University of South Bohemia, Faculty of Science

Department of Parasitology

Ph.D. Thesis

Transcriptomic and functional analysis of salivary proteins from the tick *Ixodes ricinus*

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ANNOTATION:

This thesis was focused on the identification and characterization of the salivary proteins from *Ixodes ricinus*, the European vector of Lyme disease and tick-borne encephalitis causative agents. In the first part of this work, the the transcriptomic approach was used in order to identify and describe *I. ricinus* salivary proteins. The second part is dealing with functional and structural characterization of the salivary protein named IRS-2 (*I. ricinus* serpin-2).

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Preface

This Ph.D. thesis is based on the following research papers that are referred to in the text as **paper I**, **II** and **III**:

I. Chmelar, J., Anderson, J.M., Mu, J., Jochim, R.C., Valenzuela, J.G., Kopecky, J., 2008: Insight into the sialome of the castor bean tick, *Ixodes ricinus*. BMC Genomics 9, 233.

II. Chmelar, J., Oliveira, C.J., Rezacova, P., Francischetti, I.M.B., Kovarova, Z., Pejler, G., Kopacek, P., Ribeiro, J.M.C., Mares, M., Kopecky, J., Kotsyfakis, M., 2010: A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation (revision submitted to Blood).

III. Kovarova, Z., **Chmelar, J**., Sanda, M., Brynda, M., Mares, M., Rezacova, P., 2010: Crystallization and diffraction analysis of the serpin IRS-2 from the hard tick *Ixodes ricinus.* Acta Crystallographica Section F (accepted).

Research objectives

- Using high-throughput transcriptomic approach
    - Identify and describe salivary transcripts of *Ixodes ricinus*, especially potential secreted effectors that can play role at the tick x host interface
    - Characterize the dynamics of the salivary proteins expression during the feeding course of *I. ricinus*
- Perform functional and structural characterization of IRS-2, serpin from the saliva of *I. ricinus*

GENERAL INTRODUCTION

1 The Model organism – *Ixodes ricinus*

1.1 *Life cycle and vector potential*

Ixodes ricinus is an acarid belonging into the order Ixodida and the family Ixodidae. It is a blood-feeding ectoparasite, infesting various mammals, birds and small reptiles. *I. ricinus* undergoes a three-stage life cycle; the six legged larva hatches from the egg and molts into the eight legged nymph after a blood meal. The nymph turns into an adult male or female after another one blood meal. Each stage feeds on different hosts, while the host choice depends mainly on the host size. Larvae feed mainly on small rodents like wood mice or voles, but can be found also on small reptiles or birds, nymphal preferences stretch to middle sized mammals like rabbits or roe-calves. Adult females feed on larger mammals like deer or adult roe deer. From the two sexes of adult ticks only females are blood feeders while males don't feed at all and they stay attached to the female with their hypostome inserted into the female gonopore. During this attachment the female becomes fertilized, which is necessary for feeding completion. After successful feeding a female tick falls off the host and lays several thousands of spherical eggs, thus completing the tick life cycle, which lasts usually for three years in nature while under laboratory conditions it can be completed in less than one year. The actual length of the cycle depends mainly on the availability of a suitable host; both nymphal and adult stages can survive more than one year without feeding so the life cycle can be accordingly prolonged.

I. ricinus is the most abundant tick species in Europe. For a long time it was presumed that it occupies biotopes with altitude up to 1000m above sea-level, but according to recent observations, it started to spread further to higher altitudes and northern latitudes [1]. The spreading of *I. ricinus* to previously tick-free zones is unfortunately accompanied by the spreading of tick-borne diseases, which has become a very serious issue during the last two decades. *I. ricinus* is the most important arthropod disease vector in Europe. It transmits the causative agents of several diseases like tick-borne encephalitis virus, *Borrelia burgdorferi sensu lato*, or *Anaplasma marginale*. Tick-borne encephalitis (TBE) is a disease caused by a virus from the Flaviviridae family with occurrence only throughout eastern and middle Europe including southern Germany, while it was not reported from the most of western European countries. The course of TBE is very serious and it can be lethal in many cases;

however protection from TBE virus is possible by commercially available vaccine. This is not possible for the second most important tick-borne disease in Europe – Lyme disease. In Europe, Lyme disease is caused by spirochetes from the complex *B. burgdorferi sensu lato*, which consists of 12 species, from which only few are considered as being involved in Lyme disease pathogenicity. Lyme disease is an important public health issue also in the U.S.A., where it is caused only by one causative agent – *Borrelia burgdorferi sensu stricto* and transmitted by two main vectors – *Ixodes scapularis* in the eastern and *Ixodes pacificus* in the western coast. Tick-borne encephalitis is not present in the U.S.A. Due to the spreading into new areas both diseases are becoming a developing threat to human health with bigger impact compared to a few decades before.

1.2 *Blood feeding and digestion*

I. ricinus is an obligatory blood feeder. Adult female's feeding course lasts for 7-10 days during which the tick increases its size 100-200 times. The feeding period consists of two distinguished phases; a slow phase, during which host skin tissue is digested by some components of tick saliva and many changes in tick physiology take place. The mRNA expression profile of different tissues is extensively altered and all tick tissues undergo rapid development and growth. The cuticle grows in order to be able to stretch during the second phase of rapid engorgement, when large amount of blood is taken. Smaller nymphs feed for about 5-6 days and their feeding period is divided between slow and rapid phase too.

Ticks developed a way of blood digestion, which is unique among other arthropods. The whole blood digestion process, except the lysis of the ingested cells, occurs intracellularly in the lumen of the midgut epithelial cells. The tick midgut is almost free from extracellular proteases and that makes it suitable for the long term storage of the blood meal. The vertebrate blood is a very rich source of nutrients with 14-16% of hemoglobin, 6-8% of plasma proteins, about 1% of minerals and there is also plenty of dissolved amino acids and carbohydrates [2]. The digestion process has been described recently at the biochemical level and it was shown that a cascade of one aspartic and several cysteine proteases are the key players in hemoglobin digestion [3]. The processing of hemoglobin demands special handling of the resulting iron ions that are toxic for the tick. The process of iron transfer and deposition employs several proteins connected in a pipeline that has been proposed and described recently by Hajdušek and colleagues [4].

1.3 *Salivary glands and salivation*

The salivary glands of *I. ricinus* consist of two grape shaped organs that are connected by ducts leading to the salivarium and eventually to the hypostome. There are several types of acini, where the saliva is stored and/or produced. Each type of acini consists of different cell types with different function. The salivary glands are the primary site for the elimination of excess water and electrolytes and they are the tissue, where the entire water balance metabolism occurs. During the starving period of a tick the salivary glands produce a hygroscopic solution that absorbs water and enables the tick to survive. The salivary glands enlarge dramatically during feeding due to the huge increase of the protein production in this tissue. This is also because of the high volume of water that originates from the ingested blood and it is returned via saliva into the vertebrate host in order to reduce the volume of the blood in tick gut. Another role of the salivary glands during feeding is to enable the tick to complete its feeding cycle by secreting a lot of different pharmacoactive molecules, mainly proteins, into the site of tick attachment. This topic is described extensively in the chapter 3.

2 Host response to tick feeding

The tick bite and the subsequent feeding are processes, recognized as threats by the host and thus a complex defense response is raised. There is hemostasis in the front defense line, which includes blood coagulation, platelet aggregation and vasoconstriction. The unspecific, innate immune response to a tick bite is also activated that triggers an inflammatory response with all its typical mediators and effectors involved. On the verge of specific and non-specific response there is complement cascade, which is also activated upon tick infestation and finally there is an important contribution from the various components of acquired immunity, because the feeding lasts for a period that is sufficient for the raise of an antigen specific reaction and also multiple tick feeding with overlapping periods on one animal is possible in nature.

2.1 *Hemostasis*

Hemostasis comprises of several processes that protect vertebrates from the blood loss after an injury. These mechanisms include coagulation, platelet aggregation and vasoconstriction and each of those mechanisms can be activated by several pathways. The coagulation process consists of an enzymatic cascade with two ways of activation – exogenous and endogenous, while several amplification points and regulatory mechanisms

are known. Exogenous or extrinsic pathway of coagulation begins with the injury of the blood vessel and the activation of tissue factor (TF), which is followed by the formation of TF/factor VIIa (FVIIa) complex. TF/FVIIa activates factor X (FX) to factor Xa and FXa activates thrombin, which cleaves fibrinogen into fibrin, the main component of the clot. Intrinsic pathway begins with the auto-activation of FXII by its contact with polyanionic surfaces; therefore this pathway is regarded as contact phase. Platelet activation plays also a pivotal role in hemostasis and several specific mediators of platelet activation and subsequent aggregation such as collagen, thrombin, cathepsin G or ADP have been uncovered. Moreover, after an injury, several vasoconstrictors, i.e. biogenic amines and leukotrienes are also released from resident mast cells and activated platelets.

2.2 *Inflammation and complement*

Inflammation is induced as a host response to an injury and the potentially accompanying bacterial infection. It is usually characterized by the four features: calor, tumor, rubor and dolor – heat, swelling, redness and pain. Heat and redness are the results of vasodilatation and increased blood flow; swelling is caused by the exudation of plasma fluid and proteins and by the extravasation of leukocytes, mainly neutrophils, from blood vessels. Finally, some of the released or activated mediators like bradykinin, serotonin or histamine cause pain and itching. Pain, itching and swelling have a direct impact on tick feeding success, because the first two usually lead to tick removal by the host and swelling can disconnect the tick from blood flow and eventually lead to tick rejection. The mechanisms of innate immunity that are involved in tick x host interaction are described in several reviews [5-7]

In the case of tick feeding, inflammation begins with the vertebrate skin tissue damage caused by tick mouthparts, after which Hageman factor activates the clotting cascade, as well as the fibrinolytic and kinin system. Mediators of these systems induce pain, vasodilatation and vascular permeability, neutrophil chemotaxis and also complement activation via plasmin. Upon blood vessel damage, platelets get into direct contact with the extracellular collagen which leads to platelet activation and release of several types of inflammation mediators. Also neutrophils are activated by various molecules, for example by ATP, released from the injured cells. Activated neutrophils degranulate and release more mediators, chemokines and also proteolytic enzymes, namely cathepsin G and elastase that are responsible for tissue remodeling and also for further amplification of the inflammatory response – cathepsin G activates the platelets via protease activated receptor-4 (PAR-4).

Activation of neutrophils is also accompanied by the generation of pain-inducing prostaglandins. Other immune cells involved in inflammation initiation and development are mast cells that are activated via the C5a and C3a compounds of complement after tick mouthpart intrusion, leading to their massive degranulation. Important mediators of inflammation such as histamine, serotonin and serine proteases are released during mast cell degranulation. Histamine and serotonin are vasodilators, notorious due to their role in allergic reaction. Chymase is the main chymotrypsin-like serine protease produced by both activated and non-activated mast cells and was proved to attract other immune cells like eosinophils and neutrophils by processing various chemokines and receptors. It can also be chemotactic by itself. Other mediators of inflammation are the cytokines TNF-α, IL-1β and IL-6 that are released from neutrophils and that are involved in the progress of inflammatory response and its amplification. Monocytes and macrophages also produce many cytokines upon inflammation, as well as chemokines and products of arachidonic acid metabolism – prostaglandins (PG) PGE2 and PGF2, thromboxanes and leukotrienes – that function as vasodilators, chemoattractants for neutrophils and inducers of platelet aggregation. Leukotrienes play an important role in smooth muscle contraction as well.

The inflammatory response is even more intense, when the injury is septic, which is the case of tick feeding. Because of bacterial presence in the site of injury, the lectin and the alternative pathway of the complement are activated and large quantities of anaphylatoxins are produced. This leads to the attraction of granulocytes and monocytes to the site of injury. All cells involved in the inflammation crosstalk through the secretion of various cytokines and other mediators that influence their function so that the inflammatory reaction is precisely orchestrated.

2.3 *Acquired immunity*

The research on the interaction between the ticks and their hosts began in the late thirties by the classic work of William Trager, who described acquired immunity against *Dermacentor variabilis* in guinea pigs, previously exposed to ticks [8]. Another significant contribution in this research field was done by Allen and Wikel in seventies, when they published interesting results about the acquired resistance to ticks and their rejection [9-13].

The feeding of *I. ricinus* adults lasts for up to 10 days, during which the host organism is exposed to various antigens of tick or bacterial origin. The first cells that come into contact with these antigens are resident dendritic cells (DCs) called Langerhans cells, an important antigen-presenting cell population from the skin. After their activation by a tick salivary

antigen, DCs migrate to the draining lymph nodes, where the presentation of the antigen to T-cells takes place. It has been demonstrated that anti-tick resistance implicates a Th1 type immune response known as delayed type of hypersensitivity (DTH). This type of DTH reaction is connected with the influx of basophils and eosinophils into the site of exposure and is usually followed by tick rejection [13-14]. Tick rejection occurs by behavioral mechanisms like scratching and also in the case that the amount of imbibed blood is low or replaced by a purulent infiltrate that disturbs tick gut environment [6]. Continuing antigen presentation leads to the production of homocytotropic IgG and IgE antibodies that sensitize granulocytes like eosinophils, basophils and also skin residential mast cells, which possess high-affinity FcεRI receptor for IgE. After the resulting cross-linking of the antibodies, granulocytes are activated. This leads to their degranulation and the release of many proinflammatory mediators, such as histamine, serotonin or the proinflammatory cytokines TNF-α and IL-6 and to the activation of Th1 lymphocytes. This type of lymphocytes is characterized by the secretion of IFN-γ, which subsequently activates the macrophages that start to produce TNF-α and IL-6 [15]. Without the help of Th1 lymphocytes and the secretion of IFN-γ, the host is not able to elicit an immune reaction that results in tick rejection. Tick salivary antigens also trigger the production of specific antibodies by B-lymphocytes; however this type of response doesn't contribute to the tick rejection.

3 Tick-host interaction

3.1 *Anti-hemostatic and anti-complement activities of tick saliva*

The interaction between a feeding tick and its vertebrate host is a complex process, during which many physiological mechanisms on both sides are employed. The host mechanisms activated during this interaction are described above. Ixodid ticks are exposed to all defensive mechanisms of the host; therefore they are under an evolutionary pressure to develop efficient countermeasures, in order to be able to complete their blood meal. After three decades of research, it is known now that the ticks are not simple "crawling syringes", but they actively contribute to changes in the site of attachment, necessary for feeding completion. Many effects of tick saliva were discovered in eighties by the work of Ribeiro and his colleagues. The saliva of the American tick *Ixodes dammini* (former name of *I. scapularis*) was shown to exhibit many activities against host defensive mechanisms. The saliva of *I. dammini*, similarly to mosquitoes or tsetse flies, inhibited ATP, ADP and PAF (platelet aggregation factor) induced platelet aggregation by degrading active ATP and ADP

into non-active AMP, which is done by apyrase. [16]. In addition, anti-platelet activity was attributed to prostaglandins of E group (e.g. PGE_2) and prostacyclins that were proved to be present in tick saliva a few years later [17]. Another observed activity that was initially attributed to PGE_2 was the inhibition of IL-2 production by T-cells [16], but in 1994, Urioste *et al.* showed that cell activation-blocking mechanism is PGE_2 independent and it is mediated by a protein [18]. These results however propose the possibility that both systems (PGE_2 and protein dependent) can contribute to the inhibition of T-cell activation. PGs and prostacyclins that act also as short term vasodilatators were found not only in *I. dammini*, but also in *Amblyomma americanum, Boophilus microplus* and *Haemaphysalis longicornis* [19-20], pointing at the conservation and the importance of PGs in ticks. More information on the synthesis and the role of tick salivary PGs can be found in a review by Bowman and Sauer [21].

The saliva of *I. dammini* displayed also anti-complement features; more specifically, in its presence, an alternative pathway of complement was disrupted, i.e. deposition of C3b and release of C3a component. The salivary protein fraction of molecular weight 49 kDa was found responsible for this effect [22]. Furthermore, the functions of rat peritoneal-derived neutrophils, i. e. anaphylatoxin-induced aggregation, FMLP-induced granule enzyme secretion, zymosan-induced superoxide secretion and phagocytosis of *B. burgdorferi* spirochetes were reduced by 40-80% when incubated with tick saliva [23]. Coagulation inhibitory activity and anti-complement activity were observed also with the saliva from *Dermacentor andersoni* [24-25].

3.2 *The modulation of host acquired immunity*

The prolonged feeding period of hard ticks is sufficient for the activation of an acquired immune response. Therefore the tick develops appropriate counter-measures to be protected from host response. *Rhipicephalus appendiculatus* was able to suppress humoral response in rabbits as a result of a putative lymphocytotoxic factor in its saliva [26]. The responsiveness of T-cells to various mitogens was changed after the incubation with saliva or salivary gland extract (SGE) from different tick species. The proliferation of T cells stimulated with concanavalin A (Con A) was strongly diminished by the incubation with *D. andersoni* SGE, while their proliferation was enhanced when stimulated by LPS. These changes were accompanied by altered production of IL-1, IL-2, IFN-γ and TNF-α [27]. Similar findings were published about *I. dammini*, whose saliva also inhibited the proliferation of T-cells in response to Con A and phytohemaglutinin. The inhibition was accompanied by a decrease of

IL-2 production. Moreover, in the presence of the saliva, the LPS-induced production of nitric oxide by macrophages was diminished [18]. Ganapamo and colleagues observed also diminished responsiveness to Con A by T-cells from mice infested with *I. ricinus* nymphs when compared with non-infested mice [28]. Similar changes in cytokine expression and production occur after infestation with ixodid ticks or after treatment of immune cells with saliva or SGE [29-31]. The type of specific host immune reaction depends on which type of T-helper (Th) lymphocytes is activated. The profile of cytokines expression suggests that the saliva or SGE from *I. ricinus* modulate immune response towards the Th2 rather than the Th1 branch of immune response [30]. Th2 response is characterized by the activation of B-cells and the production of specific antibodies. At the level of cytokines, this response results in increased levels of IL-4 and IL-10, while the levels of IFN-γ and IL-2 are diminished. Th1 response is characteristic for the opposite action, i.e. up regulation of IFN-γ and IL-2 and decrease of IL-4 and IL-10. The main function of Th1 lymphocytes is to orchestrate an inflammatory response since Th1 specific cytokines further enhance the production of inflammatory cytokines TNF-α and IL-6 by macrophages [32]. The polarization of host response towards Th2 was also observed after treatment of human PBMC [33] and mouse splenocytes [34] with *I. ricinus* SGE. The suggestion was that the down regulation of IFN-γ expression and the polarization towards Th2 response are mediated by the increased levels of IL-10 [35]. However another group reported contrasting results to this hypothesis; more specifically IL-10 was inhibited after treatment with *I. ricinus* SGE [36]. In addition, IL-2 binding activity was detected in the saliva of *I. scapularis* that was attributed to an unidentified protein, as trypsinised saliva failed to exhibit the same effect [37].

It was shown later that there are more cell types involved in the observed mechanism and that the polarization starts with antigen presenting cells, mainly dendritic cells in the skin (DCs). Cavassani and colleagues showed that DCs differentiation, LPS dependent activation and subsequent potential of DCs to present antigens are impaired when incubated with the saliva of *Rhipicephalus sanguineus* [38]. The migration, maturation and function of DC were affected by *I. ricinus* saliva that resulted in the polarization towards Th2 immune response, while the polarization towards anti-tick effective Th1 and Th17 response was impaired [39].

In summary, ixodid tick salivary secretion can alter all host defensive mechanisms, including wound healing related processes such as coagulation, platelet aggregation and vasoconstriction, innate immunity related responses that include inflammation, complement cascade, macrophage ability to phagocytose and also natural killer cells activity. Acquired immunity is also modulated so that instead of an anti-tick effective Th1 response to tick

feeding an ineffective Th2 response is observed. In the following section, the progress in the identification of pharmacologically active salivary constituents is discussed.

4 The identification of tick salivary proteins

4.1 *The salivary content*

Tick saliva is a mixture of various bioactive molecules including a large portion of proteins. The identification of salivary constituents with an active role in the tick-host interaction became important since they can be possible targets for the development of anti-tick vaccines. To date, more than 50 tick salivary components were identified and at least partially characterized at the molecular and biochemical level. Despite this progress, a lot of salivary proteins still wait for their characterization. The current working hypothesis predicts more or less a specific function for each tick salivary protein, although most likely more than one bioactive protein can account for the same activity of tick saliva. The salivary protein identification began in the early nineties using mainly biochemical methods for the purification and molecular identification of an activity detected in crude saliva or salivary glands. The experimental design usually followed the path from revealing a salivary function in crude extracts to the identification of the protein that account for the function through a series of chromatographical steps. With the technological developments in the field of molecular biology, a more effective reverse approach (from gene to protein and then to function) became more available and started to be used more often for the description of pharmacoactive molecules originating from tick saliva. The identification of coding sequences, followed by the production and characterization of recombinant proteins, brought valuable information about the function of tick salivary proteins. At the beginning of the 21st century, a high-throughput approach, called sialotranscriptomics (sialo stands for saliva in Greek), was employed for the massive identification of salivary transcripts, creating lists of genes from which any researcher could pick a gene of particular interest and focus the research effort in its functional characterization. High throughput approaches, although effective, have also some technical drawbacks, since they were not able to identify many transcripts belonging to gene families that play a role for tick feeding success. As a result, some groups of proteins that are present in all eukaryotes were not found in tick salivary glands by this methodology. The following sections should bring more insight into the progress of tick salivary protein identification.

4.2 *Before sialomes*

Before the employment of basic molecular biology methods like PCR and molecular cloning in tick research, only a few proteins had been really characterized and investigated in details. If we summarize the activities of the proteins and small chemical compounds identified before the sialomic approach that account for a given effect of saliva in the vertebrate host, these could be divided in several groups:

- Small compounds such as ATP and prostaglandins
- Enzymes, including metalloproteases, apyrases, antioxidant enzymes and kininases
- Inhibitors of proteases
- Calcium-binding proteins
- Amine-binding proteins
- Host cytokine homologs
- IgG binding proteins
- Cytokine-binding proteins
- Neurotoxins
- Platelet aggregation antagonists
- Coagulation antagonists
- Immunomodulators with undisclosed mechanism of action

The first identified tick salivary protein that initiated further interest in tick salivary biochemistry, is an about 60 amino acid long peptide purified from the soft tick *Ornithodoros moubata* [40]. This peptide displayed very specific inhibitory activity against factor Xa, which is a thrombin activator from plasma and a key enzyme in the blood coagulation cascade. The peptide was called TAP (tick anti-coagulant peptide) and further characterization was done on the recombinant protein that was produced in the yeast *Saccharomyces cerevisiae* [41]. TAP is also the first recombinant peptide ever prepared from the tick with a rather painful procedure of construct preparation and protein expression [42]. Several other peptides were isolated from *O. moubata* since relatively large amounts of SGE can be obtained from these ticks. The list of peptides includes moubatin, which displayed inhibitory activity against collagen induced platelet aggregation. The protein was initially isolated from tick saliva and later prepared in recombinant form [43-44]. Another platelet aggregation inhibitor with a size of 6 kDa, called disagregin, binds directly to the platelet fibrinogen receptor, and it was also discovered in *O. moubata* saliva [45].

Another activity that can be attributed to salivary protein component(s) is the binding of host IgG, which results in their neutralization and return back to the host. Proteins of the size of 23 and 57 kDa from the SGE of *Rhipicephalus appendiculatus* were identified as contributors to this IgG binding activity [46]. Proteins with similar activity were reported also from the ixodid ticks *Ixodes holocyclus* and *Amblyomma variegatum* [47]. However further analysis and characterization of these proteins and their activities at the molecular level has not been done to date.

Using a chromatographical fractionation of *D. andersoni* saliva, the fraction of 36-43 kDa was identified as inhibitory for ConA induced mouse splenocyte proliferation [48], apparently accounting also for the effect that had been observed with *D. andersoni* SGE in 1992 [27]. The native protein that was responsible for the inhibition of splenocyte proliferation, named Da-p36 was isolated from the salivary glands using antibody-based purification and its cDNA was cloned and characterized [49]. Finally, recombinant Da-p36 was produced both in bacterial and insect expression systems and its immunomodulatory activity was confirmed *in vitro* [50].

This chapter does not provide the description of all proteins identified before sialomics, as it will be discussed in the following sections together with the input from transcriptomic projects. This chapter aims to provide just an insight into the experimental design followed in the past decades in the field of tick x host interaction research, before the development of high-throughput methods.

4.3 *The development of transcriptomic approaches*

During the classical approach of the tick salivary effectors identification, everything would start with the observation of an effect of the saliva or SGE on the host. Then, usually, native effectors were purified using size fractionation or antibody based chromatography. The purified protein was partially sequenced by Edman degradation, degenerated primers were designed and the DNA probe was prepared by PCR. In order to get the full-length coding DNA, a cDNA library from salivary glands or whole animals had to be screened by the produced probe. After the successful isolation of different clones, the full-length sequence was obtained and the recombinant protein could be produced. This approach was always focused on one protein only.

When using the most recent reverse approach, everything starts with cDNA library construction and the sequencing of randomly selected clones, which are bioinformatically analyzed and their possible function is predicted *in silico*. Sometimes the libraries are

subtracted in order to find differences in gene expression between two stages or different physiological conditions. Another possibility is the massive sequencing of cDNAs and the use of bioinformatics to organize the obtained data. All sequences are automatically compared with millions of transcripts or proteins from several available databases like NCBI GenBank, Pfam (Protein families database), SMART (Simple Modular Architecture Research Tool) or GO (gene ontology) and the results are presented usually in a single table. The employment of high-throughput approaches brought several surprises in the field. First, it revealed that tick saliva is much more complex mixture then anticipated; that salivary glands produce hundreds of different proteins, many of which are novel and unique among all known organisms in the sense that no similar proteins or genes can be found in public gene databases such as non-redundant database of NCBI (National Centre for Biotechnology Information). The second notable finding was that the most abundant tick salivary proteins form so called multigenic families with even 20 or 30 members. These proteins can display redundancy in their function that can contribute to the evasion of host defensive mechanisms such as immune recognition. Indeed it is now known for some families that different members are expressed as the feeding progresses. The third surprise and challenge that remains to date is that we cannot even predict the possible function of a vast majority of proteins, identified by sialotranscriptomics [51].

A detailed description of all proteins and protein families revealed by sialotranscriptomics would significantly broaden the extent of this thesis and would exceed its purpose. For those interested in this field, an excellent review that summarizes and classifies all known data from tick transcriptomes is available [51]. Transcriptomic data for *I. ricinus* are analyzed in details in **paper I** [52] that is part of this thesis. Therefore only the most abundant and most notable proteins and protein families discussed with the focus on the recent knowledge about *Ixodes sp.* gene families.

Proline and glycine rich proteins (collagen-like) - This family consists of 3.5-4.5 kDa proteins with around 30% content of Gly and Pro amino acids. It is apparently the largest multigenic protein family from the genus *Ixodes*; however its function is not known. Based on the primary structure, proteins from this group can form some kind of glue or can interfere with the collagen receptor on platelets [53]. No member from this group has been characterized thus far.

Basic tail secreted proteins (BTSP) – Another very abundant group in tick saliva comprises of rather small proteins with their molecular weight varying between 10 and 15 kDa. They all bear a tail consisting of mainly lysines. Furthermore these proteins bear a single

domain containing 6 conserved cysteines. Because lysine is coded by 3 adenines, those tails were thought to be poly A regions in non-coding 3' parts of mRNAs. However, the description of a high number of related proteins with such tails and stop codon after these regions supported the existence of this family. Moreover, the automatic processing of raw sequences most likely resulted in identifying, some members of this family as lacking basic tail, because the poly A region coding for lysines was trimmed by the software as non coding poly A region of mRNA. Several members of this group were identified as anticoagulants [54]; therefore we can expect a similar function from the other members of this group as well. Similar protein families of the size around 18.7 kDa that contain two domains, identical with the one from BTSP, but without a basic tail, were identified in all three transcriptomes from the three *Ixodes* species. These proteins were not characterized to date, so their function remains unknown.

Kunitz domain-containing proteins – This group of potential serine protease inhibitors is also very abundant in all *Ixodes* species. In *I. scapularis*, this group was divided among three subgroups based on the number of Kunitz domains; monolaris subgroup comprises of single Kunitz domain proteins, bilaris proteins contain 2 Kunitz domains and penthalaris 5 Kunitz domains. Several Kunitz domain-containing proteins were characterized and demonstrated as anticoagulants with different target proteases as described in the section 5.1. A more detailed description of the Kunitz family is also given in the section 5.4.

Lipocalins - Lipocalins form a large, diverse and ubiquitous protein family with relatively low similarity at amino acid level. On the other hand, the family is very conserved at the level of tertiary structure. Their structural features make them able to bind hydrophobic ligands inside the cavity of their molecule. They are abundant in tick saliva, where they function as histamine and serotonin binding proteins. The binding of these two mediators helps to suppress host inflammatory response against tick feeding. Another function can be the inhibition of the complement cascade as was shown with *O. moubata* [55] or the binding of leukotrienes [56].

Enzymes – The most abundant enzymes in the saliva of ixodid ticks are probably metalloproteases with fibrin(ogen)olytic and thus anti-hemostatic activity. Metalloproteases also form a relatively large multigenic family. Other notable enzymes found in tick salivary glands are carboxypeptidases, serine proteases, chitinases or dipeptidyl peptidases (kininases). From non-proteolytic enzymes it is worthy to mention phospholipase A_2, which has been demonstrated as the main pain causing agent from wasp and honey-bee venom.

Cystatins – Cystatins are inhibitors of cysteine proteases like papain and legumain. Some members were identified as immunomodulators and inhibitors of inflammation, most likely due to the inhibition of cathepsin S and L, enzymes involved in matrix degradation by fibroblasts. There are 15 cystatins in *I. scapularis* genome. Possibly not all of them are involved in tick x host interaction, since cysteine proteases play a role in blood digestion in the tick midgut, a hypothesis which is further supported by the expression profile of two cystatins during feeding course [57].

Anti-complement proteins (ISAC family) – Few of the members of this multigenic family has been identified as anti-complement proteins. A detailed description of the family is provided in the chapter 5.2.

Ixodegrins – Ixodegrins are proteins containing an Arg-Gly-Asp (RGD) or a Lys-Gly-Asp (KGD) domain that indicates their ability to block the binding of fibrin to platelets. Therefore RGD or KGD domain containing proteins can be considered as potential inhibitors of platelet aggregation. Some members of this group display similarity to the snake venom peptide dendroaspin [58], while other members contain a prokineticin-like domain that is associated with several physiological functions in the host [59].

More than 20 other groups could be mentioned from *Ixodes sp.* Some proteins display similarities to protease inhibitors, e.g. proteins containing TIL domain (trypsin inhibitor like) or carboxypeptidase inhibitors. Antimicrobial activities can be attributed also to some families, based on their similarity to known proteins or based on experimental data (defensins, peptidoglycan recognition proteins); however in the majority of those smaller groups the available information is limited to their molecular weight and amino acid sequence, because these proteins display no similarity to any known sequences in any database available. The description of these groups is included in the corresponding publications and their supplementary data [52-53, 60-61]

This chapter aims to provide an overview of the most abundant and interesting protein families that were found in the sialotranscriptomes from *I. ricinus, I. scapularis* and *I. pacificus*. There are many more sialotranscriptomic works done on other tick species, from which we know that each species and genera evolved some specific proteins that cannot be found in others. As one good example, the immunosuppressive protein Da-36p from *D. andersoni* has homologs in *Dermacentor sp.*, but no homologs in other tick species. Apparently, each species developed various counter-measures against host defense; some of them are common for all ticks, such as Kunitz domain inhibitors, and some others are unique for each taxon.

4.4 *The genome of* Ixodes scapularis

A new era in tick research began, when the genome of *I. scapularis* was publically available [62]. The genome, together with bioinformatic tools is available online at http://iscapularis.vectorbase.org. Everybody can now pick a gene of interest and find all its homologs in the genome. The genomic information helped greatly to improve our knowledge about the complexity of tick protein families. Unfortunately the genome of *I. scapularis* has certain characteristics that complicate its proper interpretation and annotation. The main problem is the very high proportion of non-coding repetitive regions on chromosomes that makes the genome assembly from the individual contigs very difficult. This often leads to incorrectly assembled coding regions. The genome is now being annotated by volunteers from the scientific community, so the available information is growing rapidly.

5 Functionally characterized proteins

In the following section, a summary of all known functionally characterized proteins from ticks is provided with emphasis on the ticks of the genus *Ixodes.* To date more than 50 tick salivary proteins with various roles in the tick-host interface were identified both from soft and hard ticks. According to their function these proteins can be divided in the following functional groups:

- Anti-hemostatic proteins
- Anti-complement proteins
- Immunomodulatory proteins, including modulators of both innate and acquired immunity

Table 1 summarizes all the known tick salivary proteins with anti-hemostatic, anti-complement and immunomodulatory features.

Name	Description	Size	Organism	Ref.
		Anti-coagulants		
Thrombin inhibitors				
Ornithodorin	Kunitz, binding to both exosites and active site	~14 kDa	*Ornithodoros moubata*	[63]
Savignin	Kunitz, binding to both exosites and active site	~14 kDa	*Ornithodoros savignyi*	[64]
Monobin	Kunitz, binding to both exosites and active site	~14 kDa	*Argas monolakensis*	[65]
Amblin	Kunitz, binding to both exosites and active site	~14 kDa	*Amblyomma hebraeum*	[66]
Boophilin	Kunitz, binding to both exosites and active site	~14 kDa	*Rhipicephalus (Boophilus) microplus*	[67]
Hemalin	Kunitz, binding to both exosites and active site	~14 kDa	*Haemaphysalis longicornis*	[68]
Madanin 1 and 2	no cysteine, binds to exosite	~7 kDa	*Haemaphysalis longicornis*	[69]
Chimadanin	no cysteine, inhibition of active site	~7 kDa	*Haemaphysalis longicornis*	[70]
NTI-I	non-competitive inhibition of active site	~3 kDa	*Hyalomma dromedari*	[71]
Microphilin	exosite binding only	~1.7 kDa	*Rhipicephalus (Boophilus) microplus*	[72]
BmAP	inhibition of active site, binding to exosite	~60 kDa	*Rhipicephalus (Boophilus) microplus*	[73]
BmGTI	binding to exosite only	26 kDa	*Rhipicephalus (Boophilus) microplus*	[74]
Variegin	C-terminus similar to hirudin binds exosite	3-4 kDa	*Amblyomma variegatum*	[75]
Americanin	competitive inhibitor	12-16 kDa	*Amblyoma americanum*	[76]
IRIS	serpin, inhibits weakly both thrombin and FXa	41 kDa	*Ixodes ricinus*	[77]
Factor Xa inhibitors				
TAP	Kunitz, binding to both exosites and active site	~7 kDa	*Ornithodoros moubata*	[40]
FXaI	Kunitz, binding to both exosites and active site	~7 kDa	*Ornithodoros savignyi*	[78]
Salp14	BTSP family, inhibits active site	~14 kDa	*Ixodes scapularis*	[54]
Unnamed	uncompetitive inhibitor of active site	~15-17 kDa	*Hyalomma truncatum*	[79]
Unnamed	uncompetitive inhibitor of active site	~15-17 kDa	*Hyalomma dromedari*	[80]
FVIIa/TF complex inhibitors				
Ixolaris	TFPI homologue, Kunitz, inhibits FVIIa/TF complex	~14 kDa	*Ixodes scapularis*	[81]
Penthalaris	TFPI homologue, Kunitz, inhibits FVIIa/TF complex	~35 kDa	*Ixodes scapularis*	[82]
Contact phase inhibitors				
Ir-CPI	Kunitz inhibitor, binds to FXIIa, FXIa and kallikrein	~7 kDa	*Ixodes ricinus*	[83]
Haemaphysalin	Kunitz inhibitor, binds to FXIIa		*Haemaphysalis longicornis*	[84]
BmTI-A/D/2	Kunitz inhibitor, inhibits kallikrein		*Rhipicephalus (Boophilus) microplus*	[85]
RsTI-Q2/Q7	Kunitz inhibitor, inhibits kallikrein		*Rhipicephalus sanguineus*	[86]
		Platelet aggregation inhibitors		
ADP hydrolysis				
Apyrase	Hydrolyses ATP and ADP to inactive AMP	~62 kDa	*Ornithodoros savignyi, Rhipicephalus (Boophilus) microplus*	[87] [88]
TXA binding				
Moubatin	Lipocalin, Binds TXA2	17 kDa	*Ornithodoros moubata*	[44]
TSGP3	Binds TXA2	16 kDa	*Ornithodoros savignyi*	[89]
Longicornin	most likely binds TXA2	16 kDa	*Haemaphysalis longicornis*	[90]
serotonin binding				
Monotonin	binds serotonin	20 kDa	*Argas monolakensis*	[65]
SHBP	binds serotonin and histamine	22 kDa	*Dermacentor reticularis*	[91]
alpha2beta1 binding				
TAI	inhibits adhesion to soluble collagen	5 kDa	*Ornithodoros moubata*	[92]
Thrombin inhibitors	**Listed in anticoagulants**			
alpha2beta3 binding				

Disagregin	binds to fibrin receptor on platelets, Kunitz	6 kDa	*Ornithodoros moubata*	[45]
Savignygrin	binds to fibrin receptor on platelets with RGD motif, Kunitz	7 kDa	*Ornithodoros savignyi*	[93]
Variabilin	binds to fibrin receptor on platelets with RGD motif	5 kDa	*Dermacentor variabilis*	[94]
Ixodegrin	binds to fibrin receptor on platelets with RGD motif	7 kDa	*Ixodes sp.*	[60]
Savignygrin-like	binds to fibrin receptor on platelets with RGD motif, Kunitz	7 kDa	*Ornithodoros coriaceus*	[95]
Monogrin	binds to fibrin receptor on platelets with RGD motif, Kunitz	10 kDa	*Argas monolakensis*	[65]
Fibrinolytic enzymes				
Metalloprotease	degrades fibrinogen and fibrin	37 kDa	*Ixodes scapularis*	[96]
Cathepsin G inhibitors				
IRS-2	inhibits cathepsin G and thrombin induced aggregation	42 kDa	*Ixodes ricinus*	**Paper II**
	Anti-complement proteins			
C3 interaction				
ISAC	interacts with C3 convertase complex, blocks C3b deposition	18.5 kDa	*Ixodes scapularis*	[97]
IRAC	homologs of ISAC, probably similar mechanism	~19 kDa	*Ixodes ricinus*	[98]
Salp20	homologs of ISAC, probably similar mechanism	~20 kDa	*Ixodes scapularis*	[99]
C5 interaction				
OMCI	lipocalin fold, blocking of C5 activation by C5 convertase	~16 kDa	*Ornithodoros moubata*	[55]
	Immunomodulators			
Salp15	inhibits T-cell prolif., binds to DC, SAT factor	15 kDa	*Ixodes scapularis*	[100]
IRIS	serpin, cytokine production modulation, inhibits T-cell proliferation	41 kDa	*Ixodes ricinus*	[101]
Sialostatin L and L2	cystatins, sial. L inhibits cathepsin L and S, anti-inflammatory, sial L2 facilitates borrelia growth	12.5 kDa	*Ixodes scapularis*	[102]
OmC2	cystatin, inhibits TNF-α and IL-12 production by DCs, inhibits T-cell proliferation	~12 kDa	*Ornithodoros moubata*	[103]
Evasin 1-4	impairs neutrophil migration, chemokines binding	~10-12 kDa	*Rhipicephalus sanguineus*	[104]
Ir-LBP	lipocalin, binds LTB4, impair neutrophil chemotaxis and activation	~22 kDa	*Ixodes ricinus*	[56]
IRS-2	serpin, anti-inflammatory, inhibits neutrophil migration	42 kDa	*Ixodes ricinus*	**Paper II**
Da-36p	inhibits mouse T-cells and splenocytes proliferation	36 kDa	*Dermacentor andersoni*	[49]
TdPI	inhibits mast cell tryptase, potentially anti-inflammatory, Kunitz		*Rhipicephalus appendiculatus*	[105]
HBP	lipocalins, histamine binding, anti-inflammatory		*Rhipicephalus appendiculatus**	[106]
Ra-KLP	K+ ion channel modulator, Kunitz	~8 kDa	*Rhipicephalus appendiculatus*	[107]
Salp25D	Antioxidant	~25 kDa	*Ixodes scapularis*	[108]
ISL929, ISL1373	Anti-neutrophil role, ADAMTS motifs	10, 11 kDa	*Ixodes scapularis*	[109]

Abbreviations: BTSP - basic tail secreted proteins; TXA - thromboxane; TFPI - tissue factor pathway inhibitor; LTB4 - Leukotriene B4; DC - dendritic cells; SAT - Saliva activated transmission, ADAMTS – a disintegrin and metalloprotease with thrombospondin;

* - Lipocalins are found in most of tick species, however only few were characterized functionally

5.1 *Anti-hemostatic proteins*

Hemostasis includes three main defensive mechanisms that primarily protect vertebrates from blood loss, namely coagulation, platelet aggregation and vasoconstriction. Tick saliva can interfere with all three mechanisms. About 25 anticoagulants were identified and at least partially characterized from tick saliva to date [110]. Because the coagulation is a process that involves multiple discrete and tightly regulated molecular events, even the tick anticoagulants

can be divided in four groups, depending on their molecular targets and mechanism of action. The most direct mechanism of action is the inhibition of thrombin that is responsible for fibrinogen cleavage. Ticks use an array of thrombin inhibitors from different protein families that vary in size. The only direct thrombin inhibitor from *Ixodes sp.* is serpin IRIS (*I. ricinus* immunosuppressor), which displayed rather weak anti-coagulative and anti-thrombin activities [77]. The second mechanism is the inhibition of factor Xa (FXa), another key protease in the coagulation cascade, responsible for the activation of thrombin from its zymogen. Besides the weak FXa inhibitor IRIS, Salp14, a member of a large multigenic protein family, found in *Ixodes sp* was proved as an inhibitor of FXa. The direct inhibitory assay with recombinant Salp14 was not performed, however its silencing by RNAi led to 60-80% reduction of anti-FXa activity in *I. scapularis* saliva [111]. The third mechanism of coagulation inhibition is the inhibition of the tissue factor (TF), the first molecular event in the coagulation cascade, activated after injury by the contact of TF with extracellular matrix. There is a natural inhibitor of tissue factor mediated coagulation in vertebrates called Tissue factor pathway inhibitor (TFPI), which is a 3 Kunitz-domain protein that interacts with TF/FVIIa complex with one domain and with FXa using the second Kunitz domain, thus creating quaternary complex TF/FVIIa/TFPI/FXa. One *I. scapularis* transcript encodes for a double Kunitz-domain homologue of TFPI, called Ixolaris that also binds to TF/FVIIa complex and that inhibits the initiation of coagulation [81]. A similar mechanism of coagulation inhibition was observed with a five Kunitz-domain protein called Penthalaris that is expressed in the salivary glands of the same tick species [82]. Ixolaris and Penthalaris can either act independently in the host or in coordination. The last mechanism of coagulation inhibition known from ticks, is the contact phase inhibition. This is also known as the inhibition of intrinsic pathway, because it is initiated by plasma proteins FXI, FXII and prekallikrein. FXII undergoes auto-activation into FXIIa after contact with polyanionic surfaces and the specific cascade continues with the activation of kallikrein and FXI. This leads eventually to the activation of FX and thrombin. A Kunitz domain-containing inhibitor of FXIIa, FXIa and kallikrein was found in *I. ricinus* and was named Ir-CPI. Moreover, when injected into bloodstream, Ir-CPI exhibited anti-thrombotic features and inhibited the formation of both venous and arterial thrombi without disturbing the clotting balance [83].

The only two characterized platelet aggregation inhibitors from *Ixodes sp.* are metalloproteases from *I. scapularis* [96] and IRS-2 (**paper II**). However, in the transcriptomes, there are many potential anti-platelet proteins, e.g. Ixodegrins – proteins containing RGD motif that possibly bind to the platelet receptor for fibrin [52]. Anti-platelet

proteins from other tick species are summarized in the Table 1 and also in the review that focused on anti-platelet molecules from arthropods [112].

5.2 *Anti-complement proteins*

In 2000, Valenzuela and colleagues identified and described a novel anti-complement protein from the salivary glands of *I. scapularis* that was named ISAC (*I. scapularis* anti-complement). ISAC is a ~18.5 kDa protein and its mechanism of complement inhibition is based on its interaction with C3 convertase and the subsequent inhibition of C3b deposition on the cell surface [97]. A relatively large multigenic family of ISAC related proteins was discovered, first in *I. scapularis* [53] and then in *I. ricinus* [98]. This protein family also appears to be very abundant in the saliva, based on the high number of obtained ESTs (expressed sequence tags) from all *Ixodes* sialotranscriptomes.

5.3 *Proteins with immunomodulatory features*

5.3.1 Salp15 and Salp25D

The discovery of Salp15 resulted from an experimental approach in which several immunodominant tick salivary proteins were identified by using the screening of expression libraries from *I. scapularis* salivary glands with anti-tick polyclonal sera [113]. The word salp stands for <u>sal</u>ivary <u>p</u>rotein and the given number represents the estimated molecular weight of the native protein. Salp15 is a 15 kDa big glycoprotein that inhibited T-cell proliferation by the interaction with T-cell receptor (TCR) and the subsequent repression of calcium fluxes. As a result, the production of IL-2 was reduced, leading to the modulation of host immune response [100]. Moreover, it was shown that the Lyme disease causative agent *Borrelia burgdorferi* binds Salp15 on its surface, thus helping the spirochete to evade antibody-mediated killing by the host [114]. In addition, Salp15 binds to DC-SIGN receptor and it inhibits the production of the proinflammatory cytokines IL-6 and TNF-α by dendritic cells activated either through Toll-like receptors or by *B. burgdorferi* [115]. Homologs of Salp15 were found in *I. ricinus* [116], but no other member from Salp15 family, except Salp15 itself, was characterized to date. Another one protein from *I. scapularis* that was demonstrated as being exploited by *B. burgdorferi* to facilitate the transmission between host an vector is antioxidant Salp25D. Ticks with RNAi-silenced Salp25D displayed lower acquisition of *B. burgdorferi* as well as the ticks that were feeding on the host immunized with this protein.

Salp25D is an antioxidant that detoxifies reactive oxygen species, thus providing a survival advantage to *B. burgdorferi* at the vector x host interface [108].

5.3.2 Sialostatin L and L2

Sialostatin L is a member of the cystatin superfamily that is expressed in the salivary glands of *I. scapularis*. Recombinant Sialostatin L inhibited the papain-like cysteine protease cathepsin L and cathepsin S and also reduced edema formation in a mouse model for acute inflammation. Sialostatin L inhibited also cathepsin C, an important activator of several proteolytic enzymes involved in immune reaction, including mast cell chymase and tryptase, neutrophil elastase, cathepsin G and protease 3 or granzyme A and B from natural killer cells and cytotoxic lymphocytes (CTL) [102]. Indeed, CTL proliferation was impaired after the treatment with Sialostatin L. Both cathepsin L and S activate cathepsin C from its precursor, therefore their inhibition may lead to the inhibition of the processes mentioned above. The mechanism of Sialostatin L function was further disclosed by Sa-Nunez et al. [117], who demonstrated the inhibitory effect on the maturation of LPS-stimulated DCs. As a consequence, CD4(+) T-cell proliferation was reduced. The studies with cathepsin L (-/-) and cathepsin S (-/-) DCs confirmed that the immunomodulatory activity is dependent on cathepsin S inhibition [117].

The second partially characterized cystatin from *I. scapularis* is Sialostatin L2 that, unlike Sialostatin L, facilitated the growth of *B. burgdorferi* in the murine skin [118]. The crystal structure of both sialostatins were determined and an unusual structure at Sialostatin L2 terminus was observed that is responsible for its specificity [118]. Moreover, the vaccination experiments revealed that Sialostatin L2 in the saliva does not induce humoral response, but animals vaccinated with recombinant protein were able to raise an effective immune response against feeding larvae [119], suggesting the importance of such "silent antigens" in tick feeding.

5.3.3 Ir-LBP

Ir-LBP originates from *I. ricinus* salivary glands and it belongs into the conserved lipocalin superfamily. Lipocalins are barrel-shaped proteins that usually act as scavengers of smaller compounds like histamine or serotonin. Ir-LBP binds leukotriene B4 (LTB4), the mediator of neutrophil-associated inflammatory response. In vitro experiments showed that Ir-LBP inhibits LTB4 induced delayed neutrophil apoptosis, a mechanism by which neutrophils prolong their life span during inflammation. Furthermore, Ir-LBP inhibited LTB4

induced neutrophil transendothelial migration [56]. Impaired chemotaxis of neutrophils into the site of inflammation can dramatically reduce the intensity of inflammatory reaction in the site of tick bite.

5.3.4 ISL 929 and ISL 1373

These two proteins from *I. scapularis* have a molecular weight of about 10 kDa each and they were described as effective inhibitors of neutrophil function. Knock-down of these proteins by RNAi led to impaired ability of tick saliva to down regulate the expression of integrins on neutrophils. As a result, the neutrophils ability to migrate to the inflamed site was not tampered after treatment with knocked-down saliva, while normal tick saliva impair this ability significantly [109]. Moreover, mice immunized with ISL 929/1373 had increased numbers of neutrophils at the site of tick attachment and a lower spirochete burden in the skin and joints 21 days after infection compared to control-immunized animals [109].

Other immunomodulators from *Ixodes sp.* are IRIS and IRS-2, serpins from *I. ricinus* that are extensively described in other chapters. The knowledge about IRIS is summarized in the chapter 6.4.2. and IRS-2 is characterized extensively in the attached **paper II**.

Finally, it is worth mentioning briefly some remarkable immunomodulators from other tick species. There is a cystatin from the soft tick *O. moubata* called OmC2 that inhibits T-cell proliferation and the production of proinflammatory cytokines TNF-α and IL-12 by mouse dendritic cells [103]. Four novel proteins from *Rhipicephalus sanguineus* called Evasin 1-4, were shown as capable to bind several types of chemokines and thus influence neutrophil migration and inflammation [104]. Several lipocalins with histamine binding properties were characterized from *Rhipicephalus appendiculatus* [106] as well as a modified Kunitz-domain inhibitor of mast cell tryptase [105].

5.4 *Serine Protease inhibitors*

Inhibitors of serine proteases play an important role during tick feeding. Most physiological processes in vertebrates, such as coagulation, complement activation, protein degradation, protein activation, signaling among cells and others, are driven by proteolysis and proteolytic enzymes. Pathological changes of tissues that could lead even to death of the organism can result from the abnormal regulation of the proteolytic activity. Under physiological conditions, proteolysis regulation is usually based on the activity of protease inhibitors that remain in balance with their target proteases. If this balance is disrupted, for

example due to the presence of an exogenous inhibitor, pathological conditions can appear. Such exogenous inhibitors can be of parasitic origin and the ticks apparently use protease inhibitors secretion in their saliva in order to facilitate their feeding.

Most of the known tick salivary protease inhibitors are described in Table 1 and in the section dealing with anticoagulants, inhibitors of platelet aggregation and immunomodulators. The first group of protease inhibitors is cystatins that were already discussed in the chapter 4.3.

Two main families of known serine protease inhibitors were found in ticks thus far, in addition to proteins or small peptides that also display inhibitory activity, but they have not been characterized extensively in the molecular level and no homologs can be found in any of available databases. The first family is highly represented in the saliva of both soft and hard ticks. It is the family of Kunitz domain-containing proteins. Kunitz domain inhibitors belong to the MEROPS inhibitor family I2, clan IB; their usual targets are proteases of the S1 family [120]. The structure of one domain usually consists of two β-strands and one α-helix. This structure is stabilized by 3 disulphide bridges that are created between six conserved cysteines. The molecular weight of one typical Kunitz domain is about 7 kDa, however many members of this family consist of several Kunitz domains. One, two and five Kunitz domain-containing inhibitors can be found in ticks. The majority of salivary transcripts are single domain putative inhibitors that differ in their amino acid sequences significantly. Many of them possess structural modifications that are the basis for their specific activities [105, 107]. In addition to the inhibitory activity, single Kunitz proteins are known also for being ion channel blockaders that are found in the venom of certain poisonous snakes [121]. Similar non-inhibitory single Kunitz domain-containing protein called Ra-KLP that modulated K+ channels activity was found in *R. appendiculatus* [107]. Two domain Kunitz proteins are tick anticoagulants and platelet aggregation inhibitors and they usually target thrombin and FXa. Double-domain Kunitz inhibitors can interact either with the enzyme active site or with the thrombin exosite I or with both, using one domain for the interaction with the exosite I while the second domain interacts with the active site of the targeted enzyme [67]. Another target for multidomain Kunitz-inhibitors is TF/FVIIa complex that activates FXa. To date, two such inhibitors from *I. scapularis* were characterized –Ixolaris and Penthalaris and both are mentioned in the chapter 5.1. Notably, only few inhibitors from Kazal superfamily have been found in ticks to date and none was identified as being secreted into the saliva. Kazal type inhibitors of thrombin were isolated for example from triatomines [110].

The second large group of serine protease inhibitors from ticks is the Serpin superfamily. The presence of serpins in tick salivary glands was shown several times before the employment of transcriptomic methods. Interestingly, the sialotranscriptomic approaches didn't reveal the expected number of serpins in salivary glands although their presence was proved by other methods. Apparently, the high-throughput methodology that was used for the analysis of tick salivary gland transcriptomes provides an insight into the salivary content, but it does not describe all the salivary transcripts, mainly due to technical restraints. The two main papers that form the basis for this dissertation are describing both approaches for the identification of *I. ricinus* salivary candidate effectors. While **paper I** took an advantage of the recently developed high-throughput, sialotranscriptomic methodology, **paper II** describes a second methodology that is complementary to the first one and that focuses on the identification and characterization of specific transcripts or proteins known to be present in tick salivary glands. Accordingly, *I. ricinus* protein IRS-2, a member of the Serpin superfamily and other family members that were not described in *I. ricinus* salivary glands by using a transcriptomic approach were cloned and characterized by molecular and biochemical methods. The next chapters describe the Serpin superfamily and its relevance for tick x host interaction in details.

6 The Serpin superfamily

The second part of the thesis is dedicated to the Serpin superfamily of serine protease inhibitors as the second main research goal of this thesis was the characterization of serpins from *I. ricinus* as potential modulators of the vertebrate host immune response. The name serpin is an acronym for serine protease inhibitor, which should originally regard to all serine protease inhibitor families. As the research in the field progressed, the name got more specific meaning and today it represents a single protein superfamily of ancient origin that is present in all organisms from viruses to vertebrates including Archea or plants. Inhibitory serpins target mainly serine proteases and also some cysteine proteases. Non-inhibitory serpins that lost their inhibitory function and gained another one have also been reported. Serpins usually function as natural regulators of physiological processes that depend on proteolytic activity. They control either proteases that activate other enzymes in proteolytic cascades (e.g. coagulation) or proteases that activate various signaling molecules such as cytokines or cell receptors. Three typical representatives of the family are α1-antitrypsin, antithrombin and chicken ovalbumin while the latter one is typical non-inhibitory serpin that functions as main storage protein in chicken egg-white.

6.1 *Structure and mechanism of inhibition*

All members of the serpin superfamily possess a single domain consisting of three β-sheets and 8-9 α-helices that is called serpin core domain. This structure provides serpins with several unique features that remained conserved among most members of the superfamily, despite their relatively low similarity at the amino acid level, which usually does not exceed 30% among distantly related organisms. Apart from well defined core domain, serpins possess reactive centre loop (RCL), which represents the most variable part of serpin. Interestingly, it has been observed that serpins from *Manduca sexta* can use splice variants similar to immunoglobulins in order to increase the functional repertoire of serpins. Those variants consist of 336 identical amino acids of core domain and 39-46 amino acids including RCL at the C-terminus vary in the different serpins [122].

Serpins naturally adopt 4 different conformational states; the **native**, **cleaved**, **latent** and **δ** stage [123]. The native state is characteristic for uncleaved, active, inhibitory serpins and that is also called **stressed,** because of the tension that is present in this conformation. The tension is released after the cleavage by the target protease and it enables the conformational change from native (stressed) to cleaved (**relaxed**) state. The transition is accompanied by the insertion of one additional β-strand (created by cleaved RCL) in the middle of β-sheet A, creating a 5-strand sheet (Figure 1). The third conformational state of serpins is the latent state, which is very similar to the relaxed state with the difference that the insertion of the fifth strand occurs without the cleavage of the RCL. This state is inactive and the serpin displays no inhibitory activity. The last state, called δ-state represents the conformation with partially inserted RCL and partially formed β-strand. This conformation was observed in native α-1-antichymotrypsin (PDB code 1QMN) and it is considered as an evidence of natural occurrence of intermediates during the loop insertion.

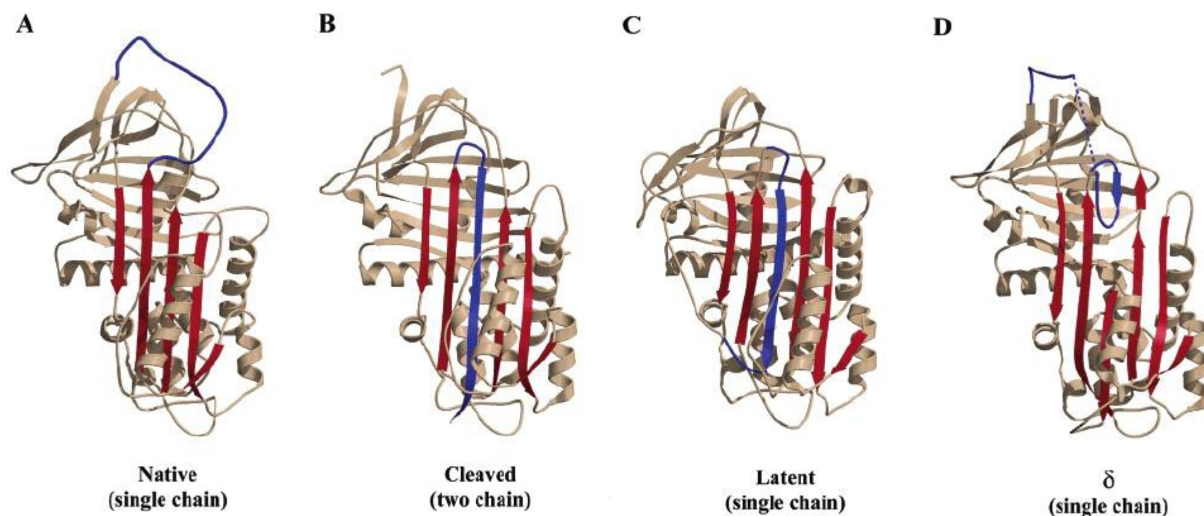

Figure 1) Comparison of four different possible serpin conformations. β-sheet A is depicted in red color and 5th β-strand that is formed by the insertion of RCL is depicted in blue. A) native, stressed state B) cleaved, relaxed state C) latent state D) δ-state. Adjusted from Gettins, 2002 [123].

In native form, RCL is easily accessible to the targeted proteases and functions as bait. Its amino acid composition and structure mimic natural substrate of the targeted protease; usually the most exposed part of RCL bears a P1 protease recognition site, after which the cleavage occurs. The cleavage is followed by the creation of a covalent bond between the serpin and the protease and subsequent conformational shift, during which the protease is attracted closer to the serpin and trapped in the covalent complex with it. The formation of serpin/protease complex results in the elimination of both proteins from the system. Therefore the mechanism of serpin inhibition is sometimes called as a "suicide inhibition". The inhibitory specificity of any serpin depends on the proper composition of three regions of the protein; the hinge – P15-P9 region of RCL, responsible for RCL mobility and successful conformational change, the breach – the initial part of the insertion of RCL into β-sheet A, and the shutter, which is located in the middle part of β-sheet A. Together with the breach, the shutter facilitates the proper opening of β-sheet for RCL insertion [124]. Sometimes, due to mutation in the hinge region or when the serpin is cleaved by a protease that is not its primary target, the complex is not formed and the serpin acts as a substrate rather than an inhibitor.

6.2 *Evolution and nomenclature*

Serpins form a superfamily that comprises more than 1500 described members to date from almost all groups of multicellular organisms and some bacteria and viruses [125]. There

are 16 phylogenetic clades of serpins named with letters *a – p* where vertebrate serpins form several functionally distinguished clades, while serpins from lower animals or plants usually form phylogenetically separated clades that follow the phylogeny of the organisms [124]. Human serpins have a well established nomenclature; each serpin name consists of the word serpin, usually with capital letters, then the letter describing the clade and the number of serpin in the clade. For example α-1-antitrypsin is designated as SERPINA1 and antithrombin is SERPINC1. The common ancestor of all serpins is not known, but provided that all serpins from multicellular organisms arose from one ancestral gene, the origin of serpins must be older than ~1.5 billion years, when plants and animals diverged. [124].

6.3 *Distribution and function*

As stated above, serpins usually function as serine protease inhibitors. As such, they regulate many physiological pathways in vertebrates, invertebrates and plants. Their occurrence can be both intracellular and extracellular, while the vertebrate serpins appear to be more conserved during their evolution than the invertebrate and plant serpins [124].

6.3.1 Human and vertebrates

There are 36 serpins in human with 29 inhibitory and 7 non inhibitory members [125]. The best studied serpin is α-1-antitrypsin, an extracellular inhibitor of neutrophil elastase; its deficiency results in emphysema and its polymerization and retention in the endoplasmic reticulum leads to cirrhosis. It is known for many serpins that their deficiency or misfolding leads to serious diseases, called serpinopathies.

Several serpins are involved in defensive and reparative mechanisms, such as coagulation or complement. Antithrombin (SERPINC1) is one example of coagulation regulator. Complement 1-inhibitor (SERPING1) regulates the cleavage of C1 component and activation of complement by classical pathway. Non-inhibitory serpins play role in hormone transportation, they are important markers in cancer with unknown function, but with lethal phenotype in serpin knock-out mice or they regulate blood pressure [126]. The detailed description of human serpin functions would exceed the purpose of this thesis.

6.3.2 Arthropods

In arthropods, serpins are found both in mandibulates and chelicerates. Insect serpins are important for the defense against pathogens and for tissue homeostasis after an injury. It was found that serpins in *Manduca sexta* regulate proteases responsible for the activation of

prophenol oxidase in plasma [127]. Prophenol oxidase pathway is the most important defensive mechanism of insects against injury and invading bacteria. Tight regulation of this pathway by serpins is known from *Drosophila sp.* in addition to *M. sexta*. Another role for serpins was discovered in *Drosophila melanogaster*, where serpins are involved in the regulation of an evolutionary conserved signaling pathway that plays role in the antimicrobial defense of this fruit fly. More specifically, a proteolytic cascade (where the regulation by serpin takes place) leads to an activation of a protein called spätzle, which is then able to bind to Toll receptor and activate transcription factor Dorsal, the homologue of mammalian NF-κB. This factor stimulates the expression of the antibacterial protein drosomycin [128]. It was shown that impaired regulation caused by serpin knock-down and following spätzle over-activation leads to spontaneous melanization, because of the excessive production of drosomycin [129]. It is also known that mosquito serpin SRPN6 inhibits plasmodium proliferation in *Anopheles stephensi*, while SRPN2 most likely facilitates midgut invasion by *Plasmodium berghei* [130]. Moreover, as short term blood feeders, mosquitoes need to overcome blood clot formation in host blood. At least three serpins are probably involved in this process, as the *Aedes aegypti* transcriptome revealed [131]. One of them was characterized as an inhibitor of Factor Xa. Interestingly, this particular serpin appears to act rather through binding in the Factor Xa exosite than through a classical serpin suicide mechanism [132]. In non-insect arthropods, serpins were studied in the horseshoe crab *Limulus polyphemus*, where the specific role of three serpins (LICI-1, LICI-2 and LICI-3) in the regulation of plasma coagulation cascade was described [133]. As shown above, this role of serpins is common for arthropods and vertebrates.

6.3.3 Plants

All plant serpins discovered up to date were considered as inhibitors. Their role can be in the protection of storage proteins of plant seeds, where their abundance is very high. Another possible function is the regulation of plant defense mechanisms [134].

6.4 *Serpins in parasitic organisms*

Serpins in parasites draw the interest, because they target host proteolytic enzymes, inhibition of which is vital or at least beneficial both for endo- and ecto-parasites. For endoparasites like helminthes, proteolysis has a direct impact by its potential to destroy parasite tissue, while ecto-parasites, i.e. bloodsucking arthropods, need inhibitors to block the activation of physiological defense mechanisms such as blood coagulation, platelet

aggregation or host inflammatory response. The latter activity is important for helminthes as well.

6.4.1 Helminthes

Blood feeding trematodes can express serpins on their surface in order to protect the parasite against proteolytic enzymes. Such a serpin was found on the surface of *Schistosoma mansoni*. The serpin is called Smpi56 and in addition to an endogenous 28-kDa protease it inhibits neutrophil elastase, which is known mainly for the killing of pathogens and remodeling of the tissues in the site of inflammation. The advantage that this serpin may provide to parasite survival is not known yet, but it can provide protection against neutrophil elastase and also against endogenous proteases, which are used by the parasite to invade the host tissues [135].

The microfilarial stages of *Brugia malayi* express serpin Bm-SPN2, which was shown to specifically inhibit neutrophil cathepsin G and elastase. This serpin forms 2% of the total ESTs from *B. malayi* microfilarial stage, whereas no similar ESTs are found in any other life stages of the parasite [136]. Another group reported that Bm-SPN2 is not an inhibitory serpin and that the previous finding was the result of an experimental artifact [137]. However, high amount of Bm-SPN2 expression during microfilarial stage suggests the connection with parasite survival and evasion of the host immune response.

6.4.2 Tick serpins

Interestingly, the herein described sialomic approach in *I. ricinus* did not reveal any serpin but numerous members were revealed mainly after the genome of *I. scapularis* was published [138-139]. It is likely that the tick salivary serpins, when introduced into the vertebrate host, can interfere with various physiological pathways and thus disrupt at least some of the host defense mechanisms. Several tick serpins were identified and described from ixodid ticks to date; however very few of them were characterized functionally. Two putative serpins have been described from *Haemaphysalis longicornis* [140], four from *R. appendiculatus* [141], 17 from *Ammblyoma americanum* [142], one from *I. ricinus* [101] and 45 genes coding for serpins was discovered in the genome of *I. scapularis* [143]. Two non-secreted serpins from *R. appendiculatus* were tested successfully in vaccination experiments [144] as well as HLS-2, another non-secreted serpin from *H. longicornis* [145]. The first and for long time the only described and functionally characterized tick serpin originates from *I. ricinus* and was named IRIS (*I. ricinus* immunosuppressor). This serpin was first detected as

up regulated upon blood feeding in tick salivary glands; different libraries from partially fed and unfed females were compared and the clones that differed in their expression in the two groups were analyzed and sequenced. Among them, the transcript regarded as Seq24 belonged into the serpin superfamily [146]. In the following work, Seq24 was renamed to IRIS, because it displayed several immunomodulatory activities [101]. Soluble protein extract from CHO-KI cells transfected with IRIS inhibited the proliferation of splenocytes from Balb/c naive mice at concentration ~6.5nM by 81% when compared with CHO-KI protein extract from non-transfected cells. The production of various cytokines by human peripheral blood mononuclear cells (PBMCs) was also affected. PBMCs treated with cell extract containing IRIS and stimulated with phytohemaglutinin showed 80% decrease in IFN-γ production. When stimulated with LPS, IFN-γ levels remained unchanged while the production of IL-10 was enhanced by 400%. Furthermore the production of IL-6, TNF-α and IL-8 was also diminished [101]. The same research group pursued IRIS characterization in 3 additional works. Prevot and colleagues showed anti-hemostatic, antifibrinolytic and anticoagulant activity of recombinant IRIS, however in quite high concentrations, where the nonspecific inhibition of proteases involved in coagulation can occur [77]. Vaccination with IRIS raised certain level of protective immune response against tick adults and nymphs by rabbits [147]. The latest contribution to this series of publications described the mechanism underlying the anti-inflammatory effect of IRIS. Authors showed that IRIS binds to monocytes/macrophages and inhibits the production of TNF-α. Interestingly this mechanism is not dependent on the serpin inhibitory function, but most likely is mediated by an exosite [148]. Despite such promising activities obtained with single serpin, IRIS does not bear a classical signal peptide, which makes it hard to believe that it is really secreted into the host with the saliva. The presence of IRIS in the ducts of salivary glands and an increase of its abundance may be explained by the cross-reactivity of polyclonal sera raised against recombinant IRIS with another serpin that is secreted from the salivary glands. The idea that IRIS is actually an intracellular serpin is supported by the phylogenetic analysis, where IRIS clusters rather with intracellular clade B than with other tick serpins with predicted signal peptide.

6.4.2.1 Novel *Ixodes ricinus* serpins

One of the main aims during my PhD. studies was to isolate and characterize salivary serpins from *I. ricinus* with clear secretion signal. Using degenerated primers that were

designed to anneal with conserved serpin domains, partial sequences of 8 different serpin clones were determined and named accordingly as IRS 1-8.

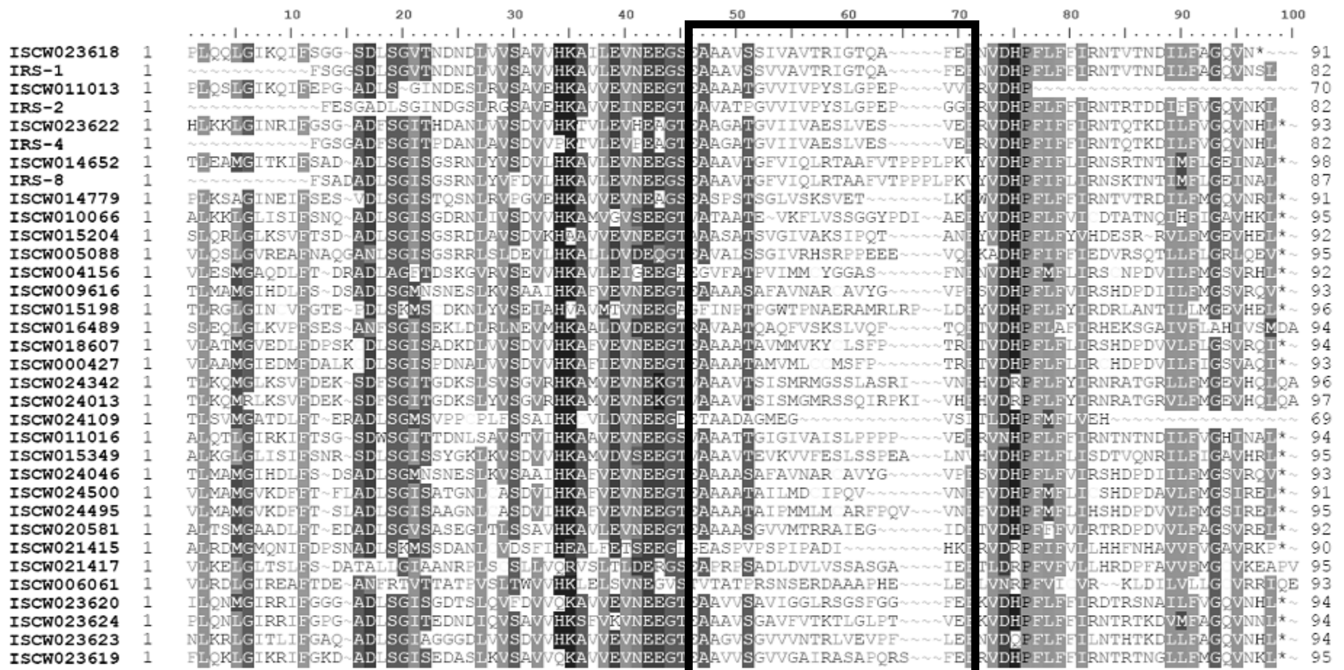

Figure 2: Alignment of C terminus of 4 serpins from *I. ricinus* salivary glands, isolated as full-length clones by screening a salivary cDNA library. The serpins from *I. ricinus* are compared with serpins, obtained from the genome of *I. scapularis*. Black rectangle represents the variable region of RCL.

The PCR with degenerated primers could amplify only small fragment that could be used for the identification, but not for the expression of recombinant protein, so another approach was employed that should result in obtaining full coding sequences. Using the same degenerated primers, a radioactively labeled probe was prepared and a cDNA library was screened for full-length clones. With this approach, 4 full length serpin sequences were cloned and deposited in GenBank – IRS-1 (DQ915842), IRS-2 (DQ915843), IRS-4 (DQ915844) and IRS-8 (DQ915845). The alignment in figure 2 shows the RCL of these 4 clones together with 30 serpin clones that can be found in the genome of *I. scapularis*. The four serpin genes were cloned into an expression vector with the aim to be produced in pure recombinant form. Each serpin required different refolding conditions because all four serpins were overexpressed as insoluble inclusion bodies in bacteria and out of the four, IRS-2 gave the best yield and resulting activity. The characterization of IRS-2 is described in detail in the **paper II**. The other serpins wait for their expression in a eukaryotic system (baculovirus) that should be more appropriate for their proper folding and functional characterization.

7 Summary of results

This Ph.D. thesis is based on 2 main research papers and one additional methodological research paper, hereby referred to as **paper I**, **paper II** and **paper III**. The topic of the thesis is the identification and characterization of the salivary proteins from the saliva of *Ixodes* ricinus, European vector of Lyme disease causative agent, *Borrelia burgdorferi sensu lato* To follow this goal, two complementary approaches were employed that resulted in the two research papers.

Paper I describes the results of high-throughput methodology that was used to identify, how the expression of proteins from the salivary glands changes during the feeding course of *I. ricinus*. Four cDNA libraries from the salivary glands of adult females were constructed. The following feeding stages were used for the construction: unfed, 24 hours, 4 days and 7 days after an attachment to the guinea pig. Almost 600 clones were randomly picked from each library plated on Petri dishes with LB agar and sequenced. From total ~2300 sequenced clones, 1881 sequences, considered as high-quality were subjected to further bioinformatic analytical pipeline. Sequences were first trimmed of the fragments belonging to cloning vector and poly A non coding regions from 3' terminus. According to clustal W based assembly tool, 1274 clusters with the identity higher than 95% at nucleotide level and consensual sequences were created. The assembly resulted in the identification of 268 contigs (clusters with 2 or more sequences) and 1006 singletons. By using BLAST (Basic Local Alignment Search Tool), these contigs and singletons were compared with 7 customized nucleotide and protein libraries, e.g. non-redundant nucleotide NCBI GenBank library, Smart (Simple modular architecture research tool), Pfam (protein families database) or gene ontology (GO). In addition, the sequences were tested for the presence of signal peptide; its presence was considered as an indirect evidence of the secretion into the saliva and the host. The resulting data were recorded in a single table with many links to text files containing data from BLAST analysis. In the following step, manual annotation of whole dataset was performed. As a result, all sequences were sorted according to their predicted function or their phylogenetic relatedness.

The transcripts can be divided among 5 basic groups. The first group comprises of transcripts coding for housekeeping proteins like cytochromes, proteasome components or ribosomal proteins (29.6% of all transcripts). The second group is formed by proteins with predicted signal peptide - putatively secreted proteins (31.7%). The third, notably most abundant group consists of unknown transcripts with no match in any of searched databases

(33.8%). Interestingly the largest portion of unknown transcripts, which exceeded 66%, was discovered in the library from unfed ticks. Those transcripts did not bear a signal peptide, suggesting some intracellular, probably housekeeping role. Small portion of transcripts (2.6%), regarded as “unknown conserved”, is formed by proteins with relatives among other organisms, however without any knowledge about their function. Last group comprises of other minor transcripts that cannot be included in another group like predicted transposon elements or reverse transcriptase-like proteins. This work was focused mainly on the identification of secreted proteins; therefore their description represents the main part of **paper I**. First interesting finding was that there is huge increase in the expression of secreted proteins as a reaction to the start of feeding and attachment to the host. More specifically, the amount of secreted proteins was 11 times higher in 24 h library than in the library from unfed ticks. Second interesting result concerns multigenic families; the data from **paper I** suggest the sequential expression of paralogs from the most abundant multigenic family as the feeding progresses. This could be explained by the attempt of the tick to change the antigenic profile of its saliva or it can be connected with the sequential organization of these genes on the chromosome. From the comparison among *I. ricinus, Ixodes scapularis* and *Ixodes pacificus* transcriptomes we can make a suggestion that, concerning the most abundant multigenic families, the same evolutionary mechanisms were applied by each species on different genes from the same family. Therefore the members of the largest families from one species cluster together rather than with orthologs from other species.

In addition to the findings stated above and to the detailed description of *I. ricinus* transcripts from the salivary glands, the work, published in **paper I**, represents the most complex overview of the changes in salivary proteins expression during the feeding course of this tick and it also founded the basis for further characterization of candidate *I. ricinus* salivary effectors.

The second approach, complementary to high-throughput methodology used in **paper I**, is the functional analysis of one specific salivary protein. In my case it was the member of the Serpin superfamily of protease inhibitors. Notably, this family was not revealed by transcriptomic approach, although its presence in ticks, in particular in *I. ricinus,* was reported before. Therefore I used the method of cDNA library screening with radioactively labeled DNA probe to find full-length serpins in the salivary glands of *I. ricinus*. The probe was prepared by using PCR with degenerated primers designed after two most conservative serpin domains. Four full-length serpin sequences were obtained and subjected to further analysis.

First, the expression profiles for all 4 serpins in 3 main tick tissues (i.e. salivary glands, midgut and ovary) were done by using qRT-PCR. The results showed that the serpin named **IRS-2** is the most upregulated upon tick feeding. Therefore I decided to pursue its further characterization that is the topic of the **paper II.** In order to disclose the potential role of IRS-2 in tick x host interaction, active recombinant IRS-2 was produced by using bacterial expression system, it was refolded, purified to more than 99% purity and concentrated to suitable concentration. The decontamination from bacterial lipopolysaccharid (LPS) was also performed, as LPS could interfere with IRS-2 activity in *in vivo* experiments. Because of the analysis complexity, not all necessary experiments were performed in our laboratory. Therefore some of the results were done in other workplaces either by our collaborators or by me. First we tested, if IRS-2 affects an inflammatory response, because the prediction of its inhibitory specificity pointed at chymase as targeted protease, which is known for its involvement in inflammation. The results from mouse paw edema experiments showed clearly that IRS-2 inhibits carrageenan induced acute inflammation in mouse paws. Moreover the levels of myeloperoxidase in the affected tissues were reduced significantly when carrageenan was co-injected with IRS-2, showing the decrease in the number of neutrophils migrated to the inflamed site. After proving the anti-inflammatory effect of IRS-2, its inhibitory profile was determined in a series of enzymatic assays *in vitro*. Out of 16 tested serine proteases, only 5 were inhibited by IRS-2. IRS-2 inhibited trypsin and one trypsin-like protease thrombin and α-chymotrypsin and two chymotrypsin-like proteases cathepsin G and chymase. The latter two were inhibited in equimolar concentration, demonstrating extremely high specificity against these two proteases. The inhibitory activity against mMCP-4, mouse equivalent of human chymase, and the formation of covalent complexes between mMCP-4 and IRS-2 were proved in *ex vivo* experiments with the population of mouse peritoneal cell-derived mast cells. IRS-2 was also shown to inhibit cathepsin G induced and in higher concentration also thrombin induced platelet aggregation. It was for the first time, when tick salivary protein affected this pathway of platelet activation. Finally, the crystal structure of cleaved IRS-2 was resolved at the resolution of 1.8Å. The structure confirmed tyrosine at the P1 site and revealed high structural similarity with α-1-antichymotrypsin, human endogenous inhibitor of the proteases targeted by IRS-2. The procedure of IRS-2 crystallization is described in detail in **paper III.** The cleavage site of IRS-2 reactive centre loop strongly resembles the cleavage site for chymase on big endothelins, precursors for important vasoconstrictors. This is first time, when parasite protease inhibitor was demonstrated to

mimic a host endogenous substrate and thus facilitates the evasion of host defensive mechanisms by parasite.

If we assume that *I. ricinus* possesses the same spectrum of serpins as *I. scapularis*, than, according to the information from the genome of *I. scapularis*, IRS-2 is the only serpin from *I. ricinus* with the specificity and the activity described in **paper II**. IRS-2 is the second functionally characterized serpin from ticks, after IRIS, another serpin from *I. ricinus.* However, since IRIS is not most likely secreted into saliva, because it lacks the signal peptide, IRS-2 is probably the first characterized tick salivary serpin that plays role at the tick x host interface.

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Appendix – attached papers

Paper I

Chmelar, J., Anderson, J.M., Mu, J., Jochim, R.C., Valenzuela, J.G., Kopecky, J., 2008: Insight into the sialome of the castor bean tick, *Ixodes ricinus*. BMC Genomics 9, 233.

Abstract

BACKGROUND: In recent years, there have been several sialome projects revealing transcripts expressed in the salivary glands of ticks, which are important vectors of several human diseases. Here, we focused on the sialome of the European vector of Lyme disease, Ixodes ricinus.

RESULTS: In the attempt to describe expressed genes and their dynamics throughout the feeding period, we constructed cDNA libraries from four different feeding stages of Ixodes ricinus females: unfed, 24 hours after attachment, four (partially fed) and seven days (fully engorged) after attachment. Approximately 600 randomly selected clones from each cDNA library were sequenced and analyzed. From a total 2304 sequenced clones, 1881 sequences forming 1274 clusters underwent subsequent functional analysis using customized bioinformatics software. Clusters were sorted according to their predicted function and quantitative comparison among the four libraries was made. We found several groups of over-expressed genes associated with feeding that posses a secretion signal and may be involved in tick attachment, feeding or evading the host immune system. Many transcripts clustered into families of related genes with stage-specific expression. Comparison to Ixodes scapularis and I. pacificus transcripts was made.

CONCLUSION: In addition to a large number of homologues of the known transcripts, we obtained several novel predicted protein sequences. Our work contributes to the growing list of proteins associated with tick feeding and sheds more light on the dynamics of the gene expression during tick feeding. Additionally, our results corroborate previous evidence of gene duplication in the evolution of ticks.

Paper II

Chmelar, J., Oliveira, C.J., Rezacova, P., Francischetti, I.M.B., Kovarova, Z., Pejler, G., Kopacek, P., Ribeiro, J.M.C., Mares, M., Kopecky, J., Kotsyfakis, M., 2010: A tick salivary protein targets cathepsin G and chymase and inhibits host inflammation and platelet aggregation (revision submitted to Blood).

Abstract

Platelet aggregation and acute inflammation are key processes in vertebrate defense to a skin injury. Recent studies uncovered the mediation of two serine proteases, cathepsin G and chymase, in both mechanisms. Working with a mouse model of acute inflammation, we revealed that an exogenous salivary protein of Ixodes ricinus, the vector of Lyme disease pathogens in Europe, extensively inhibits edema formation and myeloperoxidase activity in the inflamed tissue. We named this tick salivary gland secreted effector as Ixodes ricinus serpin-2 (IRS-2) and we show that it primarily inhibits cathepsin G and chymase, while in higher molar excess it affects thrombin activity as well. Moreover, we disclosed the ability of IRS-2 to inhibit cathepsin G- and thrombin-induced platelet aggregation and we resolved its crystal structure at a resolution of 1.8 Å, which is the first for a serpin originating from a parasite. Also for the first time an ectoparasite protein is shown to exhibit such pharmacological effects and target specificity. The stringent specificity and the biological activities of IRS-2 combined with the knowledge of its structure can be the basis for the development of future pharmaceutical applications.

Paper III

Kovarova, Z., **Chmelar, J**., Sanda, M., Brynda, M., Mares, M., Rezacova, P., 2010: Crystallization and diffraction analysis of the serpin IRS-2 from the hard tick *Ixodes ricinus.* Acta Crystallographica Section F (accepted).

Abstract

IRS-2 from the hard tick Ixodes ricinus belongs to the serpin family of protease inhibitors. It is produced in the salivary glands of the tick and its antiinflammatory activity suggests that it plays a role in parasite–host interaction. Recombinant IRS-2 prepared by heterologous expression in a bacterial system was crystallized using the hanging-drop vapour-diffusion method. The crystals belonged to the primitive tetragonal space group P43 and diffracted to 1.8 A resolution. Mass-spectrometric and electrophoretic analyses revealed that IRS-2 was

cleaved by contaminating proteases during crystallization. This processing of IRS-2 mimicked the specific cleavage of the serpin by its target protease and resulted in a more stable form [the so-called relaxed (R) conformation], which produced well diffracting crystals. Activity profiling with specific substrates and inhibitors demonstrated traces of serine and cysteine proteases in the protein stock solution.