tick species where they were identified. Antimicrobial activity of homologous proteins might be connected to a tick species vectorial-capabily. *Amblyomma hebraeum* is the main vector of different *Rickettsia*

species, and *Riphicephalus (Boophilus) microplus* is known as a main vector of different species of *Anaplasma* (genus of rickettsiales bacteria) and *Babesia* (protozoan parasite of the blood). The various species of hard ticks from *Ixodes ricinus* complex most frequently infected with various microorganisms and viruses, of which some are substantial pathogens to humans. Pathogens transmitted to humans include mainly the causative agents of Lyme borreliosis and the tick-borne encephalitis, rarely *Rickettsia* and *Anaplasma* species, occasionally *Francisella* spp*, Babesia* spp or *Bartonella* spp. (Stanek, 2009).

The diversity of invading pathogens influences the specialization of antimicrobial proteins against certain pathogens, therefore the antimicrobial proteins from the same protein family of different ticks posses different activity *in vitro.* For example, longicin, defensin from *Haemaphysalis longicornis,* displayed bactericidal (against both G^+ and G^- bacteria) and fungicidal activity (Tsuji *et al.,* 2007), while the other defensins are active only against G^+ bacteria (Nakajima *et al.,* 2003a; Saito *et al.*, 2009).

Because the antimicrobial activity of HM_sca_1 was not proved in this study other possible function was tested.

There are several studies presenting salivary protein possessing the inhibition function against broad spektrum of proteases (e.g. Kotsyfakis *et al.,* 2006; Salat *et al.,* 2010; Chmelar *et al.,* 2010). The presence of gene expression mainly in the salivary gland and the small similarity to elastase inhibitors (28,7%) indicates that other potential function of the recombinant might be the inhibition of proteases. Unfortunately, the results of this test didn't matched our expectations.

7. CONCLUSION

A comprehensive analysis of antibacterial abilities of two proteins from *I. ricinus* (ricinusin) and *I. scapularis* (HM protein) that belong to a novel family of antimicrobial histidine-rich peptides didn't brought any positive results regardless of predicted functional properties based on sequence similarity, secondary structure similarity, concerved amino acids defined and concerved protein motif presented. HM protein also failed to show protease inhibitor activity in corresponding test that was used for analysis of alternative activity described for related proteins from this family. Further analysis under different conditions are obviously required to discover the functional abilities of ricinusin and HM protein.

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9. SUPPLEMENT

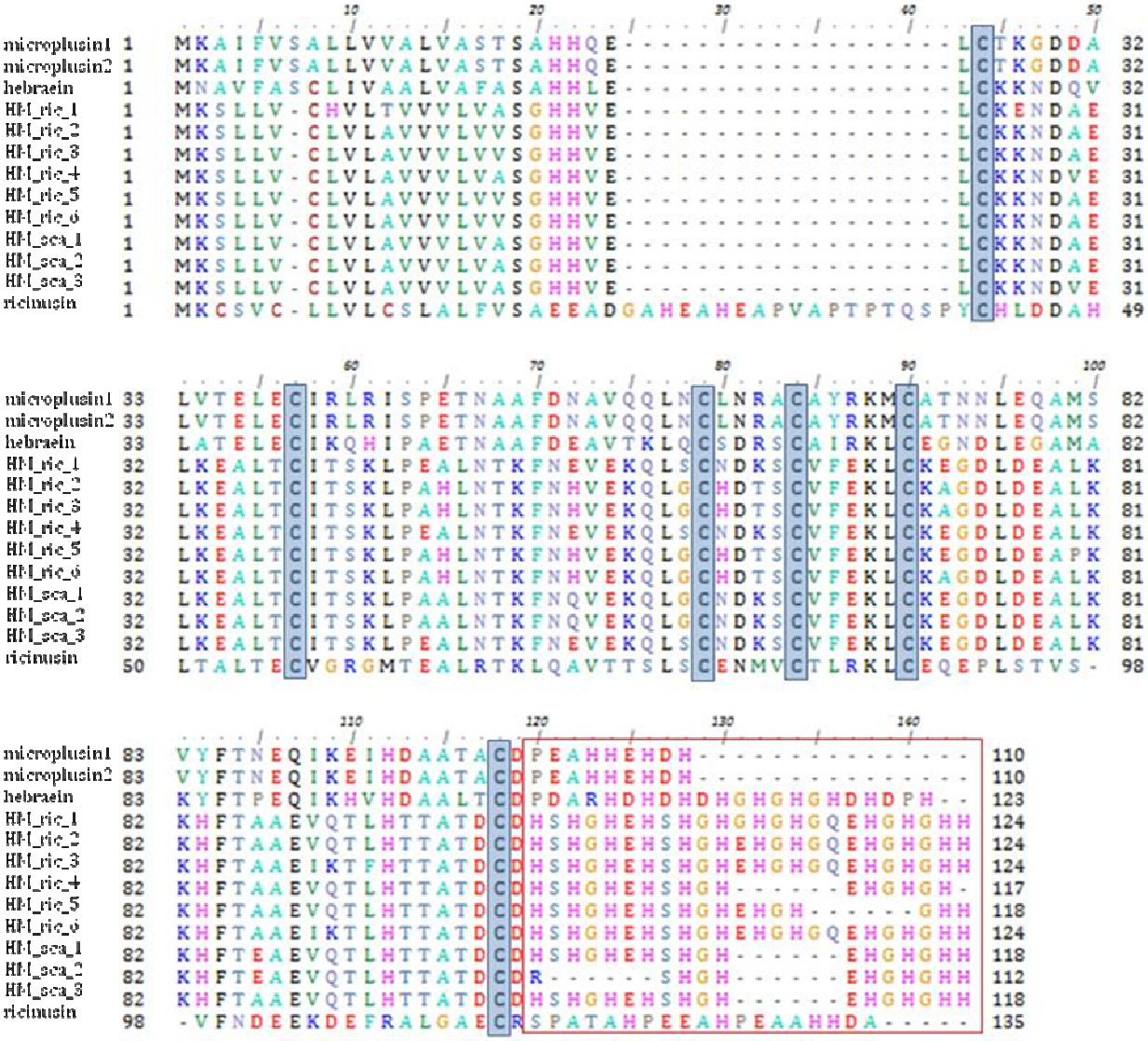

Fig.17. Amino acid alignment showing similarity of cystein motif and histidine rich C-terminus of microplusin (1 and 2), hebraein, ricinusin and HM sequences identified in this study.

six cysteins forming disulphide bonds

histidine-rich C-terminus