CHARLES UNIVERSITY, FACULTY OF SCIENCE,

DEPARTMENT OF PARASITOLOGY

Ph.D. study programme: Parasitology

Summary of the Ph.D. Thesis

Bioactive molecules involved in blood processing by haematophagous monogeneans of the family Diplozoidae

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Prague, 2019

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Abstract

Monogeneans from the family Diplozoidae (subclass Heteronchoinea) are bloodfeeding ectoparasites inhabiting gills of common carp. Digestion of blood in diplozoids is an intracellular process taking place in gut cells within lysosomal cycle in the presence of parasite's peptidases. However, information about the blood digestion comes only from ultrastructural and histochemical analyses. Therefore, I have focused in this work on biochemical and molecular characteristics of bioactive molecules which may participate in blood processing by *E. nipponicum* adults, especially cysteine peptidases of cathepsin L- and B- types, aspartic peptidases of cathepsin D- type, and Kunitz- type inhibitors of serine peptidases.

In homogenates and excretory/secretory (E/S) products of *E. nipponicum* adults, an activity of cysteine peptidases of cathepsins L-type dominated, followed by an activity of cathepsin D-like aspartic peptidases and a minor cathepsin B-like activity. Inhibitors of the abovementioned peptidase types completely blocked hemoglobinolytic activity in the samples. In the transcriptome of *E. nipponicum* adults, ten cathepsin L-coding transcripts were found and only one cathepsin B-coding transcript. Primary structures of the encoded enzymes were bioinformatically and phylogenetically compared. Two abundant cathepsins L (EnCL1, EnCL3) were expressed in *P. pastoris*/*E. coli* expression systems and then biochemically and functionally characterized. Both enzymes were localized inside the gut cells and also in gut lumen of the adult worm. The results suggested probable extracellular phase of blood digestion in *E. nipponicum* and also the presence of a wide range of cathepsins L with different structural characteristics and also with likely different functions.

In addition to peptidases participating in the degradation of blood proteins, haematophagous parasites also need bioactive molecules inhibiting coagulation during the blood intake. To regulate the haemostasis, they often use inhibitors of serine peptidases which participate in the coagulation cascade. These include, e.g., Kunitz-type inhibitors which were also found in the transcriptome of adult *E. nipponicum*. One of them (named EnKT1) was chosen for heterologous expression in *E. coli* system. Recombinant EnKT1 inhibited peptidolytic activity of human factor Xa, plasmin and plasma kallikrein *in vitro*. Its anticoagulation activity based on inhibition of factor Xa was confirmed *in vitro* by tromboelastography. Despite the ability to inhibit plasmin, its impact on fibrinolysis was not confirmed. On the other hand, EnKT1 effectively inhibited cytolytic activity of fish complement *in vitro*. By means of *in situ* hybridization and immunohistochemistry, the EnKT1 was localized inside the digestive cells of the parasite. It possesses a secretory signal sequence and was detected in E/S products of the worms. Based on these characteristics, we suppose that EnKT1 serves as a bifunctional inhibitor dininishing coagulation of ingested blood in the parasite's gut and, simultaneously, protecting cells of the gastrodermis from complement attack. It is the first example of a parasite-originated Kunitz protein with confirmed inhibitory effect on complement cascade in host’s blood.

Introduction

Eudiplozoon nipponicum is a blood-feeding monogenan (family Diplozoidae) which parasitizes mainly on the gills of common carp, a fish of high economic importance in many countries of Asia and Europe. Information about the blood digestion in monogeneans used to be available from ultrastructural and histochemical analyses only. Although it has been assumed that digestion in blood-feeding monogeneans is similar to digestion in other blood-feeding platyhelminths, the knowledge of the biochemistry of digestion and participating digestive enzymes in monogeneans is rather poor. Monogenea, as well as other blood-feeding parasites, also need to overcome clotting of host's blood. And serine peptidases are enzymes that participate in haemostasis or complement cascade. Therefore, inhibitors belonging to the families of anexins, serpins or Kunitz domain proteins are utilised to regulate the activity of such host's enzymes. Only few Kunitz type inhibitors were identified in endoparasitic helminths, but no peptidase inhibitors have been detected in monogeneans so far. In this work, we focused on molecular and biochemical characterization of a Kunitz domain inhibitor and several peptidases, which probably participate in blood intake and blood digestion in adult *E. nipponicum*.

Aims of the study

The main objectives of the study:

- Determination, identification and ratios of peptidase activities in soluble protein extract and excretory/secretory products of adult *E. nipponicum*.
- Determination of inhibitory activities in soluble protein extract of adult *E. nipponicum* against serine peptidases from the coagulation cascade (factor X, thrombin) and fibrinolytic system (plasmin).
- Search for cathepsin L transcripts and Kunitz inhibitors in the transcriptome of *E. nipponicum* adult.
- Preparation of recombinant *E. nipponicum* proteins (cathepsins L1, L3 and EnKT1)
- Biochemical and functional characterization of recombinant cathepsins L1/L3 and EnKT1.
- Localization of the proteins in adult *E. nipponicum* (imunolocalization/ RNA in situ hybridization)

Summary and conclusions

This work represents the first comprehensive exploration and functional description of bioactive molecules involved in the blood uptake and digestion in blood-feeding monogeneans. From a biochemical and molecular point of view, it is a neglected group of ectoparasitic helminths, although some species may cause great devastation in fish aquaculture. Unfortunately, the lack of available experimental data from other monogeneans excludes any comparisons. Information is available only from ultrastructural and histochemical analyses and, just recently, also from genomic/transcriptomic data from a few species. The thesis is divided into two parts, the first part being focused on proteolytic enzymes involved in blood digestion and the second part focused on a Kunitz inhibitor from adult *Eudiplozoon nipponicum* with an anticoagulation and complement-inhibiting effect.

Protein digestion is a major proteolytic process in blood-feeding parasites. The first part of the Ph.D. thesis is focused on peptidases, potential digestive enzymes. We have shown that the main proteolytic activities in *E. nipponicum* are attributed to cysteine peptidases, especially to cathepsins L. However, the results show that both cysteine and aspartic peptidases are involved in haemoglobin degradation. The same information is known from other parasites such as *Schistosoma mansoni* (Caffrey *et al.,* 2004) or *Ixodes ricinus* (Sojka *et al.,* 2013). Cathepsin L proteolytic activities outweigh cathepsin D activities and consequently activities of cathepsin B. Two complete sequences of cathepsins L *E. nipponicum* (EnCL1 and EnCL3) were obtained by means of degenerated primers and PCR methods. The data were summarized in **Jedličková *et al.* (2016)** published in **Parasitology**.

Further study focused on major activities of cathepsins L, particularly on detailed characterization of the two recombinantly expressed cathepsins L. Cathepsins L were produced both in yeast and bacteria. They showed substrate preferences typical for cathepsins L, differing only in activity pH profiles against fluorogenic substrates. Both were able to cleave haemoglobin, albumin, collagen, IgG, and fibrinogen. Cathepsins L were detected within hematin (digestive) cells as well as in the lumen of the intestine by imunolocalization and RNA *in situ* hybridization. From the available literature, it was assumed from ultrastructural analyses that blood digestion takes place intracellularly in the lysosomal cycle within the digestive cells (Smyth *et* Halton, 1983, a; b) like in ticks (Sonenshine, 1991). However, it can be seen from the results of this work that the first phase of digestion is likely to occur extracellularly in the lumen of the intestine in the presence of secreted peptidases. Similarly to the digestion in *Fasciola hepatica*, the first phase of digestion seems to occur extracellularly in the lumen of the intestine and is followed by intracellular digestion within the digestive cells (Robinson *et al.,* 2008).

Transcriptome analysis of adult monogenean *E. nipponicum* revealed a broad spectrum of cathepsins L (10 transcripts), only one cathepsin B and three cathepsins D. This corresponds with the proportion of enzymes in other blood-feeding helminths where, e.g., in *F. hepatica,* 27 cysteine peptidases were detected and only 1 aspartic peptidase (cathepsin D). Fourteen cysteine peptidases have been identified in *Fasciola gigantica* (Kašný *et al.,* 2009; Rawlings *et al.,* 2010) and 9 in *Fascioloides magna* (Cantacessi *et al.,* 2012). Some cathepsins L from the transcriptome of *E. nipponicum* showed structural differences from typical cathepsins L. This is due to the composition of the S2 pocket of the active site, which is somewhat more variable and resembles cathepsins S or B in some cases. Wide presence of cathepsins L in *E. nipponicum* may indicate the importance of these enzymes in the parasite life cycle. *F. hepatica* uses these enzymes in digestion, tissue migration, escape from immune response, metacercarial excystation, or in egg production (Tort *et al.,* 1999, Dalton *et al.,* 2006). The secretion of proteolytic enzymes by *E. nipponicum* into the external environment, which occurs in *F. hepatica* during tissue migration, appears to be unlikely, since it is an ectoparasite. Therefore, in addition to digestion, cathepsins L could eventually be released into the blood of the host and might play a role in interaction between the host and the parasite. In addition, the production of a broad spectrum of overlapping peptidases allows the parasite to degrade host macromolecules more effectively. These results were concluded in **Jedličková *et al.* (2018)** and published in **Parasites and Vectors**.

The blood intake and digestion interact with the coagulation system of the host. Therefore, the extracts of *E. nipponicum* were tested for the presence of antihaemostatic molecules. In a sample of soluble protein extracts of adults *E. nipponicum* we found inhibitory activities against peptidases included in the coagulation cascade, such as factor Xa and thrombin, as well as plasmin, which takes part in fibrinolysis. Therefore, in transcriptome analyses of adult worms, we focused on Kunitz-type inhibitors where at least six Kunitz inhibitors were discovered (unpublished). One of them (EnKT1) showed high similarity with textilinin-1, an antihemorrhagic factor from the venom of the Eastern brown snake *Pseudonaja textilis*. Therefore, EnKT1 was produced in recombinant form and biochemically and functionally characterized. EnKT1 showed inhibitory activity against factor Xa, but even higher activity was recorded against human plasmin and plasma kallikrein *in vitro*. EnKT1 anticoagulant activity was confirmed by thromboelastography, while antifibrinolytic activity was not proven. EnKT1 was detected within intestinal digestive cells by signal-amplified antibodies and RNA *in situ* hybridization. It also contains a signal sequence and its presence has been confirmed in excretory/secretory products. These results indicate that it may be a potential anticoagulant directed against factor Xa. Furthermore, its ability to inhibit fish complement *in vitro* was demonstrated. In this case, it is the first known parasite Kunitz protein with the ability to block

complement. EnKT1 blocked human plasma kallikrein *in vitro* assays. In the course of the tests, we encountered the linking of coagulation, fibrinolysis and complement cascades (Rittirsch *et al.,* 2008). Afterward they found that these cascades work closely together. Therefore, the ability of EnKT1 to inhibit both plasmin and factor Xa, which may act as C3 and C5 convertases, may be a key element in preventing complement activation and may work in the protection of gut cells or tissues of the parasite. We offered a hypothesis that it could serve as a bifunctional protein that blocks blood coagulation during blood suction by the parasite before it is cleaved by peptidases while simultaneously protecting intestinal cells from complement activity. These results were summarized in **Jedličková *et al.* (2019)** published in **International Journal for Parasitology**.

So, it is clear that monogeneans have a variety of tools that interact with their hosts. Therefore, a detailed description of the structure and function of these molecules can lead to elucidation of the interactions between the parasite and the host, mainly in terms of pathogenesis and fish immunity. But also, new molecules can be found with a potential application in biomedicine or in the development of vaccines targeted against fish parasites.

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Jedličková L, Dvořáková H, Kašný M, Ilgová J, Potěšil D, Zdráhal Z, Mikeš L. Major acid endopeptidases of the blood-feeding monogenean *Eudiplozoon nipponicum* (Heteronchoinea: Diplozoidae). Parasitology. 2016 Apr;143(4):494-506. IF2016=2,713 doi: 10.1017/S0031182015001808.

Abstract

In parasitic flatworms, acid endopeptidases are involved in crucial processes, including digestion, invasion, interactions with the host immune system, etc. In haematophagous monogeneans, however, no solid information has been available about the occurrence of these enzymes. Here we aimed to identify major cysteine and aspartic endopeptidase activities in *Eudiplozoon nipponicum*, an invasive haematophagous parasite of common carp. Employing biochemical, proteomic and molecular tools, we found that cysteine peptidase activities prevailed in soluble protein extracts and excretory/secre-tory products (ESP) of *E. nipponicum*; the major part was cathepsin L-like in nature supplemented with cathepsin B-like activity. Significant activity of the aspartic cathepsin D also occurred in soluble protein extracts. The degradation of haemoglobin in the presence of ESP and worm protein extracts was completely inhibited by a combination of cysteine and aspartic peptidase inhibitors, and diminished by particular cathepsin L, B and D inhibitors. Mass spectrometry revealed several tryptic peptides in ESP matching to two translated sequences of cathepsin L genes, which were amplified from cDNA of *E. nipponicum* and bioinformatically annotated. The dominance of cysteine peptidases of cathe-psin L type in *E. nipponicum* resembles the situation in, e.g. fasciolid trematodes.

Jedličková L, Dvořáková H, Dvořák J, Kašný M, Ulrychová L, Vorel J, Žárský V, Mikeš L. Cysteine peptidases of *Eudiplozoon nipponicum*: A broad repertoire of structurally assorted cathepsins L in contrast to the scarcity of cathepsins B in an invasive species of haematophagous monogenean of common carp. Parasite and Vectors. 2018 Mar 6;11(1):142. IF2018=3,163 doi: 10.1186/s13071-018-2666-2.

Abstract

Background: Cysteine peptidases of clan CA, family C1 account for a major part of proteolytic activity in the haematophagous monogenean *Eudiplozoon nipponicum*. The full spectrum of cysteine cathepsins is, however, unknown and their particular biochemical properties, tissue localisation, and involvement in parasite-host relationships are yet to be explored.

Methods: Sequences of cathepsins L and B (EnCL and EnCB) were mined *from E. nipponicum* transcriptome and analysed bioinformatically. Genes encoding two EnCLs and one EnCB were cloned and recombinant proteins produced in vitro. The enzymes were purified by chromatography and their activity towards selected substrates was characterised. Antibodies and specific RNA probes were employed for localisation of the enzymes/transcripts in tissues of *E. nipponicum* adults.

Results: Transcriptomic analysis revealed a set of ten distinct transcripts that encode EnCLs. The enzymes are significantly variable in their active sites, specifically the S2 subsites responsible for interaction with substrates. Some of them display unusual structural features that resemble cathepsins B and S. Two recombinant EnCLs had different pH activity profiles against both synthetic and macromolecular substrates, and were able to hydrolyse blood proteins and collagen I. They were localised in the haematin cells of the worm's digestive tract and in gut lumen. The EnCB showed similarity with cathepsin B2 of *Schistosoma mansoni*. It displays molecular features typical of cathepsins B, including an occluding loop responsible for its exopeptidase activity. Although the EnCB hydrolysed haemoglobin in vitro, it was localised in the vitelline cells of the parasite and not the digestive tract.

Conclusions: To our knowledge, this study represents the first complex bioinformatic and biochemical characterisation of cysteine peptidases in a monogenean. *Eudiplozoon nipponicum* adults express a variety of CLs, which are the most abundant peptidases in the worms. The properties and localisation of the two heterologously expressed EnCLs indicate a central role in the (partially extracellular?) digestion of host blood proteins. High variability of substrate-binding sites in the set of EnCLs suggests specific adaptation to a range of biological processes that require proteolysis. Surprisingly, a single cathepsin B is expressed by the parasite and it is not involved in digestion, but probably in vitellogenesis.

Jedličková L, Dvořák J, Hrachovinová I, Kašný M, Ulrychová L, Mikeš L. A novel Kunitz protein with proposed dual function from *Eudiplozoon nipponicum* (Monogenea) impairs haemostasis and action of complement *in vitro*. International Journal for Parasitology. 2019 Apr;49(5):337-346. IF2017=3,078 doi: 10.1016/j.ijpara.2018.11.010.

Abstract

Serine peptidases are involved in many physiological processes including digestion, haemostasis and complement cascade. Parasites regulate activities of host serine peptidases to their own benefit, employing various inhibitors, many of which belong to the Kunitz-type protein family. In this study, we confirrmed the presence of potential anticoagulants in protein extracts of the haematophagous monogenean *Eudiplozoon nipponicum* which parasitizes the common carp. We then focused on a Kunitz protein (EnKT1) discovered in the *E. nipponicum* transcriptome, which structurally resembles textilinin-1, an antihemorrhagic snake venom factor from *Pseudonaja textili*s. The protein was recombinantly expressed, purified and biochemically characterised. The recombinant EnKT1 did inhibit *in vitro* activity of Factor Xa of the coagulation cascade, but exhibited a higher activity against plasmin and plasma kallikrein, which participate in fibrinolysis, production of kinins, and complement activation. Anti-coagulation properties of EnKT1 based on the inhibition of Factor Xa were connfirmed by thromboelastography, but no effect on fibrinolysis was observed. Moreover, we discovered that EnKT1 significantly impairs the function of fish complement, possibly by inhibiting plasmin or Factor Xa which can act as a C3 and C5 convertase. We localised Enkt1 transcripts and protein within haematin digestive cells of the parasite by RNA *in situ* hybridisation and immunohistochemistry, respectively. Based on these results, we suggest that the secretory Kunitz protein of *E. nipponicum* has a dual function. In particular, it impairs both haemostasis and complement activation *in vitro*, and thus might facilitate digestion of a host‘s blood and protect a parasite‘s gastrodermis from damage by the complement. This study presents, to our knowledge, the first characterisation of a Kunitz protein from monogeneans and the first example of a parasite Kunitz inhibitor that impairs the function of the complement.

Curriculum vitae

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Czech Science Foundation (Grant No. P506/12/1258): **Host-parasite interactions in blood-feeding diplozoid monogeneans: Investigation of a highly specialized adaptation to parasitism** (2012-2016), as a member of the team.

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Congress presentations:

2013 – 20th Helminthological days, Štědronín u Orlíku, Czech Republic
L. Jedličková, H. Dvořáková, M. Kašný and L. Mikeš: Peptidases of monogeneans of the family Diplozoidae (oral presentation).

2014 – 21st Helminthological days, Stráž nad Nežárkou, Czech Republic
L. Jedličková, H. Dvořáková, M. Kašný and L. Mikeš: Digestive peptidases from monogeneans of the family Diplozoidae (oral presentation).

2014 – V4 Parasitological Meeting: Parasites in the Heart of Europe, Stará Lesná, Slovakia
L. Jedličková, H. Dvořáková, M. Kašný and L. Mikeš: Biochemical and molecular characterization of peptidases from monogenean parasites of the family Diplozoidae (Heteronchoinea) (oral presentation).

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L. Jedličková, J. Dvořák, J. P. Dalton,, I. Hrachovinová, M. Kašný, L. Mikeš: *Eudiplozoon nipponicum* (Monogenea): inhibitory peptidáz Kunitzova typu jako modulátory hemostázy a imunity. (passive participation)

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