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Isoenzymes of cathepsin L-type peptidases in the tick *Ixodes ricinus*

Bachelor Thesis

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Co-Supervisor: RNDr. Petr Kopacek, CSc.

Ceske Budejovice 2018

Annotation:
The aim of this work is to partially characterize two isoenzymes of cysteine peptidases cathepsin L1 and L3 from the European Lyme disease vector tick *Ixodes ricinus*. The aims are (i) to verify different expressions of these isoenzymes in tick tissues and developmental stages on mRNA, protein and enzyme activity levels; (ii) to recombinantly express and purify the isoenzymes and raise isoenzyme type specific antibodies in mice and rabbits, verify their specifity using Western blotting and further use them for double immunohistochemical labeling of the tick gut tissue sections.

Affirmation:
I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section. I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defense in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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České Budějovice, 10.12.2018 Katharina Perfahl

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1. Introduction

1.1 Ticks

Ticks are worldwide distributed blood-sucking ectoparasites of terrestrial vertebrates. They are arachnids, related to mites (order Acari) and are well known as vectors of numerous pathogens (from viruses to helminthes) causing disease to animals and also humans (Sonenshine, 1991).

The family of hard ticks (Ixodidae) follows a uniform life cycle, consisting of eggs and three active stages: larvae, nymphs and adults. Adult ticks feed for several days on the host and can transmit pathogens in this time. Fertilized females committing rapid engorgement, lay down a large clutch of eggs and die. Most ticks of the Ixodidae family have to parasitize three different hosts during their entire life cycle (Sonenshine, 1991).

The model tick used in this work, *Ixodes ricinus,* is commonly found in Europe, Middle East and North Africa. The larvae feed on mice or voles, the nymphs parasitize birds or small and big mammals and the adult is found feeding mainly on deer, foxes, sheep, dogs and humans (Sonenshine, 1991).

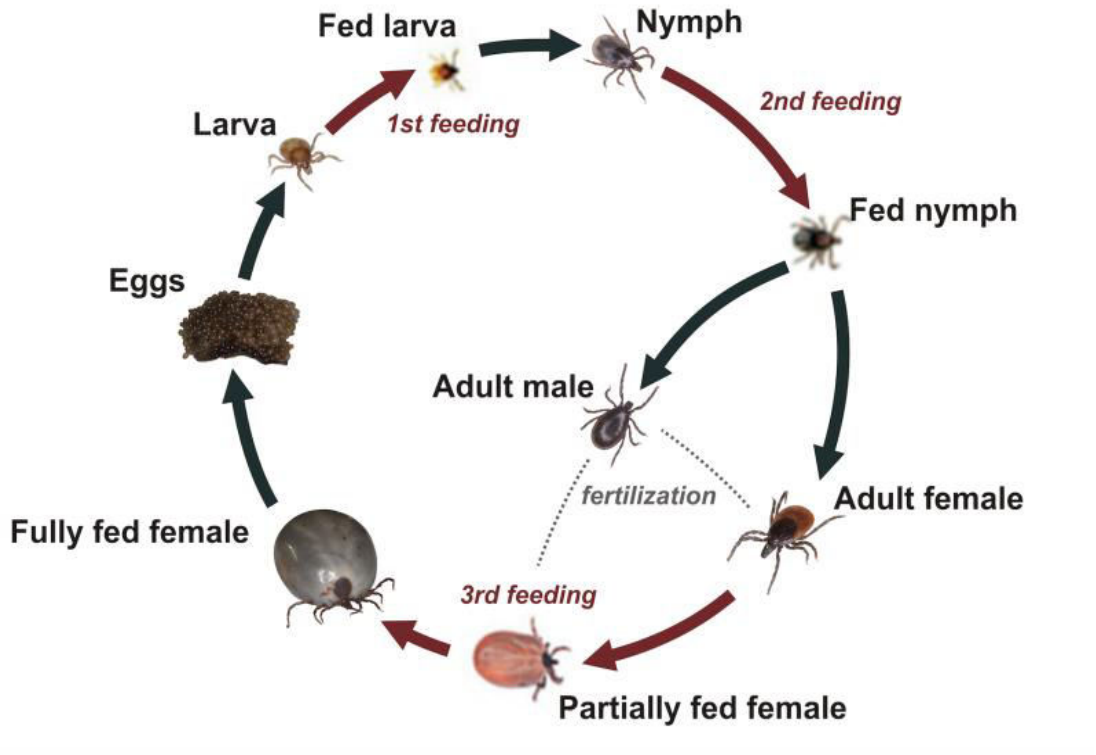

Figure 1: **Illustration of the life cycle of the three-host tick *Ixodes ricinus***
(Sojka *et al.,* 2013)

Due to the wide distribution area and the availability of different vertebrate hosts, *Ixodes ricinus* is the most important arthropod disease vector throughout Europe. This tick is the major vector of Lyme disease caused by the spirochetes of the genus *Borrelia* and is also known for transmitting the tick borne encephalitis virus (TBEV). Among other different pathogens transmitted by this tick species belong bacteria (Borrelia, e.g. *Rickettsiales* and *Anaplasma phagocytophilum* causing ehrlichiosis), protozoans (e.g. *Babesia divergens B. microti*) and spirochetes of the *Borrelia burgdorferi sensu lato*, which leads to Lyme disease. (Rizzoli *et al.*, 2011).

1.2 *I. ricinus* feeding strategy

The blood feeding process, also called hematophagy, differs significantly between the soft and hard ticks as well as between immature and adult stages of hard ticks (Nava et al., 2009; Sonenshine, 2014). Feeding of *Ixodes ricinus* adults (females, males do not feed on blood) can be divided in three stages: 1) preparative attachment, 2) slow-feeding period and 3) rapid engorgement. During the preparative phase, that takes place in the first 24-36 hours of the feeding process, the female attaches to the host by cement proteins and starts to feed with insertion of tick mouthparts into the host skin (Coons *et al.*, 1986; Sonenshine, 1991). After that, the slow-feeding phase is initiated and the tick ingests a small amount of host blood. Only mated females are able to step into the rapid engorgement phase occurring in the last 12-24 hours of the feeding course, when about 2/3 of the total blood meal is imbibed and the rate of digestion increases significantly (Weiss and Kaufman, 2004). When rapid engorgement is completed, *I. ricinus* female drops off the host and continues with digestion of imbibed blood. In contrary to insect blood feeders, digestion occurs intracellularly within the tick gut epithelium. Most of the blood imbibed during rapid engorgement serves to generate energy and provides enormous nutrient supplies for sexual reproduction - production of eggs - which in the case of *I. ricinus* is enormous (thousands of eggs per one female) and represents the main reason for a widespread existence of this tick (Sonenshine, 1991).

1.3 Digestive Phases

As stated above ticks digest intracellularly in the acidic lysosome-like vesicles of digestive cells and the tick gut lumen serves only as a meal storage space (Sonenshine, 2014; Sojka *et al.*, 2013). This is in contrast to the digestive systems of blood-feeding insects, where protein digestion proceeds rapidly and is maintained mainly by serine proteases operating in the neutral or slightly alkaline environment of gut lumen.

Two major proteins - hemoglobin and albumin - represent for about 80% of total protein component of vertebrate host blood. Thus, they are supposedly the main source of amino acids for ticks. Tick digestive gut cells take up blood proteins by heterophagy. Albumin is taken up by fluid-phase endocytosis and is directed towards a population of small acidic vesicles. In contrast, hemoglobin seems to be recognized by yet unidentified cell-surface receptors, which target it towards a population of large endosomal vesicles (Lara *et al.*, 2005). It is assumed that the receptor-mediated endocytosis and a separate processing pathway for hemoglobin evolved as a part of a mandatory heme detoxification mechanism, since the waste amounts of hemoglobin released heme are toxic in their unbound form, due to their reactivity forming of free oxygen radicals (Lara *et al.*, 2003; Graca-Souza *et al.*, 2006).

Based on the changes in the histology of gut epithelium, processing of host blood can be divided into three digestive phases; 1) slow-feeding, 2) rapid engorgement and 3) post-detachment period. Even though many previous studies describe various gut cell types (digestive, secretory, etc.) in different tick species, it can be also assumed that there is only a single type of tick gut cell, whose population undergoes unsynchronized developmental phases of growth, proteosynthesis, secretion, digestion and heme detoxification (Coons *et al.*, 1986, Sojka *et al.*, 2013).

1.4 Digestive Proteases

Previously, a dynamic network of multienzymes of acidic aspartic and cysteine peptidases that is capable of processing the imbibed host blood has been identified. Individual enzymes process hemoglobin to peptides of different size (Sojka *et al.*, 2013). The detailed model of host blood protein processing by tick endolysosomal digestive peptidases is shown in Figure 2.

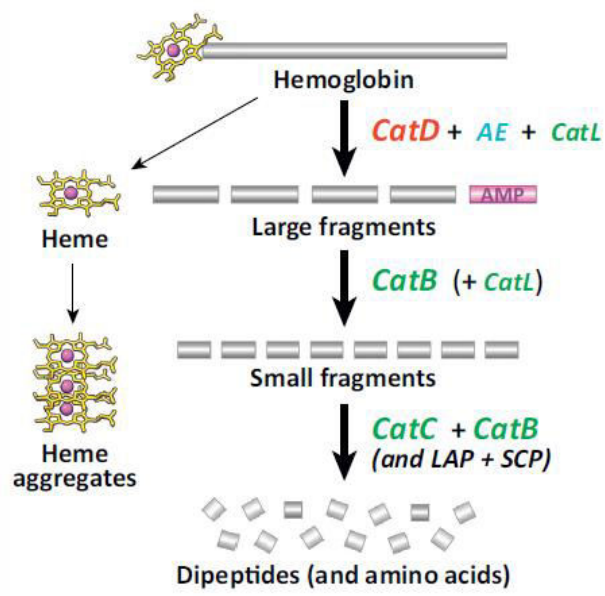

Figure 2: **Current model of proteolytic degradation inside the digestive cells of partially engorged female guts** (Sojka *et al.*, 2013)

Once in the endolysosomal system, hemoglobin is initially cleaved by the endopeptidase activity of cathepsin D (*Ir*CD1, Sojka *et al.*, 2012) and this cleavage is further supported by endopeptidases cathepsin L (*Ir*CL1, Franta *et al.*, 2011) and legumain (*Ir*AE1, Sojka *et al.*, 2007). For the cleavage into the secondary smaller fragments, cathepsin B is the major contributor with additional minor activity of cathepsin L. Cleavage into dipeptides and amino acids is further maintained by the activities of cathepsin C and cathepsin B with the final contribution of serine carboxypeptidase (SCP) and leucine aminopeptidase (LAP) (Horn *et al.*, 2009). Upon digestion of hemoglobin, unbound heme groups form aggregates that accumulate in a specialized membrane delimited organelle - the hemosome (Lara *et al.*, 2003). Recently, novel RNA seq data (Perner *et al.*, 2016) as well as our in-lab observations revealed that the tick genome encodes for a higher number of listed endo- and exopeptidase isoenzymes of various transcriptional regulation indicating different roles in the tick body (Hartmann *et al*, 2018).

1.4.1 Cathepsin L

Cathepsin L belongs to the clan CA, C1 family, subfamily A (C01.032). It can be found in most eukaryotic cells and plays a major role in intracellular protein catabolism, as well as antigen processing, tumor invasion and metastasis, bone resorption, and turnover of intracellular and secreted proteins involved in growth regulation (Kane *et al.*, 1990). Cathepsin L, similarly to other papain family members, is capable of pH dependent autocatalytic activation and degrades hemoglobin with an acidic pH optimum (Yamaji *et al.*, 2009). Besides hemoglobin, it degrades nearly all proteins, including receptors, enzymes and transcription factors. Its deficiency in mice was shown to be a molecular defect of the previously known furless mouse (Wright *et al.*, 2003).

In the tick digestive system, *I. ricinus* cathepsin L (*Ir*CL) is the second major contributor to the hydrolytic activities resulting in large hemoglobin fragments. *Ir*CL is capable of substituting *Ir*CD when it is specifically inhibited (Horn *et al*., 2009). The proenzyme has a mass of about 40 kDa and the mature form has a molecular mass of about 28 kDa. *Ir*CL cleaves hemoglobin to large fragments, but also participates in the cleavage into small peptides with a pH optimum in the acidic range at pH 4.0 (Franta et al., 2011).

1.5 Peptidase Activity

Cleavage preferences of *Ir*CL1 are mainly amino acids with polar side chains at P1, with the highest preference for arginine and bulky aromatic residues at P2 (Franta *et al*., 2011).

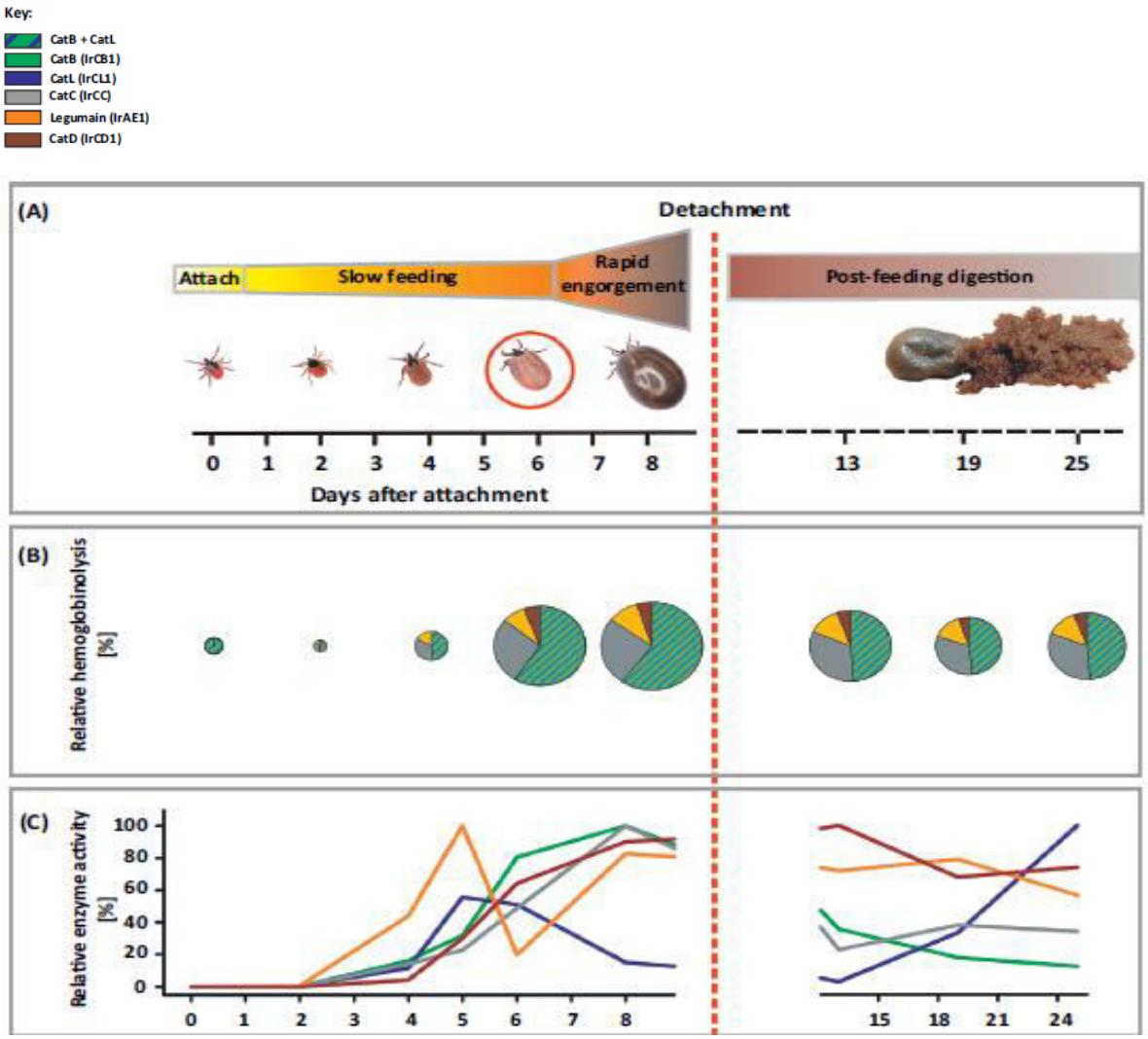

Figure 3: **Comparison of the activity profiles of the different peptidases according to the time course of feeding; panel A**: feeding and digestive phases of *Ixodes ricinus* females and their optical appearance **panel B**: relative hemoglobinolytic activities in gut tissue extracts shown in pie charts (cathepsin B and L were determined together due to the lack of a specific inhibitor for cathepsin L) **panel C**: dynamic activity profiles of the different peptidases (determined using specific fluorogenic substrates and shielding inhibitors to eliminate interference from other peptidases) (Sojka *et al*., 2013)

Three isoforms of Cathepsin-L like enzymes have been identified from *I. ricinus* and closely related *Ixodes scapularis* genomes, EST (expressed sequence tag) and RNA sequence datasets and have been tagged as *Ir*CL1-3. *Ir*CL1 was found to be responsible for the intestinal hemoglobinolytic cathepsin L activity in partially engorged females and it is also expressed in the salivary glands, ovaries and malphagian tubules (Franta *et al.*, 2011). *Ir*CL1 and *Ir*CL3 can be found in the gut, whereas the site of *Ir*CL2 expression remains unknown (Sojka *et al.*, 2008). Relative enzyme activity profiles of tick gut tissue extracts using specific fluorescent substrates and inhibitors show two significant peaks for *Ir*CL (Figure 3), which could account for two different isoforms of cathepsin L being expressed in semiengorged and fully fed *I. ricinus* females. This work represents a partial contribution to the functional and biochemical characterization of the two gut associated cathepsin L forms in *Ixodes ricinus Ir*CL1 and *Ir*CL3 in order to determine their specific functional roles in the slow and upon the rapid feeding periods.

2. Objectives:

-Cloning, expression and purification of recombinant *Ir*CL3 protein in a bacterial expression system

- Verification of recombinant *Ir*CL3 activity using fluorescent peptidyl substrates and cysteine protease inhibitors

- Preparation of *Ir*CL3 specific polyclonal antibodies in rabbits

- Verification of *Ir*CL1 versus *Ir*CL3 antibody specifity using Western blotting of recombinantly expressed *Ir*CL1 and *Ir*CL3 as well as native tick gut extracts

- Multiplex Western blot expression profiling of *Ir*CL1 and *Ir*CL3 over tick gut in different timepoints of tick feeding and digestion using the verified isoenzyme specific primary sera and commercial fluorescently labeled secondary antibodies

- Multiplex immunohistochemistry of tick gut cells using the isoform specific antibodies

3. Materials and Methods

3.1 Experimental animals

Experimental *I. ricinus* ticks were obtained from a laboratory colony maintained at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences (BC CAS), Ceske Budejovice, Czech Republic. Adult females were fed on rabbits and guinea pigs. All laboratory animals were treated in accordance with the Animal Protection Laws of the Czech Republic No. 246/1992 Sb., ethics approval issued by the ethical committees at the BC CAS, the State Veterinary Administration, and the Central Commission for Animal Welfare under protocol No. 095/2012.

3.2 Dissection and preparation of the gut tissue

Females of *Ixodes ricinus* were fed on guinea-pigs (*Cavia porcellus)* at 24°C and atmospheric humidity (approx. 95%) for the estimated time and then forcedly removed. For post-feeding intervals animals were kept in glass vials, 24°C, 95% humidity, 12/12 light/dark photoperiod. Gut tissue was extracted by dissection of collected female individuals under a binary microscope. Tissue was extensively washed in phosphate buffered saline (PBS). For RNA isolation, the washed tissue was placed in microtubes containing 500µl of TRI reagent (Sigma-Aldrich).

3.3 RNA isolation and preparation of cDNA templates

Total RNA was isolated from tick developmental stages and female tissues using the NucleoSpin® RNA kit (Macherey-Nagel). Purification of the RNA samples was done by using the TRI Reagent (Sigma - Aldrich) following the provided protocol. The quality of isolated total RNA samples was checked electrophoretically on 1% agarose gel in 1x TBE buffer. Gut isolated RNA samples were diluted to a final concentration of 200 ng/µl and used for cDNA synthesis via the Transcriptor High Fidelity cDNA Synthesis kit (Roche) using the oligo$(dT)_{18}$ primer. Quality of the synthetized cDNA was checked by PCR amplification of the housekeeping gene *elongation factor 1* (*I. scapulari*s translation elongation factor EF-1 alpha/Tu, GenBank accession No. XM_002411102).

3.4 DNA amplification and purification

Selected *Ir*CL3 fragments for the expression of *Ir*CL3 in the pET100 D TOPO® expression vector (see chapter 3.5) were PCR-amplified using the High-Fidelity polymerase (Thermo Fisher™, Fermantas), gene-specific primers forward

5'-CACCGAGAATGAGGTCCTGGAGGTTGAG-3' and reverse 5'-TTACACGAGTGGGTAGCTGGCG-3' (adaptor for directional cloning into pET100 underlined) and the gut tissue cDNA as a template. Annealing temperatures were specified using gradient PCR (40, 50, 55, 60 and 65°C annealing temperatures) in the T100 PCR cycler (BioRad). Upon 35 cycles, PCR reactions were placed on 1% agarose gel containing Ethidium Bromide and electrophoretically separated (125 V, 100 mA, 30 minutes). DNA Bands were visualized under UV light and were selected according to the expected band size from the gel with the Nucleospin® Gel and PCR Clean-up Kit (Macherey-Nagel) following the protocol provided by the manufacturer. The resulting isolated and purified PCR amplicon was immediately used for cloning into the pET 100 D TOPO® expression plasmid vector.

3.5 Cloning, transformation and isolation of plasmid DNA

For the recombinant expression of the *Ir*CL3 proenzyme, the Champion™ pET Directional TOPO® Expression kit (Invitrogen™) with the expression vector pET100 D TOPO® (Thermo Fisher™) was used in the identical manner to previous cloning and expression of *Ir*CL1 (Franta *et al.*, 2011). Briefly, 3 µl of the purified *Ir*CL3 PCR amplicon extracted from agarose gel (chapter 3.4) were mixed with 1 µl of salt solution,1 µl water and 1 µl of TOPO® vector, placed in a tube, mixed gently and incubated for 10 minutes at room temperature (RT). All 6 µl of the TOPO isomerase ligation reaction were heatshock (42°C, 1 min) transformed to One Shot® TOP10 Chemically Competent *E. coli*. 250 µl of warm (37°C) SOC medium was added and the tube was shaken horizontally at 200 rpm, 37°C for 40 minutes. The solution was used to inoculate two different LB/ampicillin plates (petri dishes with LB/agarose medium containing ampicillin, prewarmed at 37°C) at two volumes: 100 µl and residual volume from the reaction, to ensure well-spaced colonies. The plates were placed to a thermal incubator and left to grow overnight at 37°C. 20 *E. coli* colonies were picked up by a sterile pipette tip (1-10 µl, Eppendorf), resuspended in 30 µl of PCR grade water and 1 µl of the suspension was used as DNA template for the 10 µl PCR verification with gene specific primers in a 25 cycle PCR reaction (polymerase mix, TopBio) followed by gel electrophoretic analysis. PCR positive colonies were labeled and the residual bacterial water suspensions (stored at 4°C in the mean time) were used to inoculate 2.5 ml of liquid LB/ampicillin for plasmid propagation in an orbital thermal shaker (37°C, 220 rpm) overnight. Plasmid DNA of several *E. coli* clones was isolated using the High Pure Plasmid isolation kit (Roche) and sequence verified for correct ligation and an uninterrupted open reading frame of the fusion recombinant protein.

3.6 Recombinant protein expression, purification and refolding

BL21 Star™ *E. coli* bacteria strain (DE3; Thermo Fisher™) were heatshock transformed (42°C, 1 min) with the sequence verified pET100 D® plasmid constructs containing the *Ir*CL3 PCR amplicon for expression according to the Champion™ pET Expression System protocol. Expression of the N-terminal $(His)_6$-tagged protein was induced with 0.1mM isopropyl-b-D thiogalactopyranoside (IPTG) in a 400 ml bacterial culture at 37°C overnight.

For the purification of the recombinant protein from isolated inclusion bodies in denaturing conditions, a 5ml HiTrap™ IMAC FF metal-chelating column charged with Co^{2+} mounted on an ÄKTA–FPLC™ system (GE Healthcare Bio-Sciences) was used together with 8M urea containing buffers utilizing denaturing conditions. Elution of purified recombinant *Ir*CL3 was performed by an increasing imidazole gradient (0-500mM). Positive fractions were analyzed by SDS-PAGE (see chapter 3.7) and pooled for protein refolding.

Refolding was done with an aliquote of the pooled fractions, when the purified *Ir*CL3 pro-enzyme in 8 M urea (denturated) was first reduced for 1 hour at 37 °C in reduction solution (50mM GSH, 8M urea) and refolded by dialysis (Dialysing tube MW 3000 Kda cut-off size, Serva) against gradually decreasing urea concentration (6,4,2,1, 0.5M) in 30% glycerol, 0.05% PEG (Polyethyleneglycol) 1500, 0.5 mM GSSG (Glutathione disulfide), pH 8, with final dialysis against plain phosphate buffered saline (PBS). Protein was afterwards mixed with 20% glycerol and stored at -20°C for further usage. Protein activity was checked in the presence of Z-FR-AMC (Bachem, Switzerland) in a plate fluorometer (M200 Pro reader; Tecan, Austria) at excitation/ fluorescence λ = 360/ 645 nm in 1 minute intervals for one hour and for specifity control samples were inhibited with E64.

3.7 Polyclonal antibody production and purification

For the preparation of isoenzyme specific antibodies 100 µg/ml of purified and refolded recombinant *Ir*CL1 (Franta *et al.*, 2011) was mixed with Freund's adjuvant (1:1 ratio) and injected to 3 laboratory mice. Immunization was repeated for three times in three weeks. For the preparation of *Ir*CL3 antibodies, a laboratory rabbit was immunized with the same concentration of purified and folded recombinant *Ir*CL3 mixed with Freund's adjuvant (1:1 ratio). *Ir*CL3 immunization was repeated for five times in 6 weeks. The blood immune sera were prepared by centrifugation removal of blood cells (1000G, 10 minutes at 4°C). Ig fraction was isolated from the sera by mixing the mice and rabbit immune sera with Na-acetate buffer (50 mM sodium

acetate buffer, pH 4.0) (1:2 ratio) in a beaker with a magnetic stirrer. A volume of 25µl of caprylic acid was added to the solution for 3 times (75 µl total) in 15 minute intervals. The solution was allowed to precipitate for 1 hour at RT. After centrifugation (10000G, 10 minutes) and filtration, dialysis (4°C, stirring, overnight) in 1l of 5 mM Na_2HPO_4 was performed. Protein concentration was measured by Bradford Assay using the BioPhotometer (Eppendorf). Resulting Ig fraction aliquots were stored at -80°C until further usage in Western Blotting and Immunohistochemistry.

3.8 SDS-PAGE and Western blot

Gut tissue samples collected from mated *Ixodes ricinus* females at different time points of feeding were homogenized in reducing SDS-PAGE sample buffer and denaturated for 10 minutes at 100°C on a dry heat block (Labnet). Upon centrifugation for 2 minutes, maximum speed in a table top centrifuge 5410 Eppendorf) a volume according to 0.1 tick guts per well was loaded on the 4-15%, Criterion™ TGX™ Stain-Free Precast Gel (Bio-Rad) along with the 100 bp PageRuler™ Prestained Protein Ladder (Thermo Scientific™). SDS PAGE was performed in 1x Tris/Glycine SDS PAGE Running Buffer (Bio-Rad, TruPAGE SDS Express Running Buffer 20x, Sigma Aldrich) at 170 V until the run was complete. A picture of the electrophoretically separated protein load was taken using the stain free UV option of the ChemiDoc Imager (Bio-Rad). After documentation of the SDS PAGE, the gel was electrotransblotted (1.7 A and 25 V for 7 minutes) onto a PVDF membrane using the Trans Blot Turbo blotting system (BioRAD). The blotted membrane was then incubated in 1% non-fat milk in PBS-Tween (0.05%). Labeling with primary antibodies was performed in the same solution containing primary antibodies (*Ir*CL1 Ig fraction mouse 1:1000 and *Ir*CL3 rabbit serum 1:1000) on an orbital shaker, 4 °C, overnight. Before applying the secondary antibodies, the membrane was washed 3x for 5 minutes each with PBS-Tween on the orbital shaker. After the washing steps, the membrane was exposed at RT to secondary antibodies AlexaFluor 546® IgG goat anti-rabbit (red) and AlexaFluor® 488 goat anti-mouse (green) (both Thermo Fischer™) diluted 1:1000 in PBS-Tween for 60 minutes. After the second washing step, conducted 3x with PBS-Tween for 5 minutes each, the membrane was visualized using the ChemiDoc MP Imaging System (Bio-Rad) with the according excitation light and emission filters set up. Colorimetric mode was used to capture prestained ladder and the overlayed picture was recorded and edited by the LabImage software (Bio-Rad).

3.9 Immunohistochemistry

Gut tissue was dissected from female ticks at different time points of their course of feeding. Microscopical semi-thin sections of the tick gut tissue were prepared using the methodology established in the laboratory of Dr. Kopacek at Institute of Parasitology CAS. Briefly, dissected tissues were fixed in a fixing buffer (4% formaldehyde + 0.1% glutaraldehyde in 0.1 M sodium phosphate, pH 7.3) at 4°C overnight. Afterwards, the samples were swirled three times for 10 minutes in the washing buffer (4% glucose in 0.1 M sodium phosphate, pH 7.3) and heated in a microwave oven (3x30 seconds, water bath, 80W). Ascending ethanol dilutions were used for sample dehydration under microwave radiation. Dried samples were infiltrated with LR White resin (London Resin Company, Ltd.) with a row of resin: ethanol (95%) dilutions: 2:1, 1:1, 1:2, 1.5h each, 4°C. Afterwards the samples were kept in pure LR White resin overnight at 4°C. The samples were then transferred to gelatin capsules (Polysciences Inc.) and filled with resin and left for 24h for polymerization at 50°C. Polymerized blocks were used to semi-thin (0.5 µm) microscopic sections of the gut tissue. For immunolabelling, the semi-thin sections were mounted on microscopy glass slides (two sections per slide) and the tissue areas were encircled with a water repellent pen. All slides were placed on wooden sticks in a wet aluminum foil tray while shaking. The gut sections were then incubated for 10 minutes in blocking buffer (1% milk in PBS-Tween), the blocking buffer was removed and the sections were washed three times with 0.3 % PBS-Tween for 5 minutes each. Primary Mox*Ir*CL1 and Rax*Ir*CL3 polyclonal antibodies were diluted in PBS-Tween in a ratio of 1:50. The diluted sera were applied on the glass with the semi-thin tick gut sections and incubated for 1h in a humid chamber. After washing 4x for 5 minutes each with plain PBS-Tween the sections were further exposed to secondary antibodies AlexaFluor® 488 anti-mouse and AlexaFluor® 546 anti-rabbit (both Thermo Fisher™) diluted 1:500 and 1:200 in PBS-Tween, respectively. Again washing was conducted 4× for 5 minutes. After the final wash with PBS-Tween the guts were counterstained with DAPI (4', 6'-diaminidino-2-phenylindole; 2.5 µg/ml, Sigma) for 5 minutes. The slides were washed again 3× for 5 minutes each with PBS-Tween, mounted with Fluoromount G (Thermo Fisher™) and visualized in the fluorescent light microscope BX 53 (Olympus). Captured images were processed with the Fluoview software (Olympus).

4. Results

4.1 *Ir*CL3 and recombinant *Ir*CL3

While *Ir*CL1 has been fully cloned and characterized previously (Franta et al., 2011), *Ir*CL3 was first identified from the RNAseq project in the laboratory of vector immunology by Dr. Jan Perner (IrSigP_112645; Perner et al., 2016) and it was fully cloned, sequence verified and submitted to the NCBI gene bank (MH036745) within the current PhD project by Mgr. David Hartmann. The natural sequence of *Ir*CL3 has a molecular size of 36.699 kDa and a pI of 4.89 and is encoded by mRNA of 1002 bp. It contains a SignalP 4.1 Server predicted signal peptide as indicated in Figure 4.

Figure 4: **Alignment of *Ixodes ricinus* L3**; active site residues are labeled in red color, the signal is labeled with dark grey, N-terminal tag is labeled in light grey

Based on the protein sequence, DNA primers were designed (see chapter 4.3) and used for the PCR amplification and cloning of the recombinant protein (*Ir*CL3$_{rec}$) into pET100. This work was performed prior to my thesis as part of a larger project in the laboratory. The final construct is shown in Figure 5 together with the native sequence in Figure 4. The recombinant protein has a molecular size of 38.844 kDa and a pI of 5.02. The sequence verified pET 100 plasmid construct was further used for expression of *Ir*CL3$_{rec}$ (see chapter 3.6).

ATGCGGGGTTCTCATCATCATCATCATCATGGTATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATCTGTACGACGATG
ACGATAAGGATCATCCCTTCACCGAGAATGAGGTCCTGGAGGTTGAGTGGGAGACCTTCAAAGCGAATTACGGAAAGTCATACTC
GTCCGAGGCTGAAGAGCAATTCAGAATGACGGTGTACATGAACAACAAACTGAAGGTCGCCAAGCACAACGAGCAATACGCGGA
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GCAGTGGTGGTCTTATGGACAACGCCTTCCAGTACATCAAGGCCAATGGAGGCATCGACACCGAGAAGAGCTATCCCTACACTGG
CGAGGATGGTCAATGCGTCTTCGACAAGTCCAACGTTGGCGCTGCCGACACTGGCTTCGTGGACGTGCAGACAGGCGACGAGACG
CAGCTCATGAAGGCTGTGGCTTCGGTCGGCCCAATATCGGTCGCCATCGACGCGAGTCACATATCTTTCCAGTTTTACGCTCAAGGC
GTCTACGACGAGCCAGCATGCTCCAGCGAAATGCTTGATCATGGTGTGCTGGCAGTTGGCTACGGCACCCTGAATGGCAAGGACT
ACTGGCTGGTGAAAAATAGCTGGGGAGCTGACTGGGGGCAGGAAGGATACATCCTGATGAGCCGAAACAAGAACAACCAGTGCG
GAATCGCAAGCAACGCCAGCTACCCACTCGTGTAAAA

Figure 5: **pET 100 DNA construct**; N-terminal tag is labeled in light grey, start codon in green and primers (mentioned in 3.4) are labeled in blue color

4.2 Expression and purification of recombinant *Ir*CL3

Expression was performed in BL21 Star™ *E. coli* bacterial strain (DE3; Thermo Fisher™) and subsequent isolation of the insoluble cellular fraction (inclusion bodies) was done by our standardized laboratory protocol. Protein purification was performed in denaturing conditions in the presence of 8 M urea by the affinity N-terminal $(His)_6$-tag to the Co^{2+}-bound beats. The process of recombinant protein affinity purification using AKTA-purifier (GE Healthcare) is shown in Figure 6. Fractions were collected from the flow-through and from the final peak resulting from the addition of 0.5 M imidazole and were further analyzed using SDS-PAGE. The fractions containing recombinant protein (E1-E9) were pooled and used for protein refolding (described in chapter 3.6). Activity was measured in the presence of ZFR-AMC in a plate fluorometer and for specifity control samples were inhibited with E64 inhibitor (Figure 7). Activity assays clearly demonstrated high activity of the *Ir*CL3$_{rec}$ protein.

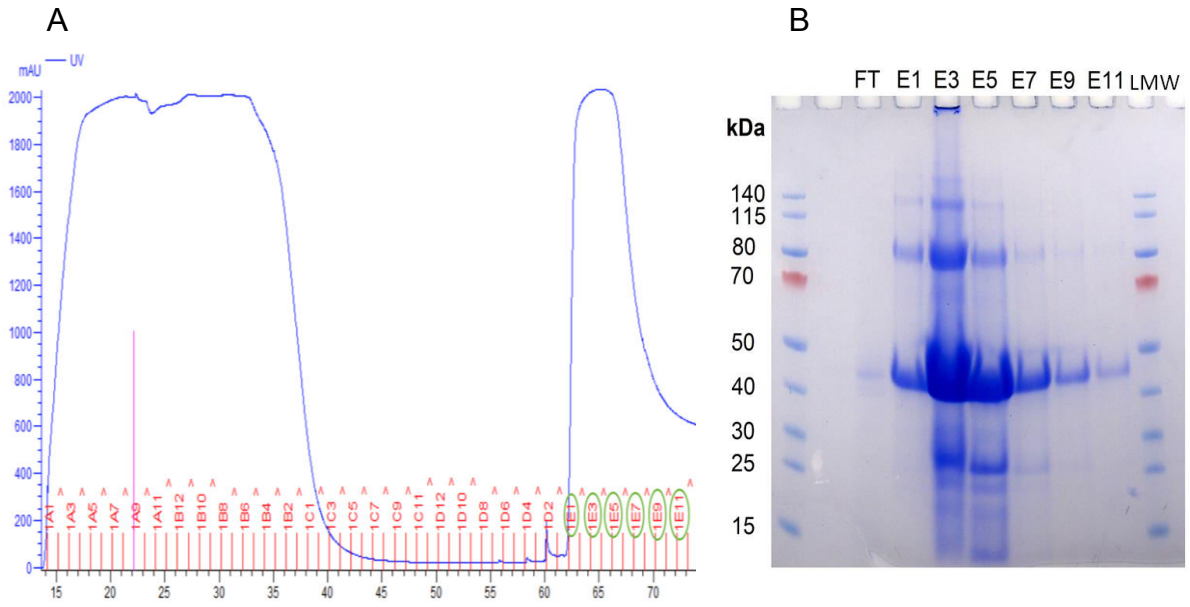

Figure 6: **Chromatography of metal affinity purification of insoluble cellular fraction of *E.coli* (panel A) and SDS-PAGE of the collected fractions (panel B)**. Purification performed on AKTA purifier (GE Healthcare) and collection of 1ml fractions in a 96 well formate on the AKTA collector are indicated. A7-FT flowthrough, E1-E11 sample elution with 0.5 M immidazole. Recombinant protein of MW 40+ kDa and multimers labeled with Coomassie Brilliant Blue on SDS-PAGE gel.

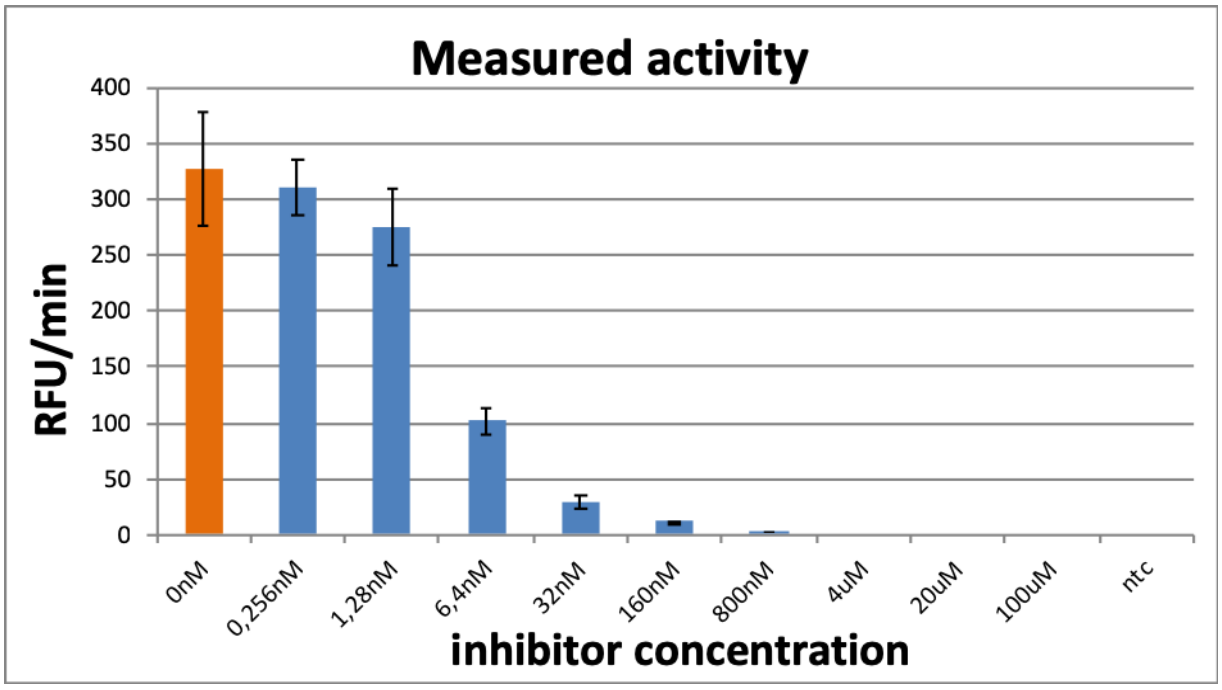

Figure 7: **Inhibition of *Ir*CL3 by increasing concentration of E64 inhibitor.** *Ir*CL3 activity was measured in assay buffer - 150 mM phosphate/citrate, 2mM DTT, pH 3.0 in the presence of 10μM ZFR-AMC substrate (Bachem). IC_{50} using the papain specific inhibitor E64 is 3nM.

4.3 Preparation of Antibodies, Western Blot and Immunohistochemistry

Preparation of polyclonal antibodies was done for *Ir*CL1 in mice and for *Ir*CL3 in rabbit with repeating immunization according to the standardized protocol (see chapter 3.7). Controls for the functionality are shown below (Figure 8 and 9) in two different ways. Firstly, SDS-PAGE and Western Blot were performed using with tick gut tissues from fully fed *I. ricinus* females. Secondly, double immunolabeling with different load amounts of the recombinant proteins in SDS-PAGE and Western Blot was done. Both functionality controls show different protein profiles for *Ir*CL1 and *Ir*CL3, therefore isoenzyme specifity of the respective polyclonal antibodies could be confirmed.

4.3.1 Preparation of antibodies and specifity verification

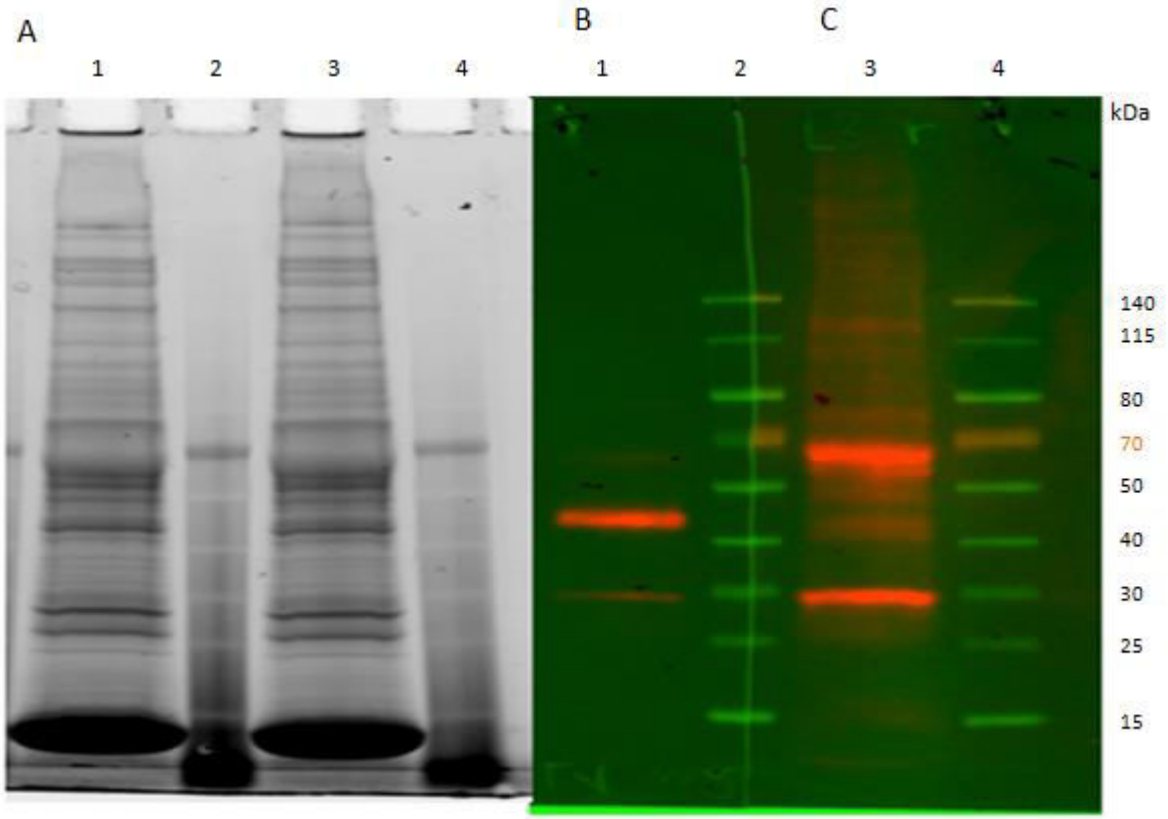

Figure 8: **Verification of antibody specifity using separate labeling on stain free TGX™ gel and Western Blot; panel A:** Stain free (Criterion™ TGX™ StainFreePrecast Gel (Bio-Rad) 4-15% SDS-PAGE gel, BioRAD) visualization of the SDS-PAGE load - identical loads used for panel B and C blotts **panel B:** Western Blot from the electroblotted SDS PAGE in panel A: primary antibodies: *Ir*CL1 IgG fraction mouse 1:3000 and secondary antibodies: AlexaFluor® 488® goat anti-mouse (Thermo Fischer™) 1:2000 **panel C:** Western Blot from the electroblotted SDS PAGE in panel A: primary antibodies: *Ir*CL3 rabbit serum 1:3000 and secondary antibodies: AlexaFluor® 488 goat anti-rabbit (Thermo Fischer™) 1:2000 **loads: (2) & (4):** PageRuler™ Prestained Protein Ladder (Fermentas, Thermo Fisher); **(1) & (3):** 0.1 guts from female fully fed *I. ricinus* ticks. Significant protein profiles are observed at same loads, indicating for specifity of the antibodies.

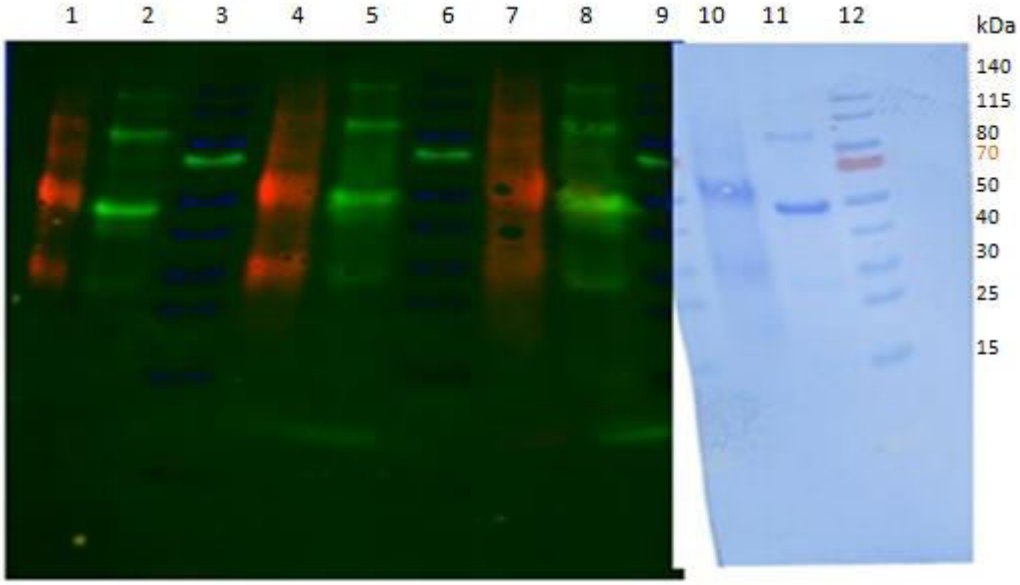

Figure 9: **Multiplex Western blot control of *Ir*CL1$_{rec}$ and *Ir*CL3$_{rec}$ primary antibody specifity**. Using recombinant *Ir*CL1 and *Ir*CL3 antigens as the load control. Antibodies: mouse anti-*Ir*CL1 and rabbit anti-*Ir*CL3 antibodies and secondary antibodies: AlexaFluor® 546 goat anti-rabbit (Lifetechnologies) and AlexaFluor® 488 goat anti-mouse (Lifetechnologies). All antibodies were applied in a dilution of 1:1000. The **loads** are as follows: **(1)** 5µl of *Ir*CL1$_{rec}$ **(2)** 5µl of *Ir*CL3$_{rec}$ **(3)** marker **(4)** 10µl of *Ir*CL1$_{rec}$ **(5)** 10µl of *Ir*CL3$_{rec}$ **(6)** marker **(7)** 15µl of *Ir*CL1$_{rec}$ **(8)** 15µl of *Ir*CL3$_{rec}$ **(9)** marker **(10)** 20µl of *Ir*CL1$_{rec}$ **(11)** 20µl of *Ir*CL3$_{rec}$ **(12)** marker; marker used: PageRuler™ Prestained Protein Ladder; the highest load was stained with Coomassie Brilliant Blue for load control; *Ir*CL1 is presented in red, whereas *Ir*CL3 can be seen in green bands. No cross-reactivity (yellow color) can be detected; thus antibodies are isoenzyme specific.

4.3.2 Multiplex Western Blot expressional profiling of *Ir*CL1 and *Ir*CL3

After successful testing of the primary antibodies for specifity to the regarding cathepsin L enzyme, double labeling with guts loaded from female *Ixodes ricinus* ticks on different time points of feeding was performed (Figure 10). Secondary antibodies were labeled with different fluorescent dyes and visualized with according excitation and emission light filter using the ChemiDoc MP Imaging System (Bio-Rad). Over the time course of feeding, a clear difference in presence and intensity of the signals for *Ir*CL1 (red) and *Ir*CL3 (green) can be seen. On the 17th day of female tick post-feeding period the intensity of *Ir*CL3 reaches its maximum and the two signals overlap and give a slightly yellow signal.

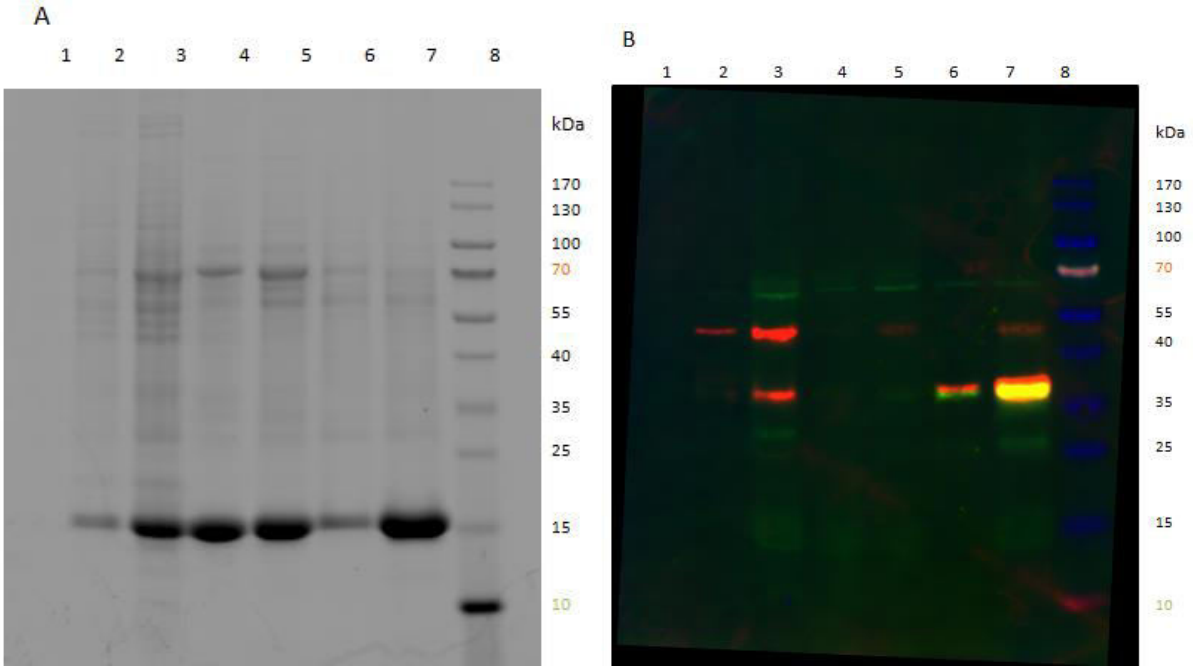

Figure 10: **Western Blot analysis of *Ir*CL1 and *Ir*CL3 protein expression in female tick gut**. **panel A:** Criterion™ TGX™ StainFreePrecast Gel (Bio-Rad) 4-15% SDS-PAGE gel, showing the load. **panel B:** Load from panel A electroblotted to a PVDF membrane and labeled with rabbit anti-*Ir*CL3 antibodies and mouse anti-*Ir*CL1 antibodies. Secondary antibodies used were AlexaFluor® 546 goat anti-rabbit (Lifetechnologies) and AlexaFluor® 488 goat anti-mouse (Lifetechnologies). All antibodies were applied in a dilution of 1:1000. The **loads** are as follows:**(1)** unfed **(2)** 3 days **(3)** 5 days **(4)** fully fed **(5)** 5 days postfeeding **(6)** 11 days postfeeding **(7)** 17 days postfeeding **(8)** marker (PageRuler™ Prestained Protein Ladder); *Ir*CL1 is presented in red, whereas *Ir*CL3 can be seen in green bands. Clear differentiation of the isoenzymes on the different days of feeding and yellow color from very strong signal of both isoenzymes on lane 7.

4.3.3 Immunohistochemistry

The previous results were additionally proofed by isoform specific antibody double labeling in multiplex immunohistochemistry (Figure 11). Gut tissues of 5th and 7th day of feeding were dissected, mounted and treated with antibodies according to chapter 3.9. The slides were visualized using a fluorescent light microscope (BX 53, Olympus). On 5th day of feeding *Ir*CL1 (green) and *Ir*CL3 (red) are both present in the cells, but on the 7th day the red signal (*Ir*CL3) increases and the green one diminishes.

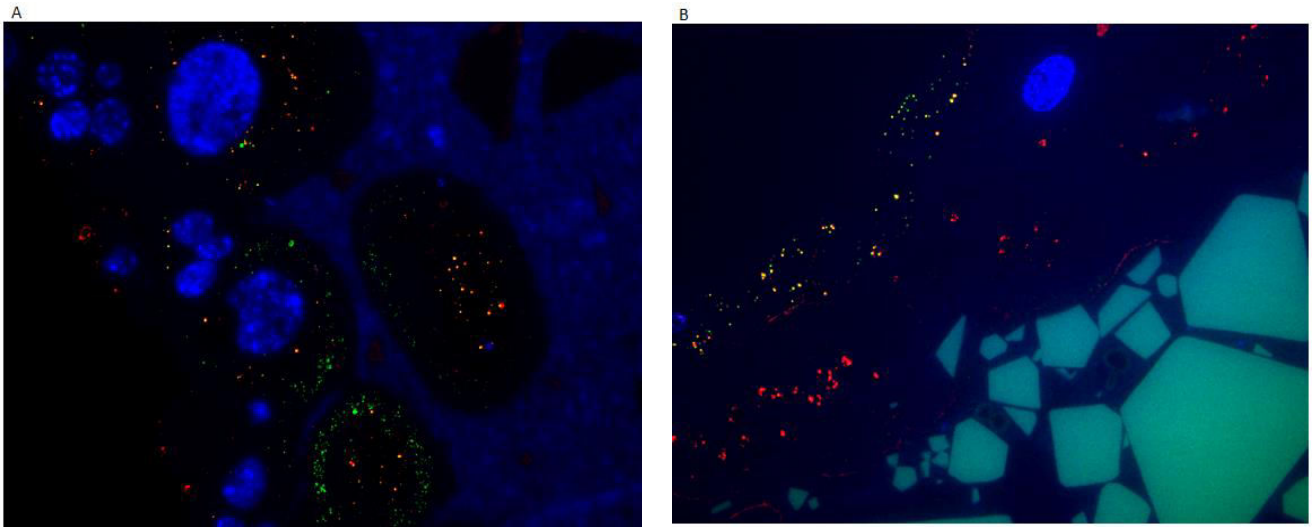

Figure 11: **Double immunostaining of *Ir*CL1 and *Ir*CL3 of the female *I.ricinus* gut semi-thin slides**. Primary Mox*Ir*CL1 and Rax*Ir*CL3 polyclonal antibodies were applied in a concentration of 1:50 in PBS-Tween. Secondary antibodies used were AlexaFluor® 546 donkey anti-rabbit (Lifetechnologies) in a concentration of 1:200 in PBS-Tween and AlexaFluor® 488 goat anti-mouse (Lifetechnologies) in a concentration of 1:500 in PBS-Tween; red dots account for *Ir*CL3 and green dots for *Ir*CL1; **panel A:** 5th day of feeding **panel B:** 7th day of feeding. The isoenzymes have distinct localization indicating a different function within the cell.

5. Discussion

Previous studies in *Ixodes ricinus* did not reflect the diversity of isoenzymes of cathepsin L and the linked multienzyme network model of acidic aspartic and cysteine peptidases, which are mainly responsible for cleavage of the hemoglobin in imbibed host blood (Hartmann *et al.*, 2017).

While digestion and processing of the host blood happens, the proteolysis synthesized heme is segregated into hemosomes. For defense against microorganisms transmitted by ingestion of the host blood, hemocidins are generated by digestion of hemoglobin (Cruz *et al.*, 2010) and enzymes involved in this are cathepsin D and L, which are upregulated during blood feeding. By investigating cathepsin L more closely recently, a time course of activity in the gut epithelium during feeding was generated (Figure 12; David Hartmann, unpublished).

Two major activity peaks can be observed at the 6th day of feeding and after the 15th day of feeding. It was assumed that for this peaks, two different isoforms of cathepsin L are responsible. To prove the involvement of the two different isoforms, sequences were isolated and antibodies were prepared successfully, that show specifity to the corresponding isoform.

When previously characterizing *Ir*CL1 (Franta *et al.*, 2011), it was detected that *Ir*CL1 is the major isoform of cathepsin L responsible for its activity in the gut homogenates from semi-engorged *I. ricinus* females. The cathepsin L enyzme activity profile indicated for an important role of only *Ir*CL1. But the recent activity assay data (Figure 13) confirms the role of the two distinct isoforms at different time points of feeding. *Ir*CL1 has its activity peak in the early feeding of female *I. ricinus* ticks, whereas *Ir*CL3 starts to appear more and more after the drop-off from the host.

The current hemoglobinolytic enzyme network model (Figure 12) shows the involvement of *Ir*CD, *Ir*CB, *Ir*CC, *Ir*AE and *Ir*CL. Cathepsin L takes a role in cleavage of hemoglobin into large and small fragments, but the role of *Ir*CL1 or *Ir*CL3 is still not completely identified. With this work, first steps into recognition and full characterization of the key roles of cathepsin L1 and cathepsin L3 were made.

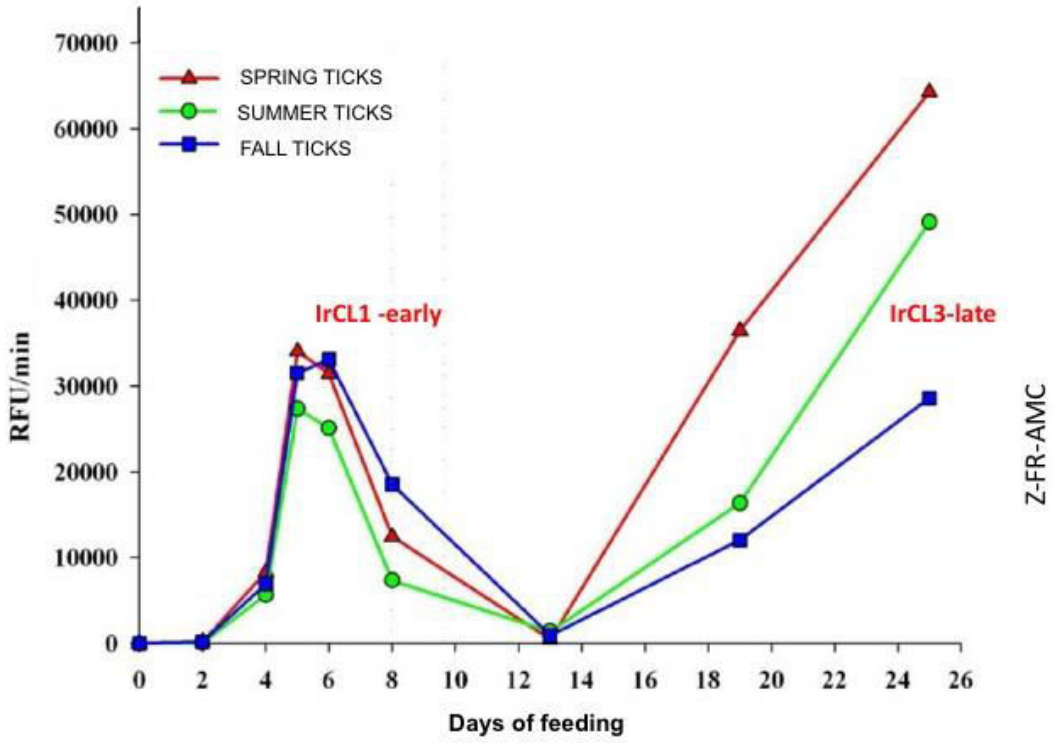

Figure 12: **Activity profile of cathepsin L in female *I.ricinus* ticks.** Female ticks collected in different seasons were analyzed for the activity of cathepsin L in the gut epithelium over the course of feeding. Red lines indicate for ticks collected in spring, green for summer and blue for ticks collected in fall. (David Hartmann, unpublished data)

6. Conclusion

The *Ixodes ricinus* is due to its wide distribution area and availability of different vertebrate hosts the most important arthropod disease vector in Europe. The importance of understanding the dynamic network of multienzymes responsible for the processing of the imbibed host blood is immense. Not only a way could be found to discourage ticks from on-host feeding, but it's also necessary for understanding of the pathogen transmission. The multienzyme network is still not completely characterized, and with this work, another step to identify the roles of the various enzymes, present in the *Ixodes ricinus* tick, was done. Cathepsin L was first to be thought as one enzyme. When taking a closer look, our lab found, that there are different isoenzymes of cathepsin L, which play a variant role in the digestion process. With preparation of *Ir*CL3 specific polyclonal antibodies and testing it against already prepared *Ir*CL1 polyclonal antibodies in Western Blotting and Immunohistochemistry, it was possible to show a significant difference in appearance with this two isoenzymes. Not only could it be confirmed, that cathepsin L is present in at least 2 isoforms, but it was also possible to demonstrate the appearance and vanishing of *Ir*CL1 and *Ir*CL3 during the time course of feeding. The obtained results show a new perspective to further understanding of the dynamic multienzyme network and provide hope for complete characterization of the whole tick machinery to develop new strategies against ticks and inhibition of tick-borne diseases.

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