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Bioaktivní molekuly zapojené do zpracování krve u hematofágních monogeneí čeledi Diplozoidae

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

DIZERTAČNÍ PRÁCE

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze dne 29. 07. 2019 …….

Mgr. Lucie Jedličková

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Obsah

Abstrakt

Monogenea z čeledi Diplozoidae (podtřída Heteronchoinea) jsou krevsající ektoparaziti nacházející se na žábrách kaprovitých ryb. Trávení krve u diplozoidů probíhá intracelulárně v lysozomálním cyklu buněk gastrodermis střeva za přítomnosti peptidáz parazita. Avšak informace o způsobu trávení krve u této skupiny pocházely dosud pouze z ultrastrukturálních a histochemických analýz. Proto jsem se v této práci zaměřila na biochemickou a molekulární charakteristiku vybraných bioaktivních molekul, které by se mohly podílet na zpracování krve dospělci *Eudiplozoon nipponicum*, a to konkrétně na cysteinové peptidázy typu katepsinů L a B, aspartické peptidázy typu katepsinu D a na inhibitory serinových peptidáz Kunitzova typu.

V homogenátech a exkrečně/sekrečních (E/S) produktech dospělců *E. nipponicum* dominovala aktivita cysteinových peptidáz typu katepsinu L, následovaná aktivitou aspartických peptidáz typu katepsinu D a minoritním podílem aktivity katepsinů B. Hemoglobinolytická aktivita těchto vzorků byla kompletně zablokována inhibitory jmenovaných typů peptidáz. V transkriptomu dospělců *E. nipponicum* byly objeveny transkripty kódující 10 katepsinů L a pouze 1 katepsin B. Primární struktury kódovaných enzymů byly bioinformaticky a fylogeneticky porovnány. Dva abundantní katepsiny L (EnCL1, EnCL3) byly exprimovány v expresních systémech *P. pastoris*/*E. coli* a následně biochemicky a funkčně charakterizovány. Oba enzymy byly lokalizovány uvnitř trávicích buněk, ale i v lumen střeva dospělého červa. Výsledky tedy odhalily pravděpodobnou extracelulární fázi trávení krve u *E. nipponicum* a zároveň přítomnost široké škály katepsinů L s rozdílnými strukturními vlastnostmi a pravděpodobně i funkcemi.

Vedle peptidáz účastnících se degradace přijatých krevních proteinů potřebují krevsající paraziti také bioaktivní molekuly zabraňující srážení nasávané krve. K regulaci hemostázy využívají často inhibitory serinových peptidáz účastnících se koagulační kaskády. Mezi ně patří např. inhibitory Kunitzova typu nalezené i v transkriptomu dospělců *E. nipponicum*. Jeden z nich (nazvaný EnKT1) byl vybrán pro heterologní expresi v systému *E. coli*. Rekombinantní EnKT1 inhiboval peptidolytickou aktivitu lidského faktoru Xa, plazminu a plazmového kallikreinu *in vitro*. Jeho antikoagulační aktivita založená na inhibici faktoru Xa byla potvrzena tromboelastograficky *in vitro*.

Navzdory schopnosti inhibovat plazmin nebyl prokázán jeho vliv na fibrinolýzu. Na druhou stranu EnKT1 efektivně blokoval cytolytickou aktivitu rybího komplementu *in vitro*. Pomocí metod *in situ* hybridizace a imunohistochemie se podařilo EnKT1 lokalizovat uvnitř trávicích buněk parazita. Protein má sekreční signální sekvenci a byl detekován v E/S produktech parazita. Na základě uvedených vlastností se lze domnívat, že EnKT1 funguje jako bifunkční inhibitor blokující koagulaci přijaté krve ve střevě parazita a zároveň chránící buňky gastrodermis před útokem komplementu. Jedná se o první Kunitz protein parazitárního původu s prokázaným inhibičním účinkem na komplementovou kaskádu v krvi hostitele.

Klíčová slova: katepsin, Kunitz inhibitor, hematofágie, monogenea, *Eudiplozoon nipponicum*, trávení krve, inhibitor peptidáz

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.

Key words: cathepsin, Kunitz inhibitor, haematophagy, monogena, *Eudiplozoon nipponicum*, blood digestion, inhibitor peptidase

Úvod

Eudiplozoon nipponicum se řadí mezi krevsající monogenea čeledi Diplozoidae. Jedná se o ektoparazitického helminta, který parazituje na žábrách kapra obecného (*Cyprinus carpio*), ryby s vysokým ekonomickým významem v mnoha zemích včetně akvakultury České republiky. *E. nipponicum* je invazní druh, který byl do Evropy zavlečen z Asie. Kromě možného patogenního účinku na své hostitele existuje o monogeneích velmi málo informací. Mnoho studií a prací zabývajících se skupinou Monogenea je zaměřeno na morfologii, ekologii, anatomii, patogenitu atp. Nicméně z molekulárního a biochemického hlediska se jedná o opomíjenou skupinu parazitů, přestože některé druhy mohou způsobit rozsáhlé škody v akvakultuře. Patogenita *E. nipponicum*, ve srovnání s některými zástupci monogeneí, je nízká a může spočívat v mechanickém poškození žaber hostitele, které slouží jako vstupní brána pro infekce do hostitele.

E. nipponicum je striktní hematofág. Hematofágie je potravní strategie, která se vyvinula u mnoha skupin organizmů. Krevsající monogenea stejně jako ostatní krevsající paraziti musí při sání překonat hemostázu hostitelské krve a hostitelskou imunitní odpověď. Proto hematofágové využívají různých antihemostatických a imunomodulačních látek, které bývají součástí jejich sekretů produkovaných různými typy žláz. Krev se dále dostává do střeva, kde dochází k trávení krve buď v lumen střeva, nebo uvnitř trávicích buněk v lysozomálním cyklu. Podle ultrastrukturálních studií tráví monogenea krev intracelulárně, podobně jako je tomu u krevsajících roztočů jako jsou klíšťata. Na trávení krve se podílí evolučně konzervovaná síť cysteinových, aspartických, případně i jiných peptidáz.

V této práci jsem se zaměřila na detailní molekulární a biochemickou charakteristiku vybraných bioaktivních molekul, které by mohly být zapojeny v příjmu a trávení krve u dospělců *E. nipponicum*. Jedná se zejména o inhibitory Kunitzova typu, které často fungují u ostatních krevsajících parazitů jako antikoagulační faktory, a o cysteinové peptidázy (katepsiny L), které hrají významnou roli u krevsajících helmintů při trávení krevních proteinů.

Literární přehled

1.1. Monogenea

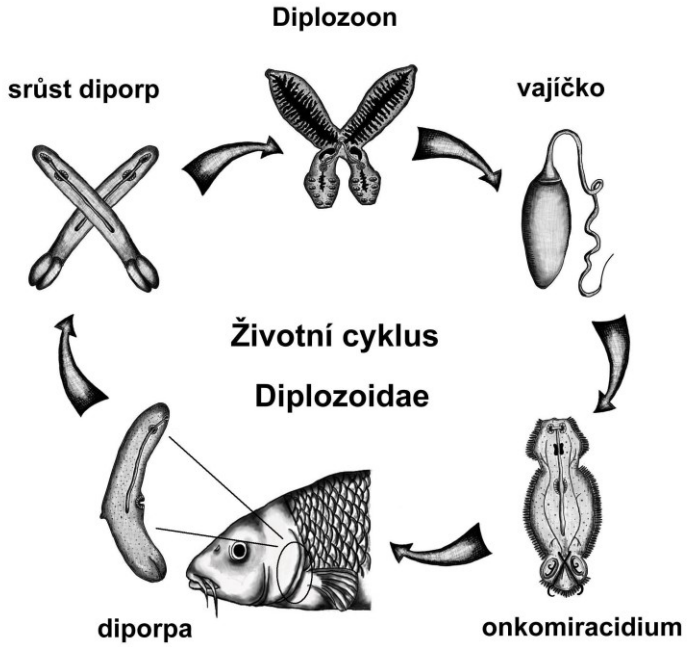
Třída Monogenea se řadí spolu s třídami Cestoda a Trematoda do podkmene Neodermata (Platyhelminthes) (Littlewood *et* Bray, 2001) a dále se dělí na dvě podtřídy: Polyonchoinea, která se živí mukusem a epiteliálními buňkami (tzv. mukofágové) a Heteronchoinea živící se krví (tzv. hematofágové) (Boeger *et* Kritsky, 2001; Buchmann *et* Bresciani, 2006). Většina monogeneí žije ektoparaziticky a vykazuje vysokou hostitelskou i orgánovou specifitu (Buchmann *et* Bresciani, 2006). Především se jedná o parazity vyskytující se na žábrách, ploutvích a kůži ryb. Mohou ale žít i endoparaziticky např. v žaludku, močovém měchýři, nebo ústní, tělní či nosní dutině (Smyth *et* Halton, 1983a). Kromě ryb je můžeme najít i u jiných druhů organizmů jako jsou např. obojživelníci, plazi, hlavonožci a u hrocha. Na konci těla mají charakteristický přichycovací orgán, zvaný opisthaptor, který může být opatřen destičkami, svorkami, háčky, nebo přísavkami (Smyth *et* Halton, 1983a; Buchmann *et* Bresciani, 2006; Whittington *et* Kearn, 2011). Vysoká intenzita infekce hematofágními druhy monogeneí a nadměrné sání krve může mít u hostitelů za následek onemocnění zvané hypochromní mikrocytická anémie (Kawatsu 1978; Buchmann *et* Bresciani, 2006; Reed *et al.*, 2009; Valigurová *et al.*, 2011).

1.1.1. Čeleď Diplozoidae

Zástupci z čeledi Diplozoidae se řadí do podtřídy Heteronchoinea. Jedná se o skupinu sladkovodních krevsajících monogeneí parazitujících na žábrách kaprovitých ryb (Matějusová *et al.*, 2001). Tato práce byla zaměřena na druh *Eudiplozoon nipponicum* vyskytující se na kapru obecném (*Cyprinus carpio*), který se řadí mezi ryby s vysokým ekonomickým významem v mnoha zemích Asie a Evropy (Kawatsu, 1978; Fisheries and Aquaculture Department, 2017).

Jedná se o oviparní hermafrodity (Obr. 1). Z vajíček, která jsou opatřena dlouhým filamentem, se líhne pohyblivá larva onkomiracidium. U onkomiracidií se objevuje opisthaptor vybavený prvním párem svorek s centrálními háčky. Ten onkomiracidium využívá k přichycení na žábry ryby a následně se larva přemění na stádium zvané

diporpa, která se už živí krví (Khotenovsky 1985 v Matějusová *et al.*, 2001; Khotenovsky 1985 v Pečínková *et al.*, 2007, Khotenovsky 1985 v Hodová *et al.* 2010, Valigurová *et al.*, 2011). Diporpa musí najít druhou diporpu, se kterou srůstá v tzv. permanentní kopulu a vyvíjí se v dospělce. Dochází tak ke sdílení nervové, trávicí, svalové a reprodukční soustavy (Zurawski *et al.*, 2001, 2003; Matějusová *et al.*, 2001; Roberts *et* Janovy, 2005; Pečínková *et al.*, 2007, Valigurová *et al.*, 2011).



Obr. 1 – Životní cyklus č. Diplozoidae

1.2. Hematofágie

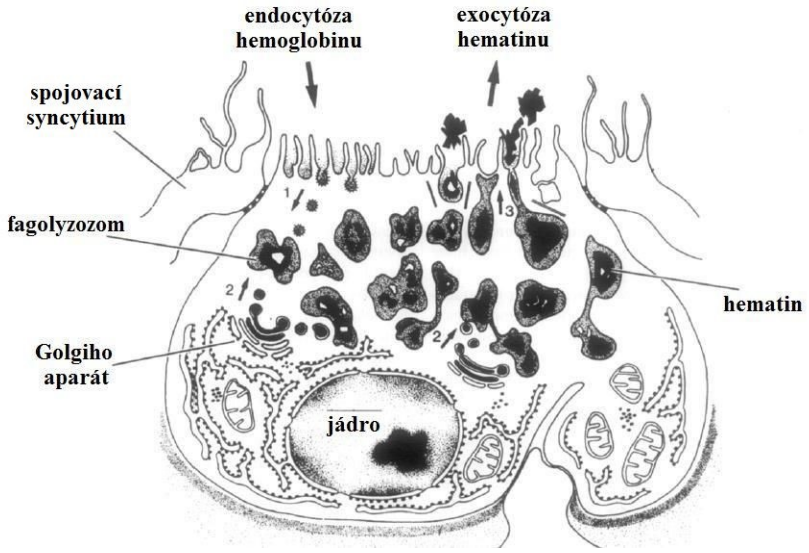
V průběhu evoluce se hematofágie vyvinula jako potravní strategie u mnoha různých skupin organizmů, jako jsou Arthropoda, Platyhelminthes, Nematoda, Annelida atd. (Mans, 2011; Azar *et* Nel, 2012). Krev je tzv. tekutý orgán, který se skládá z buněčné složky a plazmy. Plazma obsahuje vodu, bílkoviny, glukózu a množství dalších anorganických a organických látek. Buněčná složka krve se nachází v plazmě a zahrnuje erytrocyty, leukocyty a trombocyty (Pecka, 2004). Krev tedy nabízí hematofágním organizmům bohatý zdroj energie a stavebních látek pro procesy jako jsou růst, vývoj a reprodukce (Caffrey *et al.*, 2004; Delcroix *et al.*, 2006; Horn *et al.*, 2009). Zároveň je zapojena v obraně organizmu proti infekcím. Proto při vzniku hematofágie bylo zapotřebí

vyvinout i různé adaptace, které umožní krevsajícím parazitům překonat komplikace související s příjmem a trávením krve. Z molekulárního hlediska paraziti produkují ve svých sekretech různé antihemostatické a imunomodulační látky, kterými dokážou potlačit ochranné mechanizmy hostitele, jako jsou např. koagulace krve, aktivace komplementu nebo tvorba zánětu (Gillespie *et al.*, 2000, Champagne, 2005). Krev je za přítomnosti peptidáz trávena ve střevě/střevních buňkách parazita (Smyth *et* Halton, 1983a; b; Caffrey *et al.*, 2004; Sojka *et al.*, 2013). Nicméně doposud nebyly žádné z těchto molekul popsány u hematofágních monogeneí.

Dále se ve své práci budu věnovat příjmu a trávení krve u monogeneí odděleně, protože se jedná o dvě široká témata. Trávení krve je uvedeno v kapitole 1.3. a příjem krve v kapitole 1.5.

1.3. Trávení krve u Heteronchoinea

Trávení krve u hematofágních monogeneí probíhá ve slepých střevech, kam je krev pumpována svalnatým farynxem. Nachází se zde gastrodermis, která se skládá z hematinových (trávicích) buněk a spojovacího syncytia (Obr. 2). Syncytium tvoří kompaktní povrch a skrz něj ústí do střeva apikální výběžky hematinových buněk. Pod gastrodermis se nachází bazální lamina a vrstva podélné a okružní svaloviny (Smyth *et* Halton, 1983a; b; Konstanzová *et al.*, 2015). Na základě původních ultrastrukturálních studií se předpokládá, že trávení hemoglobinu probíhá intracelulárně v lysozomálním cyklu uvnitř hematinových buněk. Hemoglobin se endocytózou dostává dovnitř buněk, kde je tráven uvnitř fagolysozomu lytickými enzymy a globiny jsou postupně tráveny na dipeptidy a jednotlivé aminokyseliny. Uvolněný hem je oxidován na nerozpustný ferriporfyrin hematin a hromadí se v buňce. Buňka se plní pigmentem, který se uvolňuje exocytózou do střeva, odkud je následně odstraněn regurgitací z těla ven (Halton, 1997; Konstanzová *et al.*, 2015).

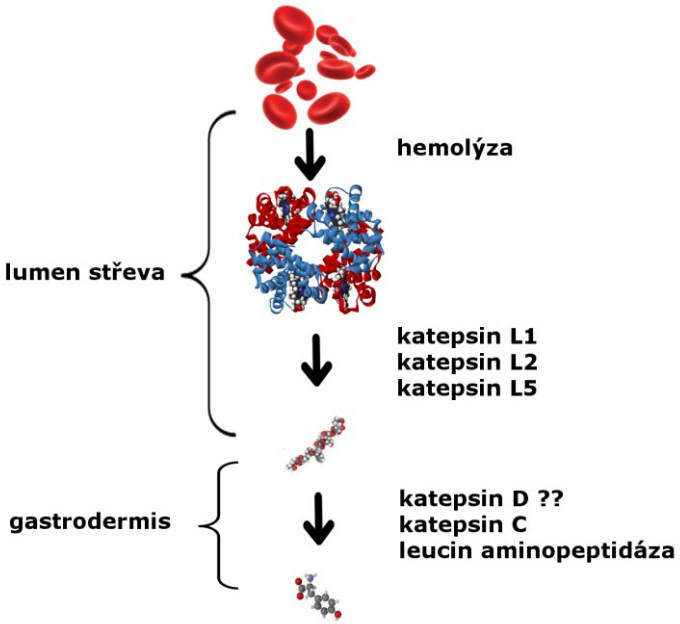


Obr. 2 – Hematinová buňka u zástupců Heteronchoinea (Smyth ***et*** Halton, 1983a, upraveno)**.**

Velmi málo informací existuje o biochemii trávení a molekulách účastnících se zpracování krve u monogeneí, na rozdíl od ostatních krevsajících parazitů jako jsou schistosomy, ankylostomy, fascioly nebo klíšťata. Do nedávna byly publikovány pouze dvě studie, které se zabývaly peptidázami u mořského mukofágního monogenea *Neobenedenia* spp., (Hirazawa *et al.*, 2006; Rao *et* Yang, 2007), ale žádné nebyly funkčně charakterizovány. Nicméně existuje předpoklad, že trávení krve u hematofágních monogeneí probíhá podobně jako u ostatních krevsajících helmintů, pouze zastoupení trávicích enzymů, fáze trávení (intracelulární/extracelulární), na které se trávicí enzymy podílejí, a místo trávení (buňky/lumen střeva) se může významně lišit. Z literatury je známo, že zúčastněné trávicí enzymy u krevsajících helmintů jsou součástí konzervované sítě cysteinových (katepsin L, B, C a legumain) a aspartických peptidáz (katepsin D) (Sajid *et* McKerrow, 2002; Williamson *et al.*, 2003, 2004; Delcroix *et al.*, 2006; McDougall, 2012). Jako příklad zde uvádím rozdílné způsoby trávení krve u fasciol, schistosom a u klíšťat.

U fasciol dochází k hemolýze v lumen střeva při kyselém pH. Gastrodermis se skládá z buněk, které opakovaně prodělávají strukturní změny podle fáze, ve které se nacházejí (absorbční/sekreční) ((Robinson *et* Threadgold 1975 v Dalton, 1999). První fáze trávení hemoglobinu probíhá **extracelulárně** v lumen střeva za přítomnosti peptidáz (katepsinu L1, L2 a L5), které jsou do lumen střeva sekretovány z buněk gastrodermis

(Halton, 1976 v Halton, 1997; McVeigh *et al.*, 2012). Hemoglobin je tráven mezi povrchovými lamelami při pH přibližně 4,5 na menší peptidy. Ty jsou pak vstřebány do buněk gastrodermis, kde dochází k **intracelulární** fázi trávení za přítomnosti katepsinu C a leucin aminopeptidázy (Obr. 3) (Acosta *et al.*, 1998; Robinson *et al.*, 2008; Lowther *et al.*, 2009). V gastrodermis byl detekován i katepsin D, který by se mohl podílet na trávení (McDougall, 2012). Při hydrolýze hemoglobinu vzniká hematin, který se hromadí převážně v lumen střeva a regurgitací je odstraněn ze střeva ven (Robinson *et* Threadgold 1975 v Halton, 1997).



Obr. 3 – Peptidázy podílející se na degradaci hemoglobinu u *Fasciola hepatica*.

U schistostosom dochází k hemolýze v jícnu, odkud se hemoglobin dostává dále do střeva (Dike 1971 v Bogitsh *et* Carter 1977). Gastrodermis u schistosom je syncytiální a trávení hemoglobinu probíhá **extracelulárně** v lumen střeva (Halton, 1997; Dalton *et al.*, 2004). Legumain, přítomný ve střevě, plní aktivační funkci a aktivuje katepsin B1, ale i katepsiny C, L1/F, L2 a D (Sajid *et al.*, 2003, Dalton *et al.*, 1996 v Caffrey *et al.*, 2004). Katepsin B1 vykazuje ve střevě jak endopeptidázovou aktivitu, tak i exopeptidázovou aktivitu (Sajid *et al.*, 2003; Caffrey *et al.*, 2004, Delcroix *et al.*, 2006). Hemoglobin je degradován na hem a globin, kdy globin je dále degradován na dipeptidy a jednotlivé aminokyseliny, které jsou následně vstřebány do gastrodermis (Halton 1997; Dalton *et al.*, 2004). V gastrodermis se katepsin L3 a leucin aminopeptidáza (LAP)

podílejí na konečném zpracování hemoglobinu (McCarthy *et al.*, 2004, Dvořák *et al.*, 2009). Hem se ve střevě oxiduje na hematin a regurgitací je následně odstraněn z lumen střeva ven (Brindley *et al.*, 1997).

U klíšťat dochází k enzymatické hemolýze v lumen střeva klíštěte (Sojka *et al.*, 2013). Hemoglobin se dostává přes receptory zprostředkovanou endocytózu do trávicích buněk střeva a dochází tak k **intracelulárnímu** trávení (Sonenshine, 1991; Coons *et al.*, 1986 v Franta *et al.*, 2011, Sojka *et al.*, 2013). Trávení hemoglobinu začíná fúzí endozomu s primárním lysozomem a vzniká tak sekundární lysozom (Sonenshine, 1991). Na degradaci hemoglobinu se podílejí cysteinové, aspartátové, serinové a metalopeptidázy (Mulenga *et al.*, 2003; Hatta *et al.*, 2006; Sojka *et al.*, 2008). Hemoglobin je degradován za přítomnosti katepsinu D, katepsinu L a legumainu na větší fragmenty, které jsou následně štěpeny za přítomnosti katepsinu B. Katepsin C se podílí na další fázi a vzniklé dipeptidy jsou degradovány karboxypeptidázou (SCP) a leucin aminopeptidázou (LAP) (Horn *et al.*, 2009). Hem se hromadí ve formě nekrystalického agregátu ve specializované organele hemozomu uvnitř trávicí buňky (Lara *et al.*, 2003). Většinou jsou velké trávicí buňky s agregátem odděleny od basální laminy a jsou uvolněny do lumen střeva a odstraněny defekací. Tyto buňky jsou následně nahrazeny novými (Sonenshine, 1991).

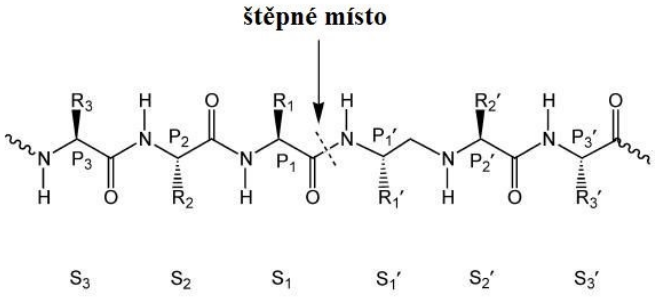
1.3.1. **Peptidázy**

Peptidázy jsou hydrolytické enzymy, které se řadí mezi významné molekuly účastnící se interakcí mezi parazitem a hostitelem. Podílejí se zejména na invazi do hostitele, trávení potravy, úniku před imunitní odpovědí a tvorbou zánětu, migrací tkáněmi atd. (Tort *et al.*, 1999; Caffrey *et al.*, 2004; Delcroix *et al.*, 2006; Kašný *et al.*, 2009). Jedná se o enzymy, které jsou esenciální pro všechny druhy organizmů (Rawlings *et* Barrett, 1999) a jsou kódovány přibližně 2 % všech exprimovaných genů (Sajid *et* McKerrow, 2002). Proto je v dnešní době věnována velká pozornost zejména trávicím peptidázám hematofágních parazitů jako jsou klíšťata (např. *Ixodes ricinus*), motolice (např. *Fasciola* spp., *Schistosoma* spp.) nebo hlístice (např. *Ancylostoma* spp.), v souvislosti s vývojem nových antiparazitárních přípravků (Dalton *et al.*, 1996, 2003; McManus *et* Dalton, 2006; Acosta *et al.*, 2008; Marcilla *et al.*, 2008).

Peptidázy hydrolyzují peptidické vazby mezi aminokyselinami proteinů a na základě místa působení se rozdělují na exopeptidázy a endopeptidázy. Endopeptidázy štěpí proteiny uvnitř polypeptidového řetězce, zatímco exopeptidázy odštěpují jednotlivé aminokyseliny nebo několik aminokyselin z jejich konce (C-/ N- konce, podle typu exopeptidázy) (Barrett *et al.*, 2004). Podle katalytického mechanizmu, který udávají postranní řetězce aminokyselin aktivního místa, můžeme peptidázy rozdělit do devíti skupin dle databáze MEROPS (Rawlings *et al.*, 2018).

1.3.1.1. Substrátová specifita peptidáz

Substrátová specifita je jednou z nejdůležitějších vlastností peptidáz, pomocí které můžeme odlišit jednu peptidázu od druhé. Nicméně u mnoha případů je specifita mnohem složitější (Rawlings *et al.*, 2008, 2010). Kolem aktivního místa se nacházejí tzv. vazebné kapsy, kde dochází k interakci s postranními řetězci aminokyselin substrátu. Vazebné kapsy jsou označovány a číslovány směrem od aktivního místa, jako Sn směrem k N-konci a Sn´ směrem k C-konci, kde n začíná 1. Postranní řetězce aminokyselin substrátu reagující s vazebnými kapsami enzymu se označují a číslují směrem k N-konci jako Pn a jdoucí k C-konci jako Pn´, kde n začíná 1. Mezi pozicí P1 a P1´ se nachází štěpné místo (aktivní místo) substrátu (Barrett *et al.*, 2004; Rawlings *et al.*, 2008) Obr 4. Dalšími důležitými faktory, které ovlivňují vazbu, jsou: polarita, náboj, prostorová dostupnost a velikost (Sajid *et* McKerrow, 2002).



Obr. 4 – Substrátová specifita peptidáz (Shinnar *et al.*, 2003, upraveno).

1.3.1.2. Regulace aktivity peptidáz

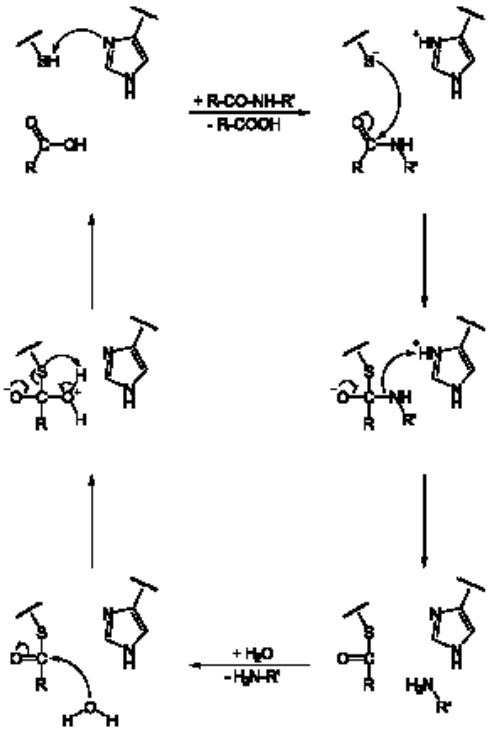
Peptidáza ve špatný čas a na špatném místě může buňku ohrozit. Proto je potřeba, aby jejich aktivita byla pod přísnou kontrolou. Poruchy regulace jejich aktivity mohou

mít za následek vznik onemocnění jako je např. rakovina, revmatoidní artritida, osteoporóza atd. (Vasiljeva *et al.*, 2007).

U organizmů existuje několik odlišných mechanizmů, které slouží k regulaci peptidázové aktivity. Mezi ně se řadí regulace genové exprese nebo směřování enzymů do speciálních kompartmentů jako jsou např. lysozomy nebo mitochondrie. Dalšími mechanizmy jsou proteolytická degradace, různé posttranslační modifikace (glykosylace, vázání kovů, přemostění S-S můstků) nebo aktivace enzymů (López-Otín *et* Bond, 2008). Většina peptidáz je syntetizována ve formě preproenzymu (zymogenů), kde proregion napomáhá ke správnému složení enzymu, dále může sloužit jako signál, který navádí enzym do intracelulárních kompartmentů nebo k sekreci a v neposlední řadě prochází skrz aktivní místo peptidázy a slouží jako endogenní inhibitor proti nechtěné aktivitě (Carmona *et al.*, 1996; Sajid *et* McKerrow, 2002). Aktivace zymogenu může být způsobena jinou peptidázou, která odštěpí prosegment, anebo může dojít k autoaktivaci při snížení pH (Caffrey *et al.*, 2004; Lowther *et al.*, 2009). Lysozomální katepsiny, až na výjimky jako jsou parazitární katepsiny, jsou aktivní v pH okolo 4 a nižším, zatímco při neutrálním pH jsou nestabilní. Pravděpodobně se jedná o regulační mechanizmus v případě uvolnění peptidáz z kompartmentů a zabraňuje tak nechtěné proteolytické aktivitě (Sajid *et* McKerrow, 2002). Mezi další významné regulační mechanizmy cysteinových peptidáz se řadí endogenní inhibitory, jako jsou cystatiny, thyropiny a serpiny, které fungují jako reverzibilní kompetitivní inhibitory (Turk *et al.*, 2012).

1.3.1.3. Cysteinové peptidázy

Obecně jsou cysteinové peptidázy syntetizovány ve formě preproenzymu, který se skládá ze signálního peptidu, prodomény a katalytické domény (Sajid *et* McKerrow, 2002). Katalytický mechanizmus cysteinových peptidáz je podobný jako u serinových peptidáz, které využívají jako nukleofil hydroxylovou skupinu serinu. Na rozdíl od nich, cysteinové peptidázy využívají jako nukleofil sulfhydrylovou skupinu aminokyseliny cysteinu (Barrett *et* Rawlings, 1996; Barrett *et al.*, 1998) (Obr. 5).



Obr. 5 - Katalytický mechanizmus cysteinových peptidáz
(https://en.wikipedia.org/wiki/Cysteine_protease).

Cysteinové peptidázy se dle databáze MEROPS dělí do 11 klanů (CA, CD, CE, CF, CL, CM, CN, CO, CP, CQ, CR). První cysteinová peptidáza z ovoce *Carica papaya* byla popsána v roce 1879 a byla nazvána papain. V klanu CA se nacházejí tzv. „papain-like“ peptidázy, které vykazují sekvenční podobnost s papainem (Barrett *et al.*, 1998). Tento klan se dále rozděluje na 41 rodin, z toho 12 rodin zahrnuje většinu známých parazitárních cysteinových peptidáz (Atkinson *et al.*, 2009, Rawlings *et al.*, 2018). Důležité parazitární peptidázy se nacházejí v C1 (katepsin L, katepsin B, katepsin C) a C2 rodině (kalpain-like) (Sajid *et* McKerrow, 2002). Typickým charakteristickým znakem pro peptidázy z klanu CA je jejich citlivost k inhibitoru E-64 (*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butan) a substrátová specifita determinovaná S2 kapsou. Peptidázové substráty obsahující arginin v P2 byly často použity k rozlišení aktivity katepsinu B a katepsinu L (Sajid *et* McKerrow, 2002). Dalším důležitým klanem cysteinových peptidáz je klan CD, kde je substrátová specifita determinována v P1 pozici. Peptidázy z klanu CD nejsou ovlivněny klasickým inhibitorem cysteinových peptidáz E-64 (Mottram *et al.*, 2003). Klan se dále dělí dle

databáze MEROPS na sedm rodin, kde se parazitární peptidázy nacházejí v rodině: C13 legumain, C14 kaspázy a C50 separázy (Mottram *et al.*, 2003; Rawlings *et al.*, 2018).

Katepsiny jsou rozdělovány na základě vysoké sekvenční podobnosti v oblasti katalytických zbytků na dvě skupiny katepsiny „L-like“ (L, V, K, S, W, F a H) a na katepsiny „B-like“. Pro katepsiny B je typická kromě endopeptidázové aktivity i exopeptidázová aktivita, za kterou je zodpovědná peptidická smyčka „occluding loop“ v katalytické doméně, kterou katepsiny L nemají (Musil *et al.*, 1991; Coulombe *et al.*, 1996; Illy *et al.*, 1997). Dále v textu se budu zabývat katepsiny L s ohledem na výsledky mé doktorské práce.

1.4. **Katepsin L**

Katepsiny L jsou členové papainové rodiny cysteinových peptidáz klanu CA (Barrett *et al.*, 1998; Rawlings *et al.*, 2010). V současné době by katepsiny neměly být považovány pouze za lysozomální peptidázy, protože se nacházejí v různých buněčných kompartmentech, ale i v extracelulárních prostorech a řadí se mezi významné multifunkční proteiny (Zhou *et al.*, 2015).

1.4.1. **Struktura**

Velikost katepsinu L se pohybuje přibližně okolo 25-30 kDa. Lidský katepsin L podobně jako papain (Kamphuis *et al.*, 1984) se skládá ze dvou domén, kde levá doména se skládá ze tří α-helixů a pravá doména je tvořena β-barelem. Aktivní místo tvoří aminokyseliny Cys, His a Asn. V centru aktivního místa se nachází His na pravé doméně, který reaguje s Cys na levé doméně a vytváří tak thiol-imidazolovou nábojovou dyádu (Gunčar *et al.*, 1999; Turk *et al.*, 2012). Tato dyáda bývá většinou stabilizována asparaginem v aktivním místě. Důležitým prvkem pro stabilizaci tetraedrálního meziproduktu v průběhu hydrolýzy je glutamin, který napomáhá formovat oxoniovou mezeru (Barrett *et al.*, 1998; Sajid *et* McKerrow, 2002).

Katepsin L je syntetizován jako preproenzym na ribozomech endoplazmatického retikula. Signální sekvence je odštěpena při vstupu do lumen endoplazmatického retikula a dochází zde k N-glykosylaci (Tao *et al.*, 1994 v Barrett *et al.*, 1998). Propeptid slouží ke

správnému složení enzymu, směřuje signál do lysozomů přes specifický receptor manóza-6-fosfát a zároveň slouží jako inhibitor enzymu (McIntyre *et al.*, 1994). Katepsiny „L-like“ obsahují ve svém proregionu konzervovaný aminokyselinový motiv ER(V/I)FNIN (písmena označují názvy aminokyselin) (Karrer *et al.*, 1993; Turk *et al.*, 2000). Katepsiny W a F mají tento motiv modifikovaný na ERFNAQ (Wex *et al.*, 1999). Tento motiv společně s motivem GNFD je pravděpodobně využíván ke vnitrobuněčnému transportu a zpracování enzymu (Vernet *et al.*, 1995; Dvořák *et al.*, 2009).

1.4.2. **Aktivita**

Jedná se o endopeptidázu, která preferuje štěpení vazby s aromatickým zbytkem v P2 pozici (Brömme *et al.*, 1994). K odlišení aktivity savčího katepsinu L od katepsinu B byl použit syntetický substrát RR-AMC, kde Arginin v P2 pozici je schopný štěpit pouze katepsin B (Sajid *et* McKerrow, 2002). Podobně je tomu i u jiných organizmů, nicméně jsou známé i výjimky, kde např. katepsin L (cruzipain) u *Trypanosoma cruzi* je schopen štěpit RR-AMC substrát (Gillmor *et al.*, 1997). Zymogen katepsinu L může být aktivován autokatalyticky (Caffrey *et al.* 2004) nebo pomocí jiné peptidázy (Sajid *et al.* 2003). U obratlovců jsou katepsiny L aktivní v kyselém prostředí, v rozmezí pH 3 - 6,5. Na druhou stranu katepsiny L u motolic jsou aktivní v širokém rozmezí pH 3-11, kde katepsin L1 u *Schistosoma mansoni* má pH optimum 6,5 a katepsiny L1 a L2 u *Fasciola hepatica* mají pH optimum 8 a 6,5. Nicméně tyto enzymy vykazují i dobrou stabilitu v neutrálním pH na rozdíl od katepsinů L obratlovců (Dalton *et al.*, 2003; Dvořák *et al.*, 2009). Katepsin L3 u *S. mansoni* byl schopen štěpit syntetický substrát FR-AMC při pH 3,5-11 s pH optimem 6,5 (Dvořák *et al.*, 2009). Široký profil pH parazitárních katepsinů je v souladu s mnoha extralysozomálními funkcemi, které byly u parazitů popsány (Sajid *et* McKerrow 2002).

1.4.3. **Katepsin L u parazitů**

Katepsiny L u parazitů jsou abundantní peptidázy a mohou hrát významnou roli např. při invazi do hostitele a tkáňové migraci, invazi imunitního systému, příjmu a trávení potravy, reprodukci a při interakcích parazita s hostitelem (Atkinson *et al.*, 2009; Kašný *et al.*, 2009; Robinson *et al.*, 2011). Z důvodu jejich velké důležitosti v průběhu životního cyklu parazita není nic neobvyklého, že se tyto enzymy vyskytují u některých organizmů ve více kopiích. Podobně jako je tomu u *Leishmania major*, kde bylo popsáno

několik katepsinů L a jiných trypanosomatid, kde např. *Leishmania mexicana* využívá více než 10 kopií katepsinů L, *Leishmania pifanoi* 8-20 kopií, *Trypanosoma cruzi* až 130 kopií (Sakanari *et al.*, 1997). Genové duplikace mohou hrát potenciální roli v evoluční historii genů a tvorbě funkční diverzity (Zhou *et al.*, 2015).

První katepsin L u helmintů byl popsán u motolice *Fasciola hepatica* (Smith *et al.*, 1993). Nyní jsou známé u všech parazitických červů. Nicméně se zdá, že u motolic se katepsiny L vyskytují ve větším množství než u hlístic (Tort *et al.*, 1999). Obecně je známo, že peptidázy parazitů jsou exprimovány v různých životních stádiích a poukazují tak na různé potřeby parazitů v průběhu jejich životního cyklu. Jako příklad zde uvádím životní cyklus motolice *F. hepatica*, u které její infekční larva využívá katepsin L3 pro penetraci hostitelské střevní stěny, juvenilní stádium využívá katepsin L1, L2 a někdy i L3 k migraci hostitelskými játry a následně dospělec používá k trávení hemoglobinu katepsiny L1, L2 a L5 (Robinson *et al.*, 2008). V tabulce 1 uvádím další katepsiny L u parazitických helmintů a jejich předpokládané nebo potvrzené funkce.

Tab. 1 – Seznam katepsinů L u parazitických helmintů dle Caffrey *et al.*, 2018, upraveno. Zeleně jsou vyznačena hlístice, žlutě motolice a modře tasemnice.

organizmus	název enzymu	předpokládaná/potvrzená funkce
Haemonchus contortus	Katepsin L	degradace hemoglobinu, fibrinogenu, kolagenu, IgG, antikoagulant
Schistosoma mansoni	SmCL1/F	degradace hemoglobinu
Schistosoma mansoni	SmCL2	účastní se produkce vajíček, degradace hemoglobinu
Schistosoma mansoni	SmCL3	degradace hemoglobinu, albuminu
Schistosoma japonicum	SjCL	degradace hemoglobinu
Fasciola hepatica	FhCL1	migrace játry, degradace hemoglobinu, IgG
Fasciola hepatica	FhCL2	migrace játry, degradace hemoglobinu, kolagenu, IgG
Fasciola hepatica	FhCL3	penetrace hostitelské střevní stěny, degradace kolagenu
Fasciola hepatica	FhCL5	degradace hemoglobinu, fibronectinu, lamininu, IgG
Paragonimus westermani	Katepsin L	degradace extracelulární matrix proteinů a IgG
Taenie solium	Katepsin L	degradace tkáně a IgG
Taenie pisiformis	Katepsin L	degradace IgG, fibronektinu
Echinococcus multilocularis	Katepsin L	degradace IgG, albuminu, kolagenu a fibronektinu

Doposud existují pouze dvě studie, které se zabývaly peptidázami u monogeneí (Hirazawa *et al.*, 2006; Rao *et* Yang, 2007) a zároveň zde byla popsána první kompletní sekvence katepsinu L u mukofágního monogenea *Neobenedenia melleni* (Rao *et* Yang, 2007). Katepsin L *N. melleni* je dlouhý 1070 pb a kóduje 335 aminokyselin, kde 17 aminokyselin tvoří signální peptid, 100 aminokyselin proregion a 218 aminokyselin aktivní enzym (Rao *et* Yang, 2007). Součástí této práce jsou dvě publikace, které se katepsiny L u monogeneí zabývají.

1.5. Příjem krve u Heteronchoinea

Trávicí soustava u monogeneí podtřídy Heteronchoinea je morfologicky a funkčně velmi dobře rozlišena. Dělí se na přední střevo a párová slepá střeva. Přední střevo začíná ústním otvorem, na který navazuje ústní dutina s bukálními přísavkami, farynx a ezofágus (Smyth *et* Halton, 1983b). Na základě ultrastrukturálních analýz bylo pozorováno, že při příjmu potravy dochází k přichycení bukálních přísavek k povrchu žaberní lamely a pomocí svalnatého farynxu, který vytvoří podtlak, dojde k jejímu protržení. Krev je následně pumpována dále do střeva (Smyth *et* Halton, 1983a; b; Hodová *et al.*, 2010). V předním střevě se nacházejí četné přídatné žlázky, kde by mohly být přítomné různé antihemostatické a imunomodulační molekuly, které by následně mohly být sekretovány do předního střeva (Smyth *et* Halton, 1983a; b; Valigurová *et al.*, 2011).

Podobně je tomu u ostatních krevsajících parazitů, kteří tyto látky obsahují ve svých slinách. Díky nim jsou paraziti schopni překonat hostitelskou hemostázu, komplement a tvorbu zánětu (Ribeiro *et* Francischetti, 2003). Mnoho antihemostatických látek je velmi dobře popsáno u krevsajícího hmyzu a klíšťat, kteří v průběhu sání mohou přenášet původce významných lidských onemocnění (Champagne *et al.*, 1995; Prevot *et al.*, 2006; Kazimírová *et* Štibrániová, 2013; Mendes-Sousa *et al.*, 2018). U helmintů byl popsán jeden z nejznámějších antikoagulantů hirudin z pijavky *Hirudo medicinalis* (Markwardt *et al.*, 1992), který je běžně využíván v medicíně (Lubenow *et* Greinacher, 2001). Také u motolic rodu *Schistosoma* byly popsány molekuly s antikoagulační a protizánětlivou aktivitou (Ranasighe *et al.*, 2015a; Ranasighe *et al.*, 2015c), které by

mohly být využity jako potenciální kandidáti na vývoj vakcín proti schistosomám (Ranasinghe *et al.*, 2017; Ranasighe *et al.*, 2015c).

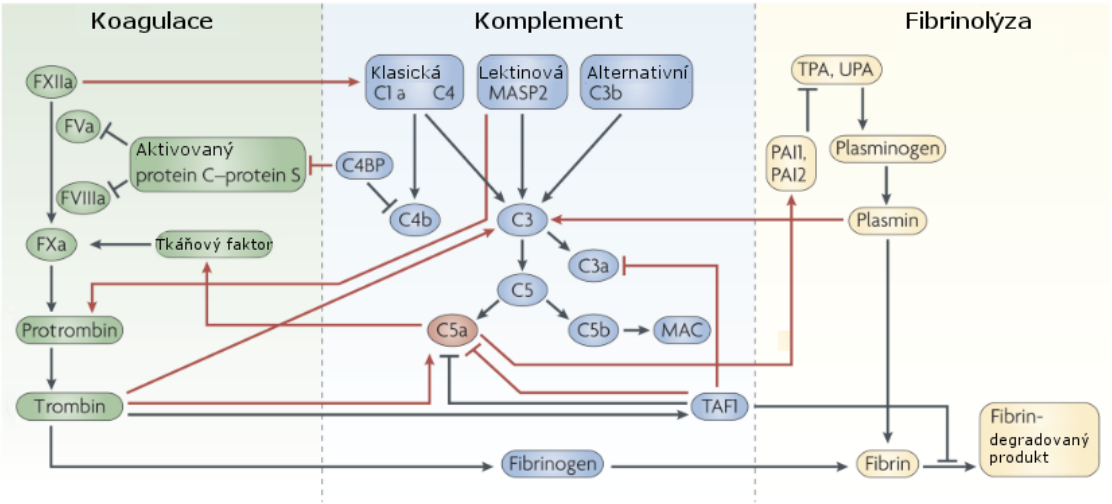
Nicméně žádná z těchto molekul dosud nebyla popsána u hematofágních monogeneí. Proto jsem se v této práci zaměřila na molekuly, které krevsající paraziti využívají k regulaci hostitelských obranných mechanizmů jako je hemostáza (kapitola 1.6.1) nebo imunitní odpověď (kapitola 1.6.2.), kterými se budu dále ve své práci zabývat.

1.6. Zánětlivá odpověď hostitele

Zánětem se označují fyziologické reakce, které slouží jako ochrana organizmu proti vzniku infekce v poškozeném místě, zároveň slouží k lokalizaci poškozeného místa a k jeho zahojení (Hořejší *et* Bartůňková, 2005). Proces zánětlivé reakce začíná zvýšením permeability cév, zvýšením adhezivity endotelií, aktivací koagulace krve/ fibrinolýzy/ kininového a komplementového systému, dále ovlivňuje autonomní nervovou soustavu a dochází i ke změně v regulaci teploty (Hořejší *et* Bartůňková, 2005; Rittirsch *et al.*, 2008). Podle rozsahu poškození a zánětu reaguje systém rozdílně. Může dojít k **septickému šoku** při vysokém průniku mikroorganizmů do krve, nebo **anafylaktickému šoku** při podnětech neinfekčního charakteru (Hořejší *et* Bartůňková, 2005).

Tradičně jsou komplement a koagulační kaskáda popisovány zvlášť, jako dva různé děje. V obou případech se jedná o proteolytické kaskády tvořené serinovými peptidázami s podobnými charakteristikami a aktivačními stimuly. Zároveň jsou tyto kaskády spolu s fibrinolýzou propojeny mnoha vzájemnými vazbami a vytváří tak složitou síť (Rittirsch *et al.*, 2008) (Obr. 6). Jednotlivé kaskády budou detailněji popsány níže. Zde uvádím pouze pár příkladů vzájemných vazeb mezi nimi. Například trombin, kromě funkce v koagulační kaskádě, je schopný generovat produkt komplementu C5a v nepřítomnosti C3 (Huber-Lang *et al.*, 2006). Na druhou stranu konečný komplex komplementu C5-9 může katalyzovat štěpení protrombinu na trombin v koagulační kaskádě i v nepřítomnosti faktoru V (Wiedmer *et al.*, 1986). Podobně je tomu u MASP2, serinové peptidázy z lektinové dráhy komplementu, která je schopná aktivovat koagulaci krve štěpením protrombinu na trombin (Krarup *et al.*, 2007). Plazmin a faktor X mohou působit jako C3 a C5 konvertáza (Amara *et al.*, 2010; Leung *et* Morser, 2016). Tyto výsledky jsou nesmírně zajímavé, už jen z důvodu, že trombin a protein C5a hrají

centrální roli uvnitř koagulační kaskády a kaskády komplementu, ale zároveň C5a a C5-9 mohou být produkovány i v nepřítomnosti aktivované dráhy komplementu (Rittirsch *et al.*, 2008).



Obr. 6 – Propojení koagulační kaskády, kaskády komplementu a fibrinolýzy (Rittirsch *et al.*, 2008).

V této práci se budu zabývat podrobněji koagulací krve a kaskádou komplementu u ryb v návaznosti na výsledky mé práce.

1.6.1. **Hemostáza**

Hemostáza je obranný proces, který při poranění organizmu zabraňuje ztrátě krve formováním fibrinové zátky. Toho je dosaženo pomocí tří mechanizmů, které spolu úzce spolupracují: cévní, trombocytární a koagulační fáze (Boon, 1993). Pro správné fungování hemostázy je zapotřebí udržovat její rovnováhu pomocí kontrolních mechanizmů jako jsou např. inhibitory a fibrinolýza (Boon, 1993; Pecka, 2004; Palta *et al.*, 2014). Narušení rovnováhy může mít za následek hyperkoagulaci, a tedy trombózu, nebo hypokoagulaci, a tedy krvácení (Rasche, 2001).

Všechny mnohobuněčné organizmy potřebují efektivní hemostatický mechanizmus jako ochranu organizmu při vzniku různých poranění. Nejinak je tomu u ryb, kde dochází často k mechanickému poškození žaber v místě, kde krev proudí v těsné blízkosti s vnějším prostředím. Proto pokud se ryba poraní a nemá účinný prostředek pro

kontrolu průtoku krve do poškozených žaber, může dojít až k jejímu vykrvácení (Tavares-Diaz *et* Oliviera, 2009).

V biochemických studiích u kostnatých ryb byly prokázány faktory vnitřní, vnější a společné cesty koagulačního systému, které naznačují, že koagulační proces u kostnatých ryb je podobný koagulačnímu systému u savců (Tavares-Diaz *et* Oliviera, 2009).

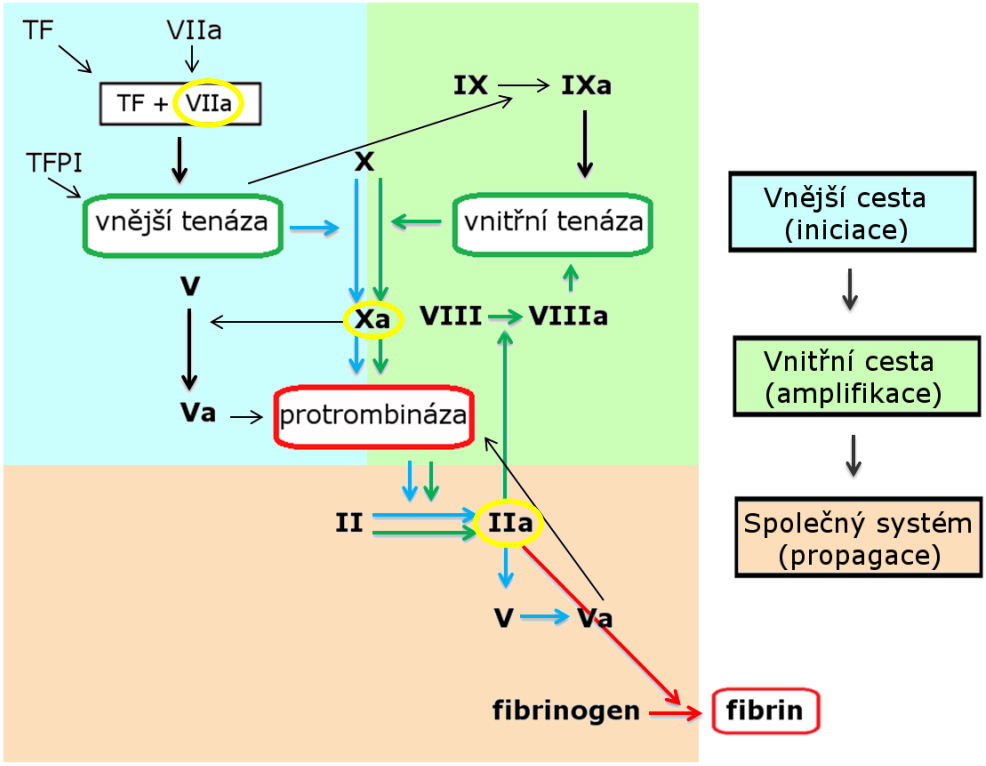
1.6.1.1. **Hemokoagulace**

Koagulační kaskáda je sled enzymatických reakcí, kde jeden faktor je substrátem druhého faktoru a dochází tak k jejich postupné aktivaci. Kaskády se účastní i látky ne-enzymatické povahy jako jsou např. vápenaté ionty. Na konci kaskády vzniká z rozpustného fibrinogenu nerozpustná fibrinová zátka (Macfarlane, 1964). Koagulační faktory jsou vždy označeny římskými číslicemi a jejich aktivované formy mají za číslem malé písmeno „a“ (Tab. 2). Všechny enzymatické koagulační faktory vyjma tkáňového faktoru se nacházejí v plazmě ve své neaktivní formě (proenzym, zymogen) a je zapotřebí jejich aktivace proteolytickým štěpením. Výjimkou je faktor VII, který se v plazmě nachází v malém množství aktivní (Broze *et* Majerus, 1980; Šlechtová, 2007).

Tab. 2 – Koagulační faktory (Trojan, 2003).

číslo	**název**
Faktor I	fibrinogen
Faktor II	protrombin
Faktor III	tkáňový tromboplastin (tkáňový faktor)
Faktor IV	vápenaté ionty (Ca^{2+})
Faktor V	proakcelerin
Faktor VII	prokonvertin
Faktor VIII	antihemofilický faktor
Faktor IX	Christmasův faktor
Faktor X	Stuartův-Powerův faktor
Faktor XI	Plasma Thromboplastin Antecedent (PTA)
Faktor XII	Hagemanův faktor
Faktor XIII	fibrin stabilizující faktor

Koagulační kaskáda může být aktivována dvěma cestami: vnější (iniciační) a vnitřní (amplifikační), které se sbíhají ve společný systém (propagační) (Obr. 7). Vnější cesta koagulace začíná vazbou faktoru VII na tkáňový faktor. Vzniká tak komplex, který spolu s Ca^{2+} a fosfolipidy dává vznik komplexu zvanému vnější tenáza, který aktivuje faktor X. Faktor X spolu s kofaktorem (faktor V), Ca^{2+} a fosfolipidy vytváří komplex zvaný protrombináza, která způsobí přeměnu protrombinu na trombin (Boon, 1993; Trojan, 2003; Pecka, 2004). Tento trombin však slouží k aktivaci vnitřní cesty a není schopen štěpit dostatečné množství fibrinogenu. Vnitřní cestou se nazývá proto, že všechny faktory jsou obsaženy v plazmě. Trombin, který vznikl působením enzymů vnější cesty, aktivuje faktor XI, faktor IX, a kofaktory faktor VIII a faktor V. Faktor IX a kofaktor (faktor VIII) spolu s destičkami a Ca^{2+} vytvářejí komplex tzv. vnitřní tenázu, která aktivuje velké množství faktoru X. Následný komplex protrombinázy je už schopen štěpit dostatečné množství protrombinu na trombin, který aktivuje fibrinogen na fibrin (Pecka, 2004; Šlechtová, 2007). Původní představa o *in vivo* aktivaci vnitřní cesty přes faktor XII byla opuštěna, jednalo se o artefakt ve studiích *in vitro* (Pecka, 2004; Šlechtová, 2007; Schmaier, 2016).



Obr. 7 – Zjednodušené schéma koagulační kaskády. TF - tkáňový faktor, TFPI - inhibitor dráhy tkáňového faktoru, římské číslice - označují koagulační faktory. Žluté kruhy označují koagulační faktory nejčastěji inhibované krevsajícími parazity.

Udržení hemostázy ve vyváženém stavu se účastní řada mechanizmů a důležitou roli zde hrají inhibitory koagulačního a fibrinolytického systému. Ty zde slouží k inhibici proteolytických enzymů hemostázy a brání tak nekontrolovanému srážení krve nebo krvácení v organizmu. Mezi přirozené inhibitory patří např. antitrombin, heparinový kofaktor, systém proteinu C, inhibitor dráhy tkáňového faktoru atd. (Pecka, 2004; Šlechtová, 2007; Palta *et al.*, 2014). Krevsající paraziti využívají některé podobné molekuly ve svých sekretech, aby mohli dokončit krmení. Mezi nejčastěji blokované faktory koagulační kaskády parazitárními molekulami se řadí faktor VIIa, faktor Xa a trombin (faktor IIa), které jsou vyznačeny v Obr. 7 (Francischetti *et al.*, 2002; Hovius *et al.*, 2008; Macedo-Ribeiro *et al.*, 2008; Tsujimoto *et al.*, 2012; Ranasinghe *et al.*, 2015a; Ranasinghe *et al.*, 2015c). Do významných parazitárních antikoagulačních molekul se řadí proteinové rodiny serpinů, anexinů nebo inhibitory Kunitzova typu (Francischetti *et al.*, 2002; Genderen *et al.*, 2008; Huntington, 2013). Inhibitory Kunitzova typu se budu podrobněji zabývat v kapitole 1.7.

1.6.2. **Imunitní systém obratlovců**

Imunitní systém slouží jako obrana organizmu před cizorodými látkami nebo i pozměněnými molekulami vlastního těla (Hořejší *et* Bartůňková, 2005). Organizmus je denně vystaven přibližně milionu potenciálních patogenů kontaktem s kůží, trávením nebo inhalací (Alberts *et al.*, 2002). Imunitu můžeme rozdělit na vrozenou (nespecifickou) a adaptivní (specifickou) (Janeway *et* Medzihov, 2002; Beutler, 2004; Tosi, 2005). Po prvním kontaktu s patogenem se adaptivní imunitní reakce objevuje pomalu. Nejdříve se musí vyvinout antigen-specifická odpověď, která spočívá v produkci protilátek a v aktivaci a rozšíření specifického klonu B/T- buněk. Proto adaptivní imunitní reakci trvá delší dobu (dny až týdny), než bude imunitní odpověď účinná. Zároveň je pro tento druh imunitní odpovědi charakteristická imunologická paměť (Alberts *et al.*, 2002; Beutler 2004; Hořejší *et* Bartůňková, 2005). Oproti tomu vrozená imunita zahrnuje rychlou a evolučně starší nespecifickou hostitelskou odpověď s rychlou akcí během prvních hodin a dní, kdy je organizmus vystaven novému patogenu. Mezi nespecifické odpovědi patří opsonizace, aktivace komplementu, fagocytóza, indukce apoptózy atd. (Alberts *et al.*, 2002; Janeway *et* Medzihov, 2002; Beutler, 2004; Tosi, 2005).

1.6.2.1. **Kaskáda komplementu**

Komplement je esenciální složka vrozeného imunitního systému a zahrnuje okolo 30 solubilních a membránově vázaných proteinů (Holland *et* Lambris, 2002; Beutler, 2004). Mezi jeho hlavní funkce patří opsonizace, chemotaxe a osmotická lýza. Některé složky mohou hrát významnou roli i v jiných procesech jako jsou aktivace B-buněk, transport antigenů do sleziny nebo sloužit jako adhezivní molekuly (Hořejší *et* Bartůňková, 2005). Dále může hrát významnou roli i ve fagocytóze nebo zánětu (Gasque, 2004). Komplement je tedy i důležitý propojující bod mezi vrozenou a adaptivní imunitou (Holland *et* Lambris, 2002; Beutler, 2004). Může být aktivovaný třemi cestami: alternativní, klasickou a lektinovou dráhou Obr. 8. Mezi hlavní složky komplementu se řadí sérové proteiny C1-C9 (Holland *et* Lambris, 2002; Beutler, 2004).

U **alternativní cesty** se samovolně štěpí klíčová složka komplementu C3 na C3b a C3a. U C3b dochází k odhalení thioesterové skupiny, která reaguje s hydroxy- a aminoskupinami v okolí. Pokud se tyto skupiny nacházejí na povrchu např. mikroorganizmu, dochází ke kovalentnímu navázání C3b na jejich povrch a ke spuštění

kaskády. K C3b se připojí faktor B, který je pomocí faktoru D štěpen na Ba a Bb. Vzniklý C3bBb, stabilizován faktorem P (properdin), tvoří tzv. **alternativní C3- konvertázu**. Tím dochází k dalšímu štěpení C3 a vzniklé C3b se váží na povrch kolem komplexu a slouží jako opsoniny nebo vytvářejí další konvertázy. Z některých molekul konvertázy vzniká složitější komplex C3bBbC3b (**alternativní C5 konvertáza**), který štěpí protein C5 na C5a a C5b (Hořejší *et* Bartůňková, 2005).

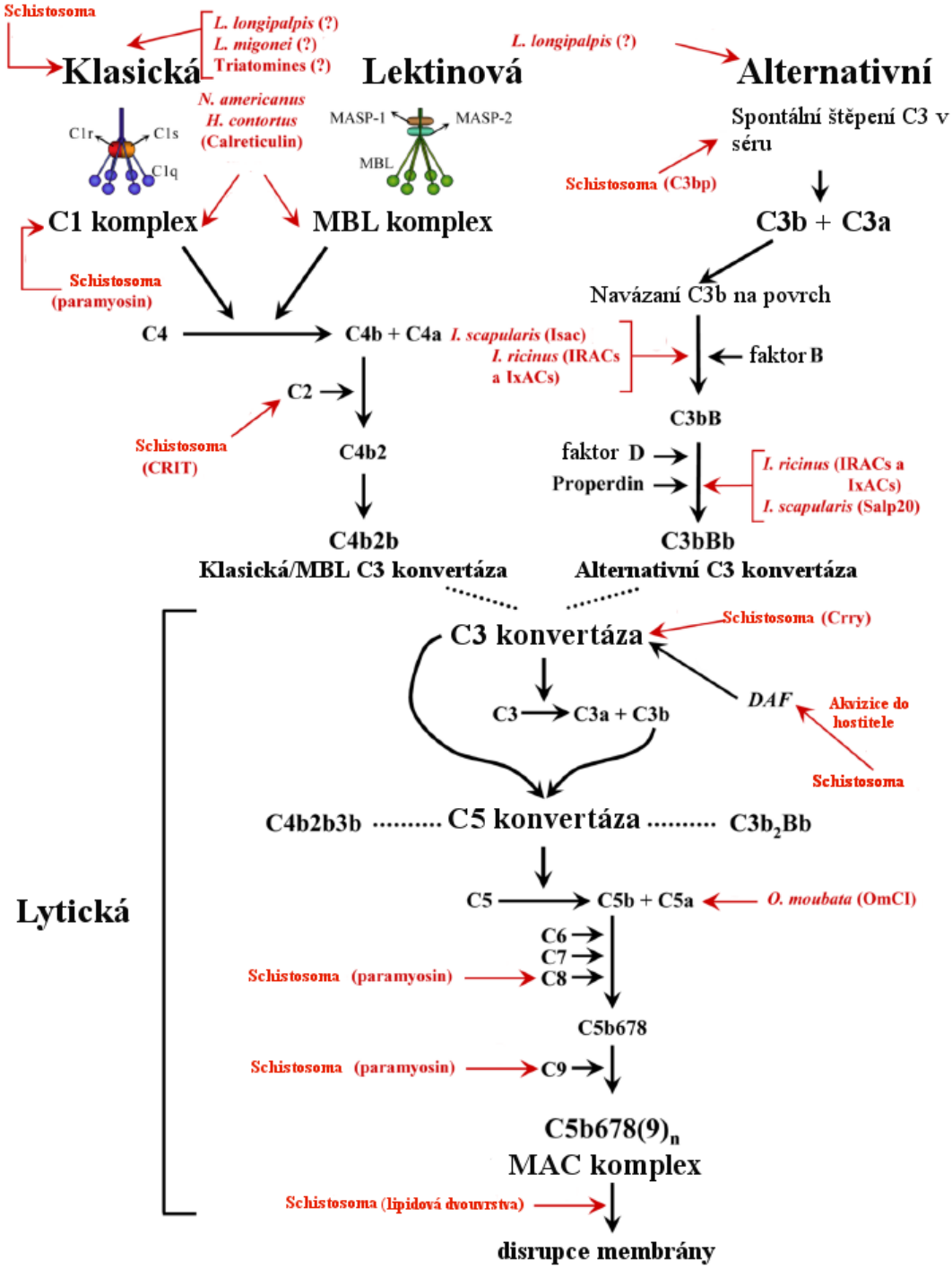
Klasická dráha je aktivována pomocí protilátek, které se váží na antigeny na povrchu mikrobů, kterým byl hostitel vystaven (Beutler, 2004). Dochází ke konformační změně protilátky a odhalení vazebného místa pro C1 protein, který změní svou konformaci a začne štěpit proteiny C4 a C2. Fragmenty C4b a C2a se vážou na povrch mikroorganizmu a tvoří **klasickou C3 konvertázu** (C4bC2a), která štěpí C3 na C3a a C3b. Následně se vytváří další enzym **klasická C5 konvertáza** (C4bC2aC3b), který štěpí C5 (Hořejší *et* Bartůňková, 2005).

Lektinová dráha je podobná klasické dráze, ale nezahrnuje protilátky. Je aktivována pomocí lektinu vázající manózu (MBL), který se váže na sacharidové struktury na povrchu mikrobů (Beutler, 2004). Po navázání lektinů se aktivují tzv. MASP peptidázy, které mají schopnost štěpit C4 a C2 (Boshra *et al.*, 2006). Dále kaskáda probíhá jako u klasické dráhy.

Všechny tři dráhy směřují ke stejnému cíli, a to k **lytické fázi** komplementové kaskády, kde při štěpení C5 vznikne C5b. C5b tvoří spolu s C6, C7, C8 a C9 komplex (membrane attack complex, MAC), který se zanoří do povrchové lipidové membrány buňky, a to vede ke komplementem zprostředkované lýze buňky (Holland *et* Lambris, 2002; Hořejší *et* Bartůňková, 2005; Zhang *et* Cui, 2014). C5a a C3a mají chemotaktické účinky pro fagocyty (Hořejší *et* Bartůňková, 2005).

Proteiny komplementové kaskády u ryb jsou strukturně velmi podobné proteinům u savců, včetně aktivace komplementové kaskády pomocí třech aktivačních drah (Holland *et* Lambris, 2002). Velmi dobře jsou charakterizovány komponenty alternativní a klasické dráhy (Sunyer *et* Lambris, 1998; Boshra *et al.*, 2004), ale velmi málo je známo o molekulách zahrnutých v lektinové dráze (Boshra *et al.*, 2006). Ryby na rozdíl od savců mají větší diverzitu ve složkách komplementu, které jsou přítomné v mnoha izoformách,

jako např. protein C3 (Sunyer *et al.*, 1996, Sunyer *et al.*, 1997, Nakao *et al.*, 2000). Hypotetizuje se, že široká diverzita komplementových složek u ryb může rozpoznat větší množství mikroorganizmů a tím rozšířit schopnosti jejich vrozené imunity (Sunyer *et al.*, 1998). Proto kombinace vyšších titrů a schopnost aktivace tohoto systému ve vysokém rozmezí teplot, spolu s různorodostí některých klíčových komponent, dělá z komplementu velice silný obranný mechanizmus u ryb (Boshra *et al.*, 2006).



Obr. 8 Kaskáda komplementu (Schroeder *et al.*, 2009, upraveno) – Tři cesty aktivace komplementu: klasická dráha, lektinová dráha, alternativní dráha. Červeně jsou vyznačeni paraziti, u kterých je známá schopnost inhibovat kaskádu komplementu. V závorce je uveden název molekuly zodpovědné za inhibici. **?** označuje molekuly, které nebyly identifikovány.

Komplement je pod přísnou kontrolou proteinů, jejichž hlavní funkcí je zabránit jeho aktivaci. Tyto inhibitory se dělí do dvou kategorií: rozpustné regulátory a regulátory membránově vázané a slouží jako přirozená ochrana buněk a tkání před nežádoucí aktivací komplementu (Zipfel *et* Skerka, 2009). Zároveň inhibice komplementu je nezbytná pro přežití všech parazitů v hostitelské tkáni nebo pro usnadnění příjmu krve. Navíc inhibice komplementu u hematofágních parazitů může přispět k přenosu patogenů (Schroeder *et al.*, 2009). Na obrázku 8 jsou uvedená místa, kde kaskádu komplementu nejčastěji blokují parazitární molekuly. Mezi tyto významné molekuly se řadí i Kunitz inhibitory, kde např. TFPI inhibuje MASP-2 v lektinové dráze (Keizer *et al.*, 2015). Podobně to bylo prokázáno u aprotininu ve studiích *in vitro* (Petersen *et al.*, 2000).

1.7. **Inhibitory Kunitzova typu**

Inhibitory Kunitzova typu jsou významné reverzibilní kompetitivní inhibitory nacházející se u mnoha různých druhů organizmů jako jsou zvířata, rostliny a mikroby. Jedná se převážně o inhibitory serinových peptidáz a ve výjimečných případech mohou inhibovat i aspartické peptidázy, cysteinové peptidázy a metalopeptidázy (Oliva *et al.*, 2010; Ranasinghe *et* McManus, 2013; Smith *et al.*, 2016).

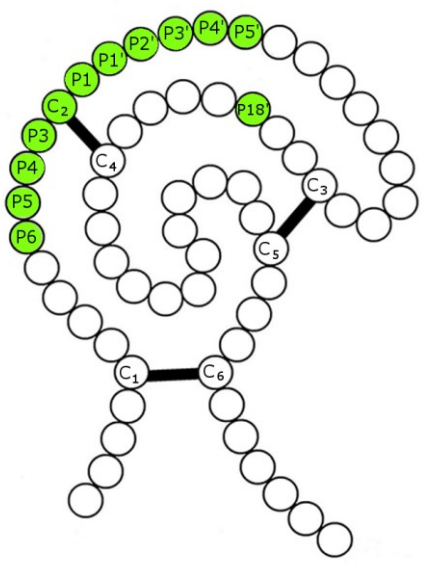
1.7.1. **Struktura**

Kunitz inhibitory jsou většinou malé proteiny o velikosti 6-8 kDa (Obr. 9), některé však mohou obsahovat i více aktivních domén a jejich velikost se může významně lišit. Například Kunitz inhibitor u *Ancylostoma caninum* obsahuje 12 aktivních domén (Hawdon *et al.*, 2003). Aktivní doména má velikost okolo 60 aminokyselinových zbytků a obsahuje šest cysteinů tvořících tři disulfidické můstky (C1-C6, C2-C4 a C3-C5), které jsou odpovědné za stabilitu proteinu (Chand *et al.*, 2004; Ranasinghe *et* McManus, 2013). Nicméně i varianty s dvěma disulfidickými můstky se běžně vyskytují mezi organizmy a nejčastěji chybí disulfidický můstek v místě C2-C4 (Dy *et al.*, 2006).

1.7.2. **Mechanizmus inhibice**

Standardní mechanizmus inhibice Kunitz proteinů je kanonická inhibice, nekovalentní interakce připomínající vazbu enzym – substrát. Kunitz inhibitor se přímo váže do aktivního místa peptidázy bez konformačních změn. Segment vázající se do

aktivního místa peptidázy se nazývá vazebná smyčka a je vysoce komplementární s aktivním místem enzymu (Krowarsch *et al.*, 2003; Ranasinghe *et* McManus, 2013). V centrálním úseku aktivní smyčky (P6-P5´) se nacházejí vysoce konzervovaná rezidua v pozici P6, P1, P5´a P18´ (Obr. 9, Chand *et al.*, 2004; Ranasinghe *et* McManus, 2013). Vazebná místa P1 a P1´ jsou důležitým determinantem peptidázové specifity (Laskowski *et* Kato, 1980; Krowarsch *et al.*, 1999; Ranasinghe *et* McManus, 2013). Typické trypsin-like inhibitory mají Arg/Lys v P1 pozici, zatímco chymotrypsin-like inhibitory mají ve stejné pozici Leu/Met (Grzesiak *et al.*, 2000).



Obr. 9 - Struktura Kunitz inhibitoru (Chand *et al.*, 2004, upraveno)**.** Zeleně vyznačeny aminokyseliny aktivního místa, **C** jsou označeny cysteiny tvořící disulfidické můstky.

U několika Kunitz inhibitorů byl popsán i nekanonický mechanizmus inhibice. Např. ornithodorin u klíšťáka *Ornithodoros moubata* se skládá ze dvou domén a je inhibitorem trombinu. Jeho C – doména se váže do bazické oblasti nazývané „exosite I“ trombinu a N – doména, tak překrývá aktivní místo trombinu. Aktivní doména ornithodorinu tedy vůbec nereaguje s aktivním místem trombinu (van de Locht *et al.*, 1996). Boophilin u klíštěte *Boophilus microplus* je schopen reagovat nekanonickým mechanizmem s trombinem a zároveň kanonickým mechanizmem s jinou peptidázou (Macedo-Ribeiro *et al.*, 2008).

1.7.3. **Funkce**

Inhibitory Kunitzova typu se mohou účastnit regulace mnoha biologických dějů v organizmech jako je např. trávení, koagulace, buněčná proliferace, formování a remodelace tkání, blokování iontových kanálů, fibrinolýza, zánět, cytotoxická aktivita, obrana proti mikrobiální infekci atd. (Shigetomi *et al.*, 2010; Lee *et al.*, 2010; Ranasinghe *et* McManus, 2013), nicméně popsány byly i jako významné bioaktivní molekuly v jedu pavouků, hadů, škorpionů, mořských sasanek atd. (Yuan *et al.*, 2008; Flight *et al.*, 2009; Millers *et al.*, 2009; Peigneur *et al.*, 2011; Zhao *et al.*, 2011). Podle jejich funkce se mohou rozdělit do pěti skupin: tělní trypsinové inhibitory, chymotrypsinové inhibitory v jedu, trypsinové inhibitory v jedu, toxiny s duální funkcí a blokátory K^+ kanálů (Yuan *et al.*, 2008).

1.7.4. **Kunitz inhibitory u parazitických helmintů**

V nedávné době byly popsány Kunitz inhibitory u endoparazitických helmintů jako je např. fasciola, schistosoma, ankylostoma a echinokok (Milstone *et al.*, 2000; Falcón *et al.*, 2014; Ranasinghe *et al.*, 2015a; b; c; Smith *et al.*, 2016; Fló e *et al.*, 2017), které mohou hrát důležitou roli při ochraně před hostitelskými trávicími enzymy, endogenní regulaci parazitárních cysteinových peptidáz, antikoagulační aktivitě a imunomodulační roli (Milstone *et al.*, 2000; Ranasinghe *et al.*, 2015a; c; Smith *et al.*, 2016; Fló *et al.*, 2017). V tabulce 3 jsou uvedeny jednotlivé Kunitz inihibitory u parazitických helmintů.

Tab. 3 – Kunitz inhibitory u parazitických helmintů. Zeleně jsou vyznačeny hlístice, žlutě motolice a modře tasemnice.

organizmus	Jméno	doména	inhibice enzymu	předpokládaná/potvrzená funkce	reference
Ancylostoma ceylanicum	AceKI	1	trypsin, chymotrypsin, elastáza	Ochrana před hostitelskými trávicími enzymy, únik před hostitelskou imunitní odpovědí	Milstone *et al.*, 2000
Ancylostoma caninum	Ac-KPI	12	trypsin, chymotrypsin	Remodelace tkání	Hawdon *et al.* 2003
Schistosoma mansoni	SmKI-1	1	trypsin, chymotrypsin, neutrofilní elastáza, Faktor X a plazmový kallikrein	Antikoagulační a protizánětlivá funkce	Ranasinghe *et al.* 2015a
Schistosoma japonicum	SjKI-1	1	trypsin, chymotrypsin, neutrofilní elastáza, Faktor X a plazmový kallikrein	Antikoagulační a protizánětlivá funkce	Ranasinghe *et al.* 2015c
Fasciola hepatica	Fh-Ktm	1	trypsin, urokináza, t-kallikrein, chymotrypsin, plazmin	Únik před hostitelskou imunitní odpovědí	Bozas *et al.* 1995 Falcón *et al.* 2014
Fasciola hepatica	FhKT1	1	katepsiny L (FhCL1, FhCL2)	Regulace endogenních cysteinových peptidáz	Smith *et al.* 2016
Echinococcus granulosus	EgKI-1	1	trypsin, chymotrypsin, pankreatitická elastáza, neutrofilní elastáza, katepsin G	Ochrana před hostitelskými trávicími enzymy, imunomodulační role	Ranasinghe *et al.* 2015b

organizmus	jméno	doména	inhibice enzymu	předpokládaná/potvrzená funkce	reference
Echinococcus granulosus	EgKI-2	1	trypsin	Ochrana před hostitelskými trávicími enzymy	Ranasinghe *et al.* 2015b
Echinococcus granulosus	EgKU-1	1	elastáza	Únik před hostitelskou imunitní odpovědí, imunomodulační role	Gonzalez *et al.* 2009, Fló *et al.* 2017
Echinococcus granulosus	EgKU-2	1	chymotrypsin	Únik před hostitelskou imunitní odpovědí, imunomodulační role	Gonzalez *et al.* 2009, Fló *et al.* 2017
Echinococcus granulosus	EgKU-3	1	chymotrypsin, elastáza	Únik před hostitelskou imunitní odpovědí, imunomodulační role	Gonzalez *et al.* 2009, Fló *et al.* 2017
Echinococcus granulosus	EgKU-4	1	trypsin	Únik před hostitelskou imunitní odpovědí, imunomodulační role, ochrana před hostitelskými trávicími enzymy	Gonzalez *et al.* 2009, Fló *et al.* 2017
Echinococcus granulosus	EgKU-5	1	trypsin	Únik před hostitelskou imunitní odpovědí, imunomodulační role, ochrana před hostitelskými trávicími enzymy	Gonzalez *et al.* 2009, Fló *et al.* 2017
Echinococcus granulosus	EgKU-6	1	trypsin	Únik před hostitelskou imunitní odpovědí, imunomodulační role, ochrana před hostitelskými trávicími enzymy	Gonzalez *et al.* 2009, Fló *et al.* 2017
Echinococcus granulosus	EgKU-7	1	trypsin	Únik před hostitelskou imunitní odpovědí, imunomodulační role, ochrana před hostitelskými trávicími enzymy	Gonzalez *et al.* 2009, Fló *et al.* 2017
Echinococcus granulosus	EgKU-8	1	trypsin, slabě chymotrypsin	Únik před hostitelskou imunitní odpovědí, imunomodulační role, ochrana před hostitelskými trávicími enzymy	Gonzalez *et al.* 2009, Fló *et al.* 2017

O Kunitz inhibitorech u monogeneí neexistují dosud žádné publikace, ale je zde předpoklad, že by mohly být sekretovány a mohly by hrát významnou roli při příjmu potravy, a to např. antikoagulační nebo imunomodulační aktivitou. Součástí této práce je publikace, která se zabývá prvním Kunitz inhibitorem u monogeneí vůbec.

Cíle práce

Tématem této dizertační práce je studium bioaktivních molekul zapojených v příjmu a zpracovaní krve u monogeneí čeledi Diplozoidae.

1. Stanovení, identifikace a poměry peptidázových aktivit v solubilním proteinovém extraktu a exkrečně sekrečních produktech dospělce *E. nipponicum*.
2. Stanovení inhibičních aktivit v solubilním proteinovém extraktu dospělce *E. nipponicum* vůči serinovým peptidázám z koagulační kaskády (faktor X, trombin) a fibrinolytického systému (plazmin).
3. Hledání transkriptů katepsinů L a Kunitz inhibitorů v transkriptomu dospělce *E. nipponicum*.
4. Příprava rekombinantních proteinů *E. nipponicum* (katepsin L1, L3 a EnKT1).
5. Biochemická a funkční charakterizace rekombinantních katepsinů L1/ L3 a EnKT1.
6. Lokalizace proteinů v dospělci *E. nipponicum* (imunolokalizace / RNA *in situ* hybridizace)

Výsledky

Výsledky této dizertační práce jsou součástí tří publikací, které vyšly v impaktovaných časopisech.

Seznam publikací:

1. Publikace: **Major acid endopeptidases of the blood-feeding monogenean *Eudiplozoon nipponicum* (Heteronchoinea: Diplozoidae).**

Jedličková L., Dvořáková H., Kašný M., Ilgová J., Potěšil D., Zdráhal Z., Mikeš L.

Parasitology. 2016 Apr;143(4):494-506. **IF2016=2,713**

doi: 10.1017/S0031182015001808.

2. Publikace: **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.**

Jedličková L., Dvořáková H., Dvořák J., Kašný M., Ulrychová L., Vorel J., Žárský V., Mikeš L.

Parasite and Vectors. 2018 Mar 6;11(1):142. **IF2018=3,163**

doi: 10.1186/s13071-018-2666-2.

3. Publikace: **A novel Kunitz protein with proposed dual function from *Eudiplozoon nipponicum* (Monogenea) impairs haemostasis and action of complement *in vitro*.**

Jedličková L., Dvořák J., Hrachovinová I., Kašný M., Ulrychová L., Mikeš L.

International Journal for Parasitology. 2019 Apr;49(5):337-346. **IF2017=3,078**

doi: 10.1016/j.ijpara.2018.11.010.

1.8. **Publikace č. 1**

Obecně je známo, že krevsající helminti při trávení krve využívají konzervovanou síť cysteinových a aspartických peptidáz, pouze zastoupení jednotlivých enzymů a místo, kde enzymy působí, se mohou značně lišit (Dalton *et al.*, 2003; Caffrey *et al.*, 2004; Delcroix *et al.*, 2006; Robinson *et al.*, 2008). Tato publikace je pilotní studií, která naznačila zastoupení kyselých peptidáz u dospělého monogenea *E. nipponicum*. Kyselé peptidázy u parazitických helmintů jsou zahrnuty v mnoha důležitých procesech, jako je trávení, invaze do hostitele, migrace hostitelskými tkáněmi, interakce s hostitelským imunitním systémem atd. (Tort *et al.*, 1999; Caffrey *et al.*, 2004; Delcroix *et al.*, 2006; Kašný *et al.*, 2009).

U monogeneí dosud neexistují žádné ucelené informace ohledně jejich proteolytické výbavy. Proto jsme v této práci využili molekulárních a biochemických metod, které prokázaly ve vzorcích *E. nipponicum* dominantní aktivitu cysteinových peptidáz, a to zejména katepsinů L převažující nad aktivitou katepsinů B. Dominantní aktivita katepsinů L byla doplněna aktivitou aspartických peptidáz, zejména katepsinem D. O procesu trávení krve u monogeneí existuje pouze velmi málo informací pocházejících z ultrastrukturálních a histochemických analýz (Smyth *et* Halton, 1983a; b; Konstanzová *et al.*, 2015). Proto jsme testovali, zda vzorky *E. nipponicum* jsou schopny degradovat hemoglobin v přítomnosti inhibitorů cysteinových a aspartických peptidáz. Degradace hemoglobinu byla zcela blokována kombinací těchto inhibitorů a snížena v případě použití specifických inhibitorů pro katepsin L, B a D. Pomocí degenerovaných primerů a PCR se podařilo získat kompletní sekvence dvou katepsinů L *E. nipponicum*. Dominantní aktivita katepsinů L ve vzorcích *E. nipponicum* připomíná podobnost s *Fasciola hepatica*, která ve svém životním cyklu využívá pět katepsinů L (Robinson *et al.*, 2008).

Můj podíl na práci spočíval ve stanovení a identifikaci poměrů aktivity cysteinových peptidáz ve vzorcích solubilního proteinového extraktu a v exkrečně sekrečních produktech *E. nipponicum*, získání sekvencí katepsinů L, porovnání jejich sekvencí, interpretaci dat a podílu na sepisování publikace.

Major acid endopeptidases of the blood-feeding monogenean *Eudiplozoon nipponicum* (Heteronchoinea: Diplozoidae).

Jedličková L., Dvořáková H., Kašný M., Ilgová J., Potěšil D., Zdráhal Z., Mikeš L.

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Major acid endopeptidases of the blood-feeding monogenean *Eudiplozoon nipponicum* (Heteronchoinea: Diplozoidae)

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SUMMARY

In parasitic flatworms, acid endopeptidases are involved in crucial processes, including digestion, invasion, intera immune system, etc. In haematophagous monogeneans, however, no solid information has been available abou these enzymes. Here we aimed to identify major cysteine and aspartic endopeptidase activities in *Eudiplozo* invasive haematophagous parasite of common carp. Employing biochemical, proteomic and molecular tools, we peptidase activities prevailed in soluble protein extracts and excretory/secre-tory products (ESP) of *E. nipponic* 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 cathepsin L type in *E. nipponicum* resembles the situation in, e.g. fasciolid trematodes.

Key words: cysteine peptidase, aspartic peptidase, protease, haematophagous monogenea, cathepsin L, cathepsin B, cathepsin D, fish parasite, common carp.

INTRODUCTION

Monogeneans of the family Diplozoidae are blood-feeding freshwater ectoparasites inhabiting the gills of cyprinid fishes. They can be significantly virulent to their hosts, causing mechanical damage to gill filaments accompanied by a risk of secondary bacterial and mycotic infections or hypochromic microcytic anaemia (Kawatsu, 1978; Buchmann and Brescini, 2006). The alimentary tract of these worms is morphologically adapted to blood uptake, although the mechanisms of blood processing in monogeneans are largely unknown. The foregut is composed of an oral opening with prominent buccal suckers, with an eversible pharynx that leads via an oesophagus into several deadend side caeca (Smyth and Halton, 1983; Valigurová et al. 2011; Konstanzová et al. 2015). Both sections of the gut are morphologically and functionally well differentiated. Numerous gland cells with proposed extracellular digestive function open into the foregut lumen.

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The chemical nature of their secretion is unknown, but it has been expected that they may produce haemolysins or peptidases, thus could be involved in preliminary degradation of complex foodstuff (e.g., blood cells) to smaller components suitable for endocytosis (Smyth and Halton, 1983; Hodová et al. 2010; Valigurová et al. 2011). The basic principles of the digestive process have been described for a few monogenean species and they were based mainly on ultrastructural and histochemical studies (Jennings, 1959; Tinsley, 1973; Halton and Stranock, 1976; Hodová et al. 2010; Valigurová et al. 2011; Konstanzová et al. 2015). According to these records, the terminal phase of digestion occurs inside specialized types of gut cells within the lysosomal cycle (Smyth and Halton, 1983). From this view, the digestion in diplozoids is more similar to haematophagous mites such as ticks (Sonenshine, 1991), than to other blood-feeding platyhelmints with their extracellular digestion (Dalton et al. 2004). The protein part of haemoglobin is broken down into peptides and amino acids (aa); the toxic haem is oxidized to haematin, exocytosed into the gut lumen and regurgitated by the worm through its oral opening (Llewellyn, 1954; Jennings, 1959; Smyth and Halton, 1983; Konstanzová et al. 2015).

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Current knowledge of monogenean peptidases involved in digestion and other processes is poor. So far, only two reports have been published concerning peptidases of marine monogeneans *Neobenedenia* spp. feeding on epidermal tissues and mucus of their hosts. A gene encoding cysteine peptidase cathepsin L in *Neobenedenia melleni* was characterized and cloned (Rao and Yang, 2007) and some activity of serine peptidases was found in homogenates of adult *Neobenedenia girellae* by zymography (Hirazawa et al. 2006). Their functions are unknown.

In general, processing of blood in sanguinivorous helminths and mites relies on an evolutionary con-served network of cysteine and aspartic peptidases (e.g., cathepsins B, L, F, C, legumain and cathepsin D) identified in trematodes – *Schistosoma mansoni*, *Schistosoma japonicum*, *Fasciola hepatica* (Caffrey and Ruppel, 1997; Dalton et al. 2003; Sajid et al. 2003; Caffrey et al. 2004; Delcroix et al. 2006; Robinson et al. 2008), nematodes – *Haemonchus contortus, Ancylostoma caninum, Necator americanus* (Williamson et al. 2003a, 2004), and in ticks – *Ixodes ricinus*, *Boophilus microplus* (Renard et al. 2000; Sojka et al. 2008). We suppose that similar molecular tools for protein hydrolysis are employed inside the digestive tract of monogeneans.

Our study represents the first direct evidence of the presence of acid endopeptidases in a haematophagous monogenean – *Eudiplozoon nipponicum*; these were identified and partially characterized by biochemical, proteomic and molecular methods.

M E T H O D S

Parasites

Living adult worms of *E. nipponicum* were carefully removed from gills of common carps (*Cyprinus carpio*) immediately after slaughter in a commercial facility of Rybářství Třeboň, Plc. The fish originated from localities (ponds) in South Bohemia, Czech Republic.

Sample preparation

Freshly collected worms were repeatedly gently washed in sterilized tap water, placed in Eppendorf tubes, frozen immediately on dry ice and stored at −80 °C. Soluble protein extracts (solPE) were pre-pared by homogenization of worms in 10 mM acetate buffer pH 5 using motor-driven teflon pellet pestle followed by 3 cycles of sonication on ice (10 W, 30 s) and centrifugation at 16 000 g for 20 min. Supernatants were collected. Excretory/secretory products (ESP) were obtained by incubation of washed adult live worms in 10 mM phosphate buffer pH 7.4 or 10 mM acetate buffer pH 6 for 3– 4 h at room temperature (RT) in Eppendorf tubes. The ability of the ectoparasitic worms to survive for several hours in selected low osmolarity buffers was verified by previous overnight incubation. Full mobility and no obvious damage of the worms were observed. ESP were concentrated on Amicon Ultra filters (MWCO 10 kDa; Millipore). Protein concentration in all samples was measured by Quant-iT Protein Assay Kit (Invitrogen). The samples were stored at −80 °C.

Living adult worms were washed several times in sterile tap water and then placed in TRIzol® Reagent (Invitrogen) for stabilization of RNA and processed or stored at −80 °C. After homogenization, total RNA was extracted following the TRIzol protocol and concentration measured in NanoDrop 1000 (Thermo Scientific). First-strand cDNA synthesis was carried out with 0.3 µg of total RNA using oligo-dT18 primer from SuperScript™ III First-Strand Synthesis kit (Invitrogen).

Peptidolytic activities, pH optima and inhibition assays

Enzyme activities in solPE and ESP were measured with a set of synthetic fluorogenic peptide substrates (Bachem): Z-Phe-Arg-AMC (FR) was used for de-tection of papain-like cysteine peptidases (acidic pH) and trypsin-like serine peptidases (neutral to basic pH), Z-Arg-Arg-AMC (RR) selectively for cathepsin B and Z-Ala-Ala-Asn-AMC (AAN) for legumain (asparaginyl endopeptidase). Abz–Lys–Pro–Ala–Glu–Phe–Nph–Arg–Leu (KPAEFnFRL), the specific substrate for cathepsin D, was kindly provided by Dr. Martin Horn, Institute of Organic Chemistry and Biochemistry, AS CR Prague (Abz = 2-aminobenzoyl; Nph and nF = nitropheny-lalanine). All assays were performed in black 96-well flat bottom plates (Nunc, Thermo Scientific). Samples (1 and 5 µg of protein per well of solPE and ESP, respectively) were pre-incubated (5 min, RT) in various buffers of pH in the range 2–10 for substrates FR, RR, AAN and pH 2–6 for KPAEFnFRL (0.1 M phosphate buffer pH 2; 0.1 M citrate pH 3; 0.1 M citrate/0.2 M phosphate pH 4–7; 0.2 M Tris–HCl pH 8; 0.2 M glycine/NaOH pH 9–10). Final volume was 100 µL. All buffers contained 2 mM dithiothreitol (DTT, Sigma-Aldrich), except for KPAEFnFRL substrate. The reactions were started by addition of particular peptidyl substrate (final concentration 50 µM) in 100 µL of the same buffer. Kinetics of the release of free fluorophors was detected by Infinite M200 flurometer (TECAN) at 28 °C for 60 min in 1 min intervals. Excitation/emission wavelengths for AMC substrates and nF substrate were 355/460 nm and 330/ 410 nm, respectively. Controls contained equal volume of appropriate buffer instead of the sample. Measurements were performed in triplicates. The quantification of cysteine and aspartic peptidase ac-tivities was performed in particular pH optima with FR and KPAEFnFRL substrates, respectively.

Table 1. Sequences of primers used for amplification of *E. nipponicum* cathepsin L genes.

Primer name	Primer sequence
cDNA fragment PCR with degenerate primers	
Cys forward primer[a]	5′-CAA/GGGNCARTGYGGITCNTGC/TTGG-3′
Asn reverse primer[b]	5′-CCANSA/TRTTYTTIACRATCCAA/GTA-3′
RACE–PCR with specific primers	
M-CL1-RACE5′	5′-CCG ACT TGG ATC ACG CCG TAT TGT TGG T-3′
M-CL1-RACE3′	5′-GAC CTT CCA ATG ACC CTG TCG TGG AGA A-3′
M-CL3-RACE5′	5′-GTG CTG CCC CTC TAG TGA TCC TGT TGT-3′
M-CL3-RACE3′	5′-CCA TGG TGT GCT GGT TGT AGG CTA TGG A-3′
GeneRacer™Forw	5′-CGA CTG GAG CAC GAG GAC ACT GA-3′
GeneRacer™Rev	5′-GCT GTC AAC GAT ACG CTA CGT AAC G-3′

[a] TGC nucleotide triplet encoding cysteine.

[b] RTT nucleotide triplet encoding asparagine.

Results were expressed as nM of substrate cleaved per minute in the presence of 1 µg of solPE total protein. Peptidase inhibitors (50 µM final concentration, Sigma-Aldrich) were used for inhibition of particular peptidase activities: E-64 (L-trans-epoxysuccinyl–leucylamido [4-guanidino] butane), an irreversible inhibitor of papain-like cysteine peptidases; CA-074 (N-(L-3-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline]), an irreversible inhibitor of cathepsin B; iCL (Arg–Lys–Leu–Leu–Trp–NH_2), a reversible inhibitor of cathepsin L; pepstatin A (isovaleryl–Val–Val–Sta–Ala–(3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid), inhibitor of aspartic peptidases. Inhibitors were mixed with samples prior to the addition of substrates and incubated for 15 min. Inhibition tests were performed in pH optimum of peptidase activity for each particular sub-strate. All measurements were performed in triplicates, repeated at least three times. Values in graphs are expressed as means with standard deviations.

Degradation of haemoglobin

Bovine haemoglobin (10 µg, Sigma Aldrich) diluted in 20 µL of particular buffers in the range of pH 3-6 (see above) containing 2 mM DTT was incubated with 3 µg of ESP or 1.5 µg of solPE proteins for 16 h at 30 °C. Control reactions contained only haemoglobin. The contribution of individual aspartic or cysteine peptidase activities to degradation of haemoglobin was evaluated by inhibition assays at pH 3 and pH 5. Pepstatin, E-64, CA-074 and iCL were used at concentrations of 10 µM. Resulting hydrolysates were mixed with reducing electrophoretic sample buffer and separated by SDS–PAGE in 4–15% gradient precast gels (Bio-Rad) which were stained with Coomassie Brilliant Blue R-250 (CBB) and scanned on GS-800 Calibrated Densitometer (Bio-Rad).

Active site-labelling of cysteine peptidases

Fluorescent Green BODIPY-DCG-04 (Greenbaum et al. 2002), an analogue of E-64 inhibitor which binds irreversibly to the active site of papain-like peptidases, was incubated with ESP (15 µg of total protein) for 1 h (20 µM DCG-04, 5 mM DTT, 5 mM $MgCl_2$) at RT in the dark. Controls were incubated for 30 min with cysteine peptidase inhibitors (10 µM) E-64, iCL or CA-074 prior to addition of the probe. SDS–PAGE of labelled samples was performed as described above. Fluorescent signal was recorded on a fluorescence scanner (Molecular Imager FX, Bio-Rad) using excitation/emission wavelengths 488/530 nm. Finally, the gels were stained by Silver Stain Plus™ Kit (Bio-Rad).

Amplification of partial DNA sequences of cysteine peptidases

cDNA obtained by reverse transcription from adult *E. nipponicum* was amplified by PCR using PPP Master Mix (Top-Bio) and degenerate primers (Table 1) designed according to the consensus sequences corresponding to the coding sequences of cysteine peptidases of several parasitic species (especially according to the active site with a cysteine residue – Cys forward primer and with an asparagine residue – Asn reverse primer) (Eakin et al. 1990; Heussler and Dobbelaere, 1996; Li et al. 2006). The PCR protocol was as follows: one cycle 5 min initial denaturation at 94 °C, then 35 cycles of denaturation at 94 °C for 1 min, primer annealing 45 °C for 1 min, extension at 72 °C for 1 min and finally one cycle 10 min final extension at 72 °C. Control reactions were performed using the same primers and cDNA obtained from gills of a noninfected carp. The amplified gene fragments of the expected length (ca. 500 bp) were electrophoresed and isolated from agarose gel (1·5%) with Gel Extraction Kit (Qiagen), subcloned into pCR2·1-TOPO cloning vector (Invitrogen) and transformed into TOP10 E. coli (Invitrogen). pCR2.1-TOPO constructs were isolated using Qiaprep Purification Kit (Qiagen) and sequenced with M13 forward and reverse primers (DNA Sequencing Laboratory, Faculty of Science, Charles University in Prague).

Obtained partial DNA sequences were compared with NCBI database using BLASTX (http://blast.ncbi.nlm.nih.gov/).

Rapid amplification of cDNA ends (RACE–PCR)

In order to amplify the 5′/3′ ends and to obtain the entire gene sequences, two pairs of gene-specific primers (M-CL1-RACE5′ + M-CL1-RACE3′, M-CL3-RACE5′ + M-CL3-RACE3′; see Table 1) were designed according to the obtained ca 500 bp sequence fragments (see above). First-strand cDNA for RACE prepared according to manufacturer′s instructions of GeneRacer™Kit (Invitrogen) was used as a template. The PCR reaction (25 μL) contained 12.5 μL of the EmeraldAmp PCR master mix (Clontech), 1 μL of the RACE cDNA (100 ng μL^{-1}), 1 μL of the M-CL1/3-RACE5′/3′ (10 μM), 3 μL of the GeneRacer primers Fwd/Rev (10 μM), 7.5 μL of ddH_20. The profile of amplification of 5′/3′ ends was: 1× 94 °C = 120 s, 5 × (94 °C = 30 s + 72 °C = 60 s), 5 × (94 °C = 30 s + 70 °C = 60 s), 35 × (94 °C = 30 s + 60 °C = 90 s + 70 °C = 90 s) and finally 1 × 72 °C = 10 min. PCR products from 5′ and 3′ RACE were cloned into pCR2.1-TOPO cloning vector as described above and submitted for sequencing with M13 forward and reverse primers (DNA Sequencing Laboratory, Faculty of Science, Charles University in Prague).

Sequence analysis

The obtained full-length cDNA sequences were blasted against sequences available in the GenBank database (NCBI) by BLASTX (http://blast.ncbi.nlm. nih.gov/). The presence of a signal sequence was predicted by the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The theoretical position of a pro-region was determined by multiple sequence alignment of cathepsins L from other organisms: human cathepsin L [GenBank: NP_001903·1], cathepsin L of *Clonorchis sinensis* [GenBank: ABK91809·1], cathepsin L of *C. carpio* [GenBank: BAD08618·1] and cathepsin L of *Neobenedenia melleni* [GenBank: ABK62794·1]. Multiple sequence alignments of aa sequences were carried out using the program Clustal Omega (http:// www.ebi.ac.uk/Tools/msa/clustalo/). Molecular mass and theoretical pI of the deduced proteins were deter-mined by the Compute pI/Mw software available at the Expasy site (http://www.expasy.ch/tools/pitool.html). Detection of potential N-glycosylation sites was performed using an online tool at NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc).

Protein identification from 1D gel bands by LC–MS/MS

ESP were separated by SDS–PAGE as described above and stained by CBB. Bands (range 25–40 kDa) were manually excised, destained and incubated each with trypsin (Promega). LC–MS/MS analyses were done using RSLCnano system (Thermo Fisher Scientific) on-line connected to Impact II Q-TOF mass spectrometer with CaptiveSpray nanoBooster ion source (Bruker). Prior to LC separation, tryptic digests were online concentrated and desalted using trapping column (100 μm × 30 mm) filled with 3.5 μm X-Bridge BEH 130 C18 sorbent (Waters). After washing of trapping column (0.1% FA), the peptides were separated (300 nl/min) using Acclaim Pepmap100 C18 column (2 μm particles, 75 μm × 500 mm; Thermo Fisher Scientific) by the 0.1% FA in water: 0.1% FA in 80% ACN gradient program.
MS data were acquired in a data-dependent strategy with 3 s long cycle time. Mass range was set to 150–2200 m/z and precursors were selected from 300 to 2000 m/z. Acquisition speed of MS and MS/MS scans was 2 and 4–16 Hz, respectively. Speed of MS/MS spectra acquisition was based on precursor intensity (low and high absolute thresholds were 10 000 and 100 000 cts, respectively). Mascot (version 2.4.1) MS/MS ion searches were done against in-house database with sequences of expected recombinant proteins. Search results obtained against in-house database were checked for false positive identifications using database search against the whole SwissProt database (version 2014_07). Mass tolerances for peptides and MS/MS fragments were 15 ppm and 0.05 Da, respectively. Oxidation of methionine, deamidation (N, Q) and propionamide (C) as variable modifications and three enzyme miss cleavages were set for all searches.

RESULTS

Activity profiling

The results of fluorometric analyses of peptidolytic activities in solPE and in ESP, pH optima and inhibition tests are shown in Fig. 1. The highest activity in both sample types was attributed to papain-like cysteine peptidases in acid buffers of pH 3–5 (optimum pH 5) with FR substrate (Fig. 1A and B). It dropped down to only ca. 20% in pH 6 and vanished above pH 7. Inhibitors E-64 and iCL suppressed the activity to zero and CA-074 by ca. 25 and 80% in the case of solPE and ESP, respectively (Fig. 1a and b).

With cathepsin B-selective substrate RR, the activity in solPE peaked in pH 5 and it was negligible in pH 6 and above (Fig. 1C). However, in ESP the highest activity appeared in pH 6, dropped to ca. 50% in pH 7 (Fig. 1D). In the case of both sample types, E-64 and iCL inhibited the peptidolytic activities nearly to zero. CA-074 reduced the activities in ESP by ca. 75% and by ca. 95% in solPE (Fig. 1c and d).
The activity of aspartic cathepsin D with was found in the range of pH 2–4 (optimum in pH 3) in solPE of worms in the presence of KPAEFnFRL

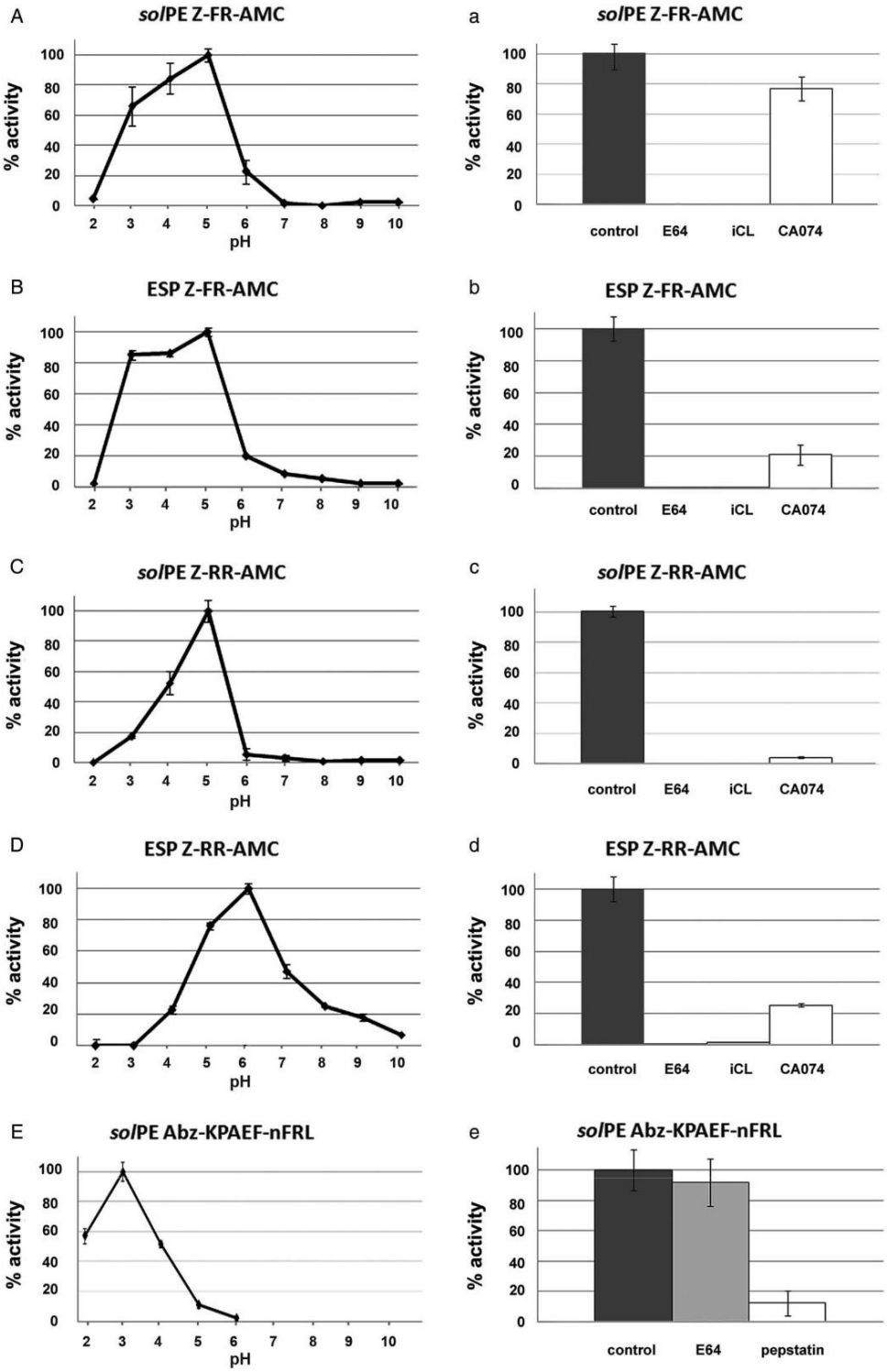

Fig. 1. The pH profiles and inhibition assays of acid endopeptidase activities. The pH optima of peptidolytic activities in soluble protein extracts (solPE) and E/S products (ESP) of *E. nipponicum* and their inhibition at corresponding pH optimum were measured with a spectrum of fluorogenic substrates: (A, a, B, b) Z-FR-AMC; (C, c, D, d) Z-RR-AMC; (E, e) KPAEF-nFRL and with selective peptidase inhibitors (E-64, CA-074, iCL, pepstatin A). The values are expressed as percentage of maximum activity in the sample (100% at pH optimum).

(Fig. 1E); this was inhibited by ca. 90% by pepstatin A (Fig. 1e). In ESP, the activity of cathepsin D was recorded occasionally in some samples (not shown). The ratio of cathepsin L + B to cathepsin D activity in solPE was approx. 4:3 (0.45 vs 0.33 nM min^{-1} μg^{-1} of protein). No activity of cysteine asparaginyl endopeptidase (legumain) was detected with AAN substrate in any sample (not shown). There was no difference in activity profiles between the samples of ESP collected at pH 7.4 and pH 6 (not shown). Therefore, a pooled mixture of samples was used for the experiments.

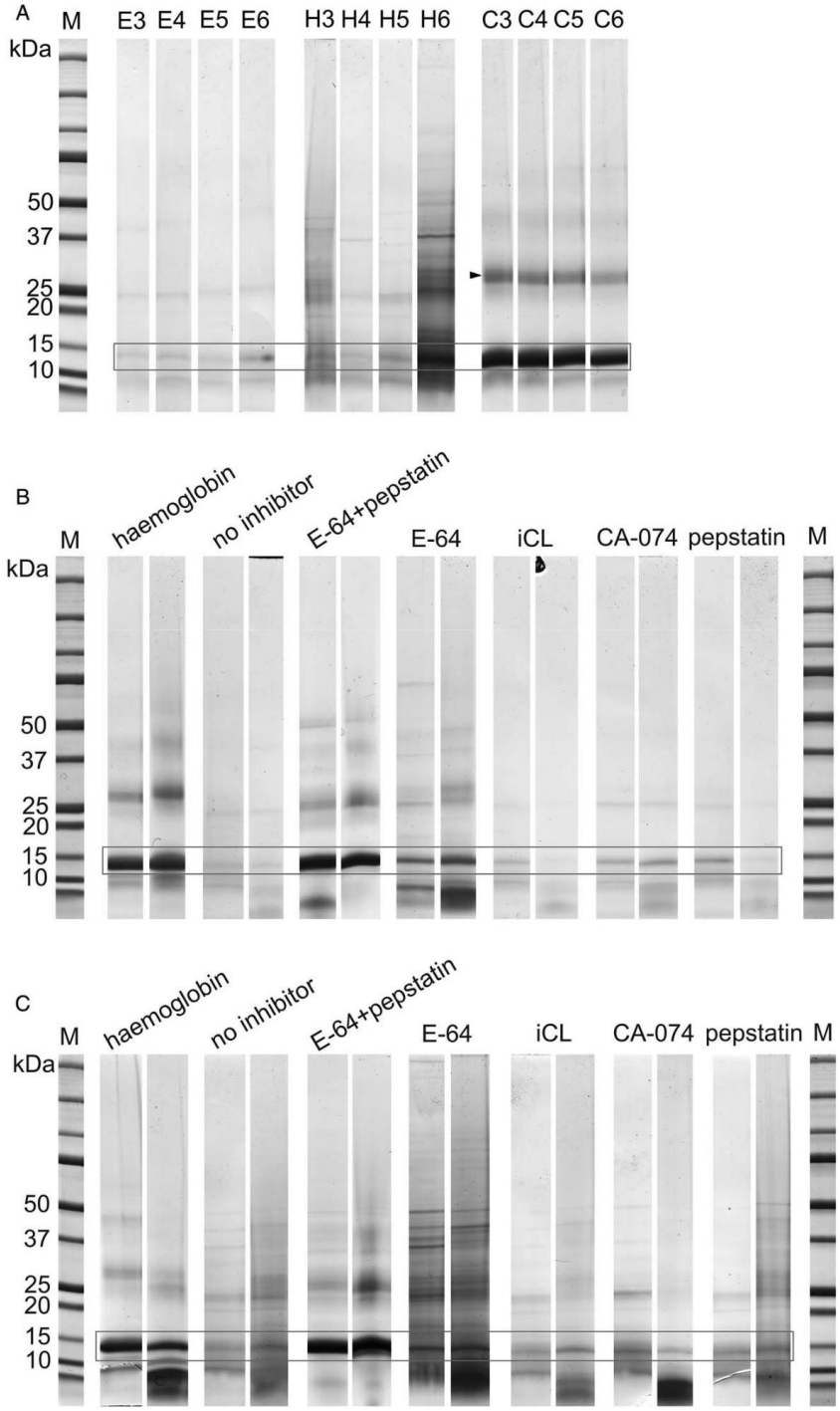

Fig. 2. The pH profile and inhibition of haemoglobin degradation by ESP and solPE. Electrophoretograms of bovine haemoglobin (Hb) after overnight incubation with E/S products and soluble protein extracts of *E. nipponicum*. (A) Degradation in the presence of ESP (lanes E3-E6) and solPE (H3-H6) at pH values 3-6. Controls of Hb in particular pH (C3-C6) contained neither ESP nor solPE. (B) Inhibition of haemoglobinolysis in the presence of ESP in pH 5 and pH 3.
(C) Inhibition of heamoglobinolysis in the presence of solPE in pH 5 and pH 3. Headings above each pair of lanes (left lane = pH 5, right lane = pH 3) indicate the type of sample: haemoglobin = controls of Hb without ESP or solPE; no inhibitor = controls of Hb with ESP or solPE; other samples contained inhibitors as labelled. M = markers of molecular size. Bands of Hb monomers are boxed in grey. Arrowhead points to alpha/beta heterodimers of Hb.

Degradation of haemoglobin

ESP as well as solPE efficiently degraded bovine haemoglobin during overnight (16 h) incubation within the pH range of 3–5 and less at pH 6 (Fig. 2A). No substantial degradation occurred above pH 7 (not shown). At pH 5 and 3, haemoglobinolytic activity was markedly (but not completely) inhibited by the general inhibitor of papain-like cysteine peptidases E-64. Lower level of inhibition

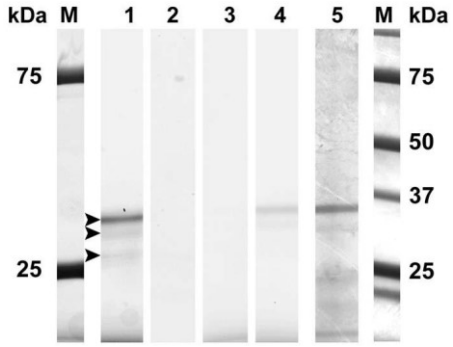

Fig. 3. Active site-labelling of papain-like peptidases in ESP by DCG-04 affinity probe. Electrophoretogram of ESP incubated with fluorescent DCG-04 and inhibitors of particular cysteine peptidases; signal (lanes 1–4) was recorded on a fluorescence scanner, excitation/emission wavelengths 488/530 nm. Lane 1 - control without any inhibitor; lane 2 - E-64; lane 3 - iCL; lane 4 - CA-074; lane 5 - silver staining of the gel. Arrowheads point to the three protein bands labelled by the probe. M = markers of molecular size.

was observed with CA-074, iCL and pepstatin alone; the latter two were less effective at pH 3 in the case of ESP. Complete inhibition at both pH values was reached only when using a mixture of E-64 and pepstatin (Fig. 2B and C). No marked differences in inhibition profiles were observed between the samples containing ESP and solPE.

Active site-labelling of cysteine peptidases

Using the irreversible affinity probe DCG-04, the presence of papain-like cysteine peptidases was demonstrated in the samples of ESP. After SDS–PAGE, three protein bands of molecular sizes between 25 and 35 kDa were demonstrated in the gels. The interaction of DCG-04 with the active sites of cysteine peptidases was efficiently inhibited by the general inhibitor of cysteine peptidases (E-64) and by the inhibitor of cathepsin L (iCL), and only partially by the inhibitor of cathepsin B (CA-074) (Fig. 3).

Amplification of *E. nipponicum* cathepsin L genes

Two amplified DNA fragments (ca. 500 bp) obtained by PCR using *E. nipponicum* cDNA as a template and primers shown in Table 1 were identified as partial sequences of cathepsin L-like cysteine peptidases. In control PCR reactions with DNA template from a non-infected carp and with the same set of primers, a sequence encoding cathepsin L was also amplified. This showed 94.8% sequence identity with cathepsin L gene from *C. carpio* [GenBank: BAD08618·1] and only 55.19 and 49.35% with the fragments amplified from cDNA of *E. nipponicum*.

Applying RACE–PCR on the basis of fragments mentioned above, whole sequences of two *E. nipponicum* cathepsin L genes were obtained, termed EnCL1 and EnCL3. Their nucleotide sequences have been deposited in the NCBI GenBank database under accession numbers [GenBank: KP793605] and [KP793606]. The EnCL1 gene has an open reading frame (ORF) of 954 bp encoding a proenzyme consisting of 317 aa (Fig. 4). Deduced propeptide region contains 93 aa and the catalytic domain (mature enzyme) 224 aa residues. No signal leader sequence has been identified. The calculated theoretical molecular weights of the zymogen/mature protein are 35/24.4 kDa and pI 5.86/6.08. EnCL1 exhibits the highest similarity (53%) to cathepsin L from the Chinese liver fluke *Clonorchis sinensis* [GenBank: ABK91809·1]. The second obtained cathepsin L sequence from *E. nipponicum* was termed EnCL3, as it has the greatest similarity to cathepsin L3 precursor of *Schistosoma mansoni* [GenBank: ABV71063·1]. The ORF of EnCL3 is made up of 1107 bp and encodes a pre-proenzyme of 368 aa (Fig. 4). The sequence is composed of a signal leader sequence (24 aa), an unusually long propeptide (120 aa) and a mature (catalytic) domain (224 aa). Expected Mr/pI values of the zymogen and mature enzyme are 38.0/ 4.8 and 24.1/4.15, respectively.

The conserved ‘ERFNIN’ motif typically present in prosequences of various papain-like cysteine peptidases, e.g. cathepsins L, K, S, but not in cathepsin B (Karrer et al. 1993), was found in EnCL3; this was slightly modified in the case of EnCL1 (ERFNVN). Another sequence motif, ‘GNFD’, that may be involved in intracellular trafficking and intramolecular processing of some papain-like cysteine peptidases (Vernet et al. 1995; Dvořák et al. 2009), is modified in the prosequences of EnCL1 and EnCL3 to ‘ANLD’ and ‘TNFD’, respectively. The catalytic domains of both enzymes include the typical catalytic triad (EnCL1/3 numbering: Cys_{118}, His_{264}, N_{284}/Cys_{169}, His_{314}, N_{335}) of papain-like cysteine peptidases and the relatively conserved flanking sequences around the catalytic residues (QGQCGSCWAFS, LD HA/ GVL, YWIVKNS/TW) (Turk et al. 2000) (Fig. 4). No potential N-linked glycosylation sites have been found throughout the sequences of both proenzymes.

The sequence identity between EnCL1 and EnCL3 is 55.21% on the aa level. Identities among *E. nipponicum* cathepsins L and selected aa sequences of cathepsins L from other organisms are summarized in Table 2. EnCL1 shares the highest number of identical aa positions (52.58%) with cathepsin L of *C. sinensis* [GenBank: ABK91809.1] and EnCL3 with cathepsin L3 of *S. mansoni* [GenBank: ABV71063.1] (52.54%). Identities of EnCL1 and EnCL3 with the only published whole sequence of cathepsin L from a monogenean, *N. melleni*, [GenBank: ABK62794.1] are only 44.01 and 41.27%, respectively.

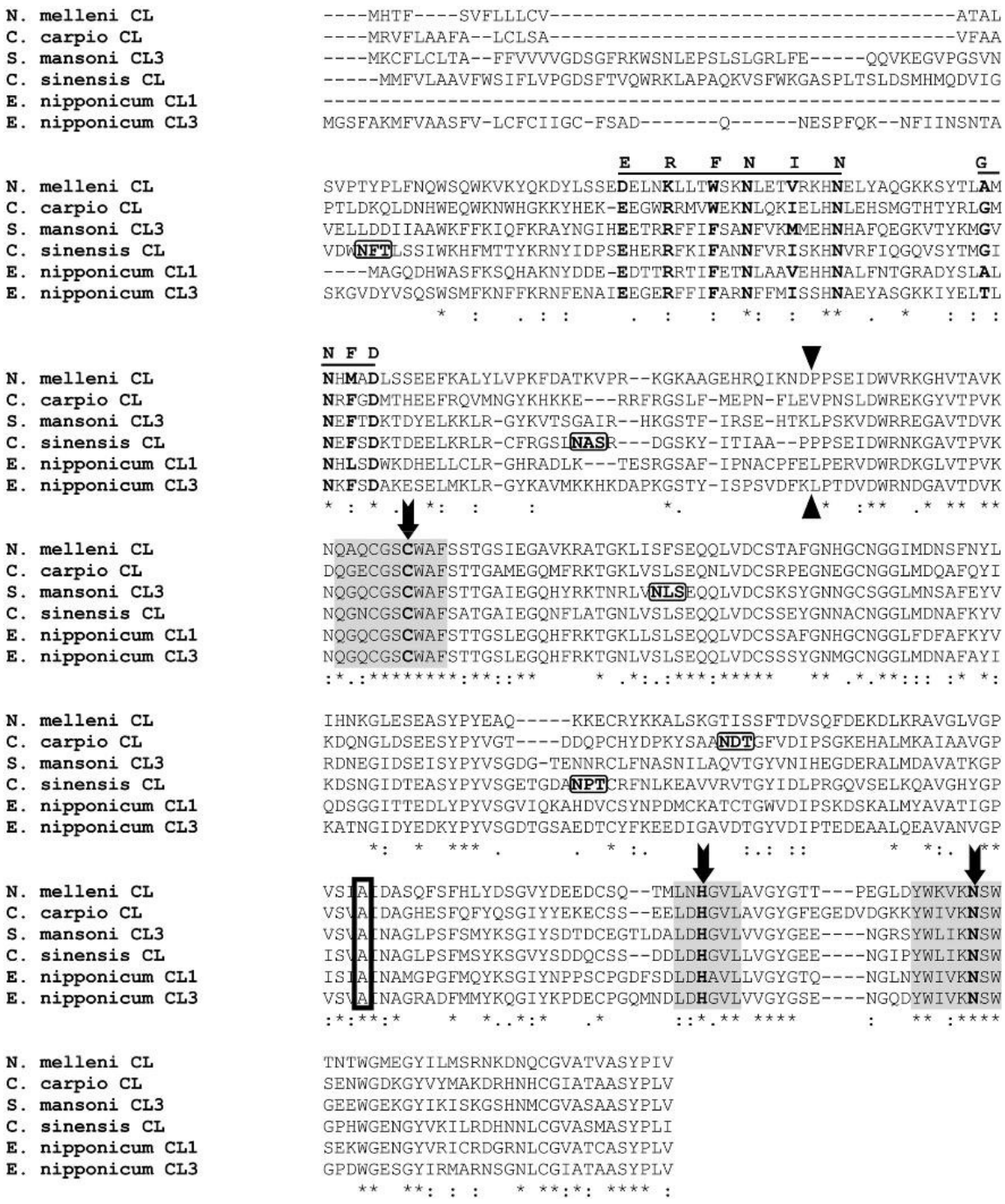

Fig. 4. Multiple sequence alignment of *Eudiplozoon nipponicum* cathepsins L with orthologs from other organisms. *Neobenedenia melleni* [GenBank:ABK62794.1]; *Cyprinus carpio* [GenBank:BAD08618.1]; *Schistosoma mansoni* CL3 [GenBank:ABV71063.1]; *Clonorchis sinensis* [GenBank:ABK91809.1]; *Eudiplozoon nipponicum* CL1 [GenBank: KP793605]; *E. nipponicum* CL3 [GenBank:KP793606]. The position of pro-region cleavage site is marked by arrowheads. ERFNIN- and GNFD-like motifs are in bold and indicated by underlined headings. Catalytic triad of the active site (C, H, N) is marked by arrows. Conserved motifs around active site residues are shaded in light grey. Tripeptides of potential N-glycosylation sites are boxed. Amino residue (A) at the base of the S2 pocket is boxed in black.

Mass spectrometry analysis of ESP of *E. nipponicum*

Both cathepsins L1 and L3, the genes of which have been amplified, were confirmed in ESP of adult *E. nipponicum* by mass spectrometry. Fifteen peptide sequences from trypsin-digested protein bands matched to the sequence of EnCL1 [GenBank: KP793605] and three peptides matched to EnCL3 [GenBank: KP793606] (Fig. 5). EnCL1

Table 2. Amino acid identities between cathepsins L of *E. nipponicum* and orthologues from other organisms.

	EnCL1		EnCL3
EnCL1	100		55·21
EnCL3		55·21	100
CsCL		52·58	51·12
SmCL3		50·80	52·54
CcCL		50·32	50·30
NmCL		44·01	41·27

Identities expressed in [%]. EnCL1, *Eudiplozoon nipponicum* [GenBank:KP793605]; EnCL3, *E. nipponicum* [GenBank:KP793606]; CsCL, *Clonorchis sinensis* [GenBank:ABK91809·1]; SmCL3, *Schistosoma mansoni* [GenBank:ABV71063·1]; CcCL, *Cyprinus carpio* [GenBank:BAD08618·1]; NmCL, *Neobenedenia melleni* [GenBank:ABK62794·1].

Fig. 5. Identification of cathepsins L1 and L3 in ESP of *E. nipponicum* by mass spectrometry. Each grey line corresponds to a single identified peptide; the sequences of some peptides partially overlap with others. (A) Fifteen tryptic peptides identified in ESP matched to the sequence of cathepsin L1 [GenBank: KP793605]. (B) Three peptides matched to the sequence of cathepsin L3 [GenBank: KP793606].

peptides were identified in nine of ten bands between ca. 24–35 kDa; EnCL3-derived peptides were recorded only above 35 kDa.

DISCUSSION

In contrast to the comprehensive knowledge concerning peptidases from various groups of blood-feeding parasites, only very limited information is available about proteolytic enzymes of haematophagous monogeneans. These neglected helminths in terms of biochemical studies have been thought to digest haemoglobin and other proteins intracellularly in the acidic lysosomes/phagolysosomes within the specialized types of gut cells, unlike other blood-feeding helmints such as schistosomes (Dalton et al. 2004), but similarly to ticks (Smyth and Halton, 1983; Sonenshine, 1991). However, it is still not clear whether the first phase of digestion in monogeneans may take place in the extracellular space of the lumen of the gut, with the participation of secreted proteases.

We aimed to identify and partially characterize dominant cysteine and aspartic endopeptidases which can be potentially involved in blood digestion or other processes in the adults of *E. nipponicum*. solPE of worms and ESP were compared in terms of peptidolytic activities. We expected that ESP may be a potential source of peptidases released into the gut lumen (either from gut epithelium or accessory glands that are opened into the front part of the digestive system), and from there excreted outside the worm by continuous regurgitation of the rests of digestion (Smyth and Halton, 1983; Hodová et al. 2010; Valigurová et al. 2011). However, the presence of peptidases originated in other tissues of the worm cannot be excluded, especially in the case of solPE from whole worms.

Activity profiling revealed that the major peptidase activity can be attributed to the cysteine class, clan CA, family C1 (MEROPS classification). This activity tested with Z-FR-AMC as a substrate at pH 5 was fully inhibited by E-64 (general inhibitor of papain-like cysteine peptidases) and the iCL, but only partially by an inhibitor specific for cathepsin B (CA-074). It is known, that in a reducing environment (presence of DTT), cathepsin L can also be inhibited by CA-074 (Steverding, 2011; Steverding et al. 2012). Besides, although iCL (the pentapeptide RKLLW) has been stated as a highly potent iCL (Brinker et al. 2000), it was able to diminish significantly the activity of recombinant cathepsin B2 of *Trichobilharzia regenti* in an unrelated previous experiment (Jedličková, unpublished). With the substrate Z-RR-AMC, which is often con-sidered as specific for cathepsin B, the relatively high peptidase activity in ESP was also fully inhibited by E-64 and iCL, but only partially by CA-074 (at pH 6). This brought some confusion on the interpretation of the relative proportion of cathepsin L/B activities.

Cathepsin B- and cathepsin L-like peptidases have been usually distinguished by their ability to degrade the substrate with an arginine in P2 position. Human cathepsin B is able to hydrolyse the substrate, whereas cathepsin L is not – a situation which is not always applicable to orthologous enzymes in other organisms (Sajid and McKerrow, 2002; Choe et al. 2006). The substrate specificity of cysteine cathepsins is thought to be determined by interactions in the S2 pocket, particularly the

glutamic acid residue in cathepsin B (Glu_{205} in human CB) and the equivalent alanine residue in cathepsin L (Ala_{205} in human CL) localized at the bottom of the S_2 pocket. The Glu residue can accommodate and stabilize the polar guanidino group of Arg in the substrate, but Ala in this position cannot bind to Arg (Sajid and McKerrow, 2002). This was confirmed for mammalian cathepsins L/B and for peptidases of numerous organisms (including parasites). However, many other parasite peptidases do not possess 'mammalian' residues in this position. For example, the ability to cleave Z-RR-AMC was described for cathepsin L-like peptidase 'cruzain' from *Trypanosoma cruzi* (Gillmor et al. 1997) and cathepsin L of *Entamoeba histolytica* (Brinen et al. 2000). On the other hand, cathepsin B from *Leishmania major* (Chan et al. 1999) and cathepsin B1·4 from *T. regenti* (Dvořák et al. 2005) cannot hydrolyse this 'typical' cathepsin B substrate. According to the sequence data of EnCL1 and EnCL3, cathepsins L identified in the ESP of *E. nipponicum* have Ala (Ala_{234} in EnCL1, Ala_{285} in EnCL3) at the bottom of the S2 pocket, and there-fore should likely not be able to cleave substrates with Arg in P2 position; however, in the light of the facts mentioned above, this must be verified experimentally with recombinant enzymes.

Cysteine peptidases in both solPE and ESP of *E. nipponicum* exhibited the greatest activity at pH 5 with the substrate Z-FR-AMC; in the presence of Z-RR-AMC the optima were pH 5 and pH 6 for solPE and ESP, respectively. The latter discrepancy could be caused by the presence of different other peptidases in solPE which is a more complex sample in terms of protein composition in comparison to ESP. The results corresponded, e.g. to pH optima measured for cysteine peptidases in secretions of adult *S. mansoni* (Dalton et al. 1996); it has been suggested that the activity against Z-FR-AMC at lower pH is predominantly due to cathepsin L, while cathepsin B activity is less significant under these conditions. The presence of at least two different cathepsins L in our samples might explain the high activity in a broader range of acidic pH 3–5. Similarly to our results, the complex sample of protein extract from the gut of *I. ricinus* ticks showed the activity in the presence of the Z-FR-AMC at acidic pH 4-6 (Horn et al. 2009). The fact that this substrate was not hydrolysed at pH > 7 suggests that trypsin-like serine peptidases were not present in the samples.

Summarizing the results, we dare to assume that the majority of cysteine peptidase activity in the samples was of cathepsin L-like nature. In addition, a smaller proportion of the activity was cathepsin B-like and sensitive to inhibition by iCL.

To verify the presence of other peptidases in solPE and ESP, substrates suitable for detection of cysteine asparaginyl endopeptidase (legumain) and aspartic cathepsin D were used. Surprisingly, no activity of legumain was detected. Legumain has been found in other bloodfeeding helminths as well as ticks (Dalton et al. 1995; Caffrey et al. 2000; Oliver et al. 2006; Sojka et al. 2007; Abdul et al. 2007). Its function is connected not only with hydrolysis of ingested host proteins, but it also plays an important role in activation of other peptidases by cleaving their prosequences (Sajid et al. 2003; Caffrey et al. 2004; Sojka et al. 2008). There is a certain possibility that this enzyme occurred in our samples as an inactive zymogen or an inactive variant. Significant activity of cathepsin D (ca. ¾ of that of cathepsins L + B) was observed in solPE, whereas only a minute activity was usually recorded in some samples of ESP (not shown). Also cathepsin D acts in the digestive process in other bloodfeeding parasites (Brindley et al. 2001; Verity et al. 2001; Banerjee et al. 2002; Williamson et al. 2003b; Boldbaatar et al. 2006; Sojka et al. 2012) and we expect its involvement in blood digestion by monogenean parasites, too. The nonpresence or low abundance of activities of these two crucial enzymes in some samples could be also possibly explained by alleged instability of their molecules and low production into the gut lumen.

In vitro degradation of haemoglobin by *E. nipponicum* peptidases is optimal at acidic pH, which corresponds with the pH optima of cysteine and aspartic peptidases. The reaction was completely blocked by a mixture of E-64 and pepstatin, less by E-64 and only partially by individual inhibitors. These results clearly confirmed that cysteine cathepsins L + B and aspartic cathepsin D are involved in haemoglobinolysis in *E. nipponicum*.

Affinity labelling with the active-site specific probe DCG-04 confirmed active cysteine peptidases in ESP, proved in three bands (between ca. 25–37 kDa), indicating the presence of various cysteine peptidases or intermediates of their activation by limited proteolysis (the sizes in gel approximately agree with theoretical MWs of proenzymes and mature parts of EnCL1 and EnCL3). The binding of the probe to all three bands was fully inhibited by both E-64 and iCL, whereas CA-074 inhibited only the band of lowest MW. This can be considered as another proof of the presence of both cathepsins L and B in ESP of *E. nipponicum*.

PCR with degenerate primers and RACE-PCR led to the amplification of *E. nipponicum* gene specific DNAs which were sequenced and identified as cathepsin L genes (named EnCL1 and EnCL3) [GenBank: KP793605, KP793606]. The propeptide of EnCL1 and EnCL3 contains some highly conserved regions typical for cathepsins L. One such a region is a variation of the $EX_3RX_2(V/I)FX_2NX_3IX_3N$ ('ERFNIN') motif (present as 'ERFNVN' in EnCL1) (Karrer et al. 1993). The residues in this motif are probably important in stabilizing the globular structure of the propeptide (Coulombe et al. 1996; Groves et al. 1998).

Another conserved motif is the GXNXFXD ('GNFD') present as 'ANLD' in EnCL1 and as 'TNFD' in EnCL3, with residues supposed to beinvolved in the control of intramolecular processing of cysteine peptidase precursors and/or participate in folding (Vernet et al. 1995; Dvořák et al. 2009). Processing is believed to be facilitated by a low pH. When pH is lowered, protonation of Asp_{36} (papain numbering) may cause a conformational change of the cysteine peptidase precursor in which the propeptide is bound less tightly into the active site, making it more susceptible for clip-off (Coulombe et al. 1996; Jerala et al. 1998). Amino acid differences in these conserved motifs in EnCL1 and EnCL3 should not affect the function.

An asparagine residue, found between the propeptide and mature domain of gut cysteine peptidases – cathepsins from other blood-feeding parasites, e.g. schistosomes and ticks, is responsible for recognition and cleavage by legumain for trans-activation (Sajid et al. 2003; Sojka et al. 2007). However, this Asn is absent in both EnCL1 and EnCL3, suggesting that pro-EnCL1 and pro-EnCL3 are rather activated by autocatalysis. The analysis of EnCL1 sequence using SignalP 4·1 software did not indicate the presence of an N-terminal signal sequence necessary for vesicle or extracellular targeting. The absence of such signal sequence has been described for a few cathepsins L from other organisms, e.g. the assassin bug *Rhodnius prolixus* cathepsin L-like protein (Lopez-Ordoñez et al. 2001). Additionally, it has been reported that human cathepsin L can be directed to a secretion pathway due to an aa sequence (-SXPXV) located at the carboxy (C) terminus of the enzyme (Chauhan et al. 1998). Since the secretion of EnCL1 was supported by our result from mass spectrometry analysis, it seems that it may be realized in an alternative way; a non-signal peptide triggered protein secretion was also predicted by SecretomeP 2·0 software tool (http://www.cbs.dtu.dk/services/SecretomeP/), but just at threshold values of the analysis. So far, it is not clear whether some cryptic (unknown) intramolecular signals are involved in secretion of this enzyme and only speculations can be made on another possibility – extracellular transport in exosomes. On the other hand, EnCL3 possesses a typical N-terminal signal sequence (24 aa). EnCL3 zymogen has an atypical long propeptide (120 aa) similarly as the human blood fluke *S. mansoni* SmCL3 (130 aa) [GenBank:ABV71063.1]. It has been suggested that these extensions may have other functions to the proenzyme, for example in protein trafficking or as binding sites for other proteins (Dvořák et al. 2009). Both EnCL1 and EnCL3 have no potential N-linked glycosylation sites in their sequences, similarly to cathepsin L of the monogenean parasite *N. melleni* [GenBank: ABK62794.1]. In general glycosylation with mannose 6-phosphate has been shown to be an important sorting signal for routing mammalian proteins into lysosomes. However, there are several experimental evidences for mannose 6-phosphate independent trafficking of proteins into lysosomes (Ni et al. 2006; Braulke and Bonifacino, 2009). According to this, it would be possible that EnCL occurrence in ESP is not connected with the discharge of phagolysosome residual content into the gut lumen or that the enzyme maybe targeted into lysosomes in an alternative manner.

Both cathepsins L were confirmed in *E. nipponicum* ESP in areas of various molecular sizes by means of mass spectrometry. Individual bands may represent partial processing or autolysis of the enzymes. According to the results, it seems that EnCL1 represents an abundant peptidase in ESP of *E. nipponicum*. In addition, the dominant protein spots found in *E. nipponicum* solPE separated by 2D electrophoresis were also identified by mass spectrometry as EnCL1 (data not shown).

Digestion of proteins in blood-feeding parasites represents a major proteolytic process. We showed that the most abundant haemoglobinolytic endopeptidase activities in *E. nipponicum* belong to the cysteine class, with cathepsin L-like activity predominating over cathepsin B-like activity. Proteomic investigations confirmed this fact. Besides, significant involvement of aspartic cathepsin D was well documented. The possibility of aimed secretion of proteolytic enzymes to the external environment by this ectoparasite of carp gills appears to be purposeless and therefore improbable. From this point of view and based on our results, we believe that cathepsins L, B and D are involved in processing of ingested host's blood in *E. nipponicum*.

A C K N O W L E D G E M E N T S

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F I N A N C I A L S U P P O R T

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REFERENCES

Abdul, A. M., Tsuji, N., Miyoshi, T., Khyrul Islam, M., Huang, X., Motobu, M. and Fujisaki, K. (2007). Characterization of asparaginyl endopeptidase, legumain induced by blood feeding in the ixodid tick Haemaphysalis longicornis. Insect Biochemistry and Molecular Biology 37, 911–922.

Banerjee, R., Liu, J., Beatty, W., Pelosof, L., Klemba, M. and Goldberg, D. E. (2002). Four plasmepsins are active in the Plasmodium fal-ciparum food vacuole, including a protease with an active-site histidine. Proceedings of the National Academy of Sciences of the United States of America 99, 990–995.

Boldbaatar, D., Sikalizyo Sikasunge, C., Battsetseg, B., Xuan, X. and Fujisaki, K. (2006). Molecular cloning and functional characterization of an aspartic protease from the hard tick Haemaphysalis longicornis. Insect Biochemistry and Molecular Biology 36, 25–36.

Braulke, T. and Bonifacino, J. S. (2009). Sorting of lysosomal proteins. Biochimica et Biophysica Acta 1793, 605–614.

Brindley, P. J., Kalinna, B. H., Wong, J. Y., Bogitsh, B. J., King, L. T., Smyth, D. J., Verity, C. K., Abbenante, G., Brinkworth, R. I., Fairlie, D. P., Smythe, M. L., Milburn, P. J., Bielefeldt-Ohmann, H., Zheng, Y. and McManus, D. P. (2001). Proteolysis of human hemoglobin by schistosome cathepsin D. Molecular and Biochemical Parasitology 112, 103–112.

Brinen, L. S., Que, X., McKerrow, J. H. and Reed, S. L. (2000). Homology modeling of Entamoeba histolytica cysteine proteinases reveals the basis for cathepsin L-like structure with cathepsin B-like specificity. Archives of Medical Research 31, S63–S64.

Brinker, A., Weber, E., Stoll, D., Voigt, J., Müller, A., Sewald, N., Jung, G., Wiesmüller, K. H. and Bohley, P. (2000). Highly potent inhibitors of human cathepsin L identified by screening combinatorial pentapeptide amide collections. European Journal of Biochemistry/ FEBS 267, 5085–5092.

Buchmann, K. and Brescini, J. (2006). Monogenea. In Fish Diseases and Disorders, Volume 1, 2nd Edn. pp. 297–344. CABI, Wallingford.

Caffrey, C. R. and Ruppel, A. (1997). Cathepsin B-like activity predominates over cathepsin L-like activity in adult Schistosoma mansoni and S. japonicum. Parasitology Research 83, 632–635.

Caffrey, C. R., Mathieu, M. A., Gaffney, A. M., Salter, J. P., Sajid, M., Lucas, K. D., Franklin, C., Bogyo, M. and McKerrow, J. H. (2000). Identification of a cDNA encoding an active asparaginyl endopeptidase of Schistosoma mansoni and its expression in Pichia pastoris. FEBS Letters 466, 244–248.

Caffrey, C. R., McKerrow, J. H., Salter, J. P. and Sajid, M. (2004). Blood “n” guts: an update on schistosome digestive peptidases. Trends in Parasitology 20, 241–248.

Coulombe, R., Grochulski, P., Sivaraman, J., Ménard, R., Mort, J. S. and Cygler, M. (1996). Structure of human procathepsin L reveals the molecular basis of inhibition by the prosegment. EMBO Journal 15, 5492–5503.

Dalton, J. P., Hola-Jamriska, L. and Brindley, P. J. (1995). Asparaginyl endopeptidase activity in adult Schistosoma mansoni. Parasitology 111, 575–580.

Dalton, J. P., Clough, K. A., Jones, M. K. and Brindley, P. J. (1996).Characterization of the cathepsin-like cysteine proteinases of Schistosoma mansoni. Infection and Immunity 64, 1328–1334.

Dalton, J. P., Neill, S. O., Stack, C., Collins, P., Walshe, A., Sekiya, M., Doyle, S., Mulcahy, G., Hoyle, D., Khaznadji, E., Moire, N., Brennan, G., Mousley, A., Kreshchenko, N., Maule, A. G. and Donnelly, S. M. (2003). Fasciola hepatica cathepsin L-like proteases: biology, function, and potential in the development of first generation liver fluke vaccines. International Journal for Parasitology 33, 1173–1181.

Dalton, J. P., Skelly, P. and Halton, D. W. (2004). Role of the tegument and gut in nutrient uptake by parasitic platyhelminths. Canadian Journal of Zooology. 82, 211–232.

Delcroix, M., Sajid, M., Caffrey, C. R., Lim, K. C., Dvořák, J., Hsieh, I., Bahgat, M., Dissous, C. and McKerrow, J. H. (2006). A mul-tienzyme network functions in intestinal protein digestion by a platyhelminth parasite. Journal of Biological Chemistry 281, 39316–39329.

Dvořák, J., Delcroix, M., Rossi, A., Vopálenský, V., Pospíšek, M., Šedinová, M., Mikeš, L., Sajid, M., Sali, A., McKerrow, J. H., Horák, P. and Caffrey, C. R. (2005). Multiple cathepsin B isoforms in schistosomula of Trichobilharzia regenti: identification, characterisation and putative role in migration and nutrition. International Journal for Parasitology 35, 895–910.

Dvořák, J., Mashiyama, S. T., Sajid, M., Braschi, S., Delcroix, M., Schneider, E. L., McKerrow, W. H., Bahgat, M., Hansell, E., Babbitt, P. C., Craik, C. S., McKerrow, J. H. and Caffrey, C. R. (2009). SmCL3, a gastrodermal cysteine protease of the human blood fluke Schistosoma mansoni. PLoS Neglected Tropical Diseases 3, e449.

Eakin, A. E., Bouvier, J., Sakanari, J. A., Craik, C. S. and McKerrow, J. H. (1990). Amplification and sequencing of genomic DNA fragments encoding cysteine proteases from protozoan parasites. Molecular and Biochemical Parasitology 39, 1–8.

Gillmor, S. A., Craik, C. S. and Fletterick, R. J. (1997). Structural determinants of specificity in the cysteine protease cruzain. Protein Science: a Publication of the Protein Society 6, 1603–1611.

Greenbaum, D., Baruch, A., Hayrapetian, L., Darula, Z., Burlingame, A., Medzihradszky, K. F. and Bogyo, M. (2002). Chemical approaches for functionally probing the proteome. Molecular & Cellular Proteomics: MCP 1, 60–68.

Groves, M. R., Coulombe, R., Jenkins, J. and Cygler, M. (1998). Structural basis for specificity of papain-like cysteine protease proregions toward their cognate enzymes. Proteins 32, 504–514.

Halton, D. W. and Stranock, S. D. (1976). The fine structure and histochemistry of the caecal epithelium of Calicotyle kröyeri (Monogenea: Monopisthocotylea). International Journal for Parasitology 6, 253–263.

Heussler, V. and Dobbelaere, D. (1996). Cloning of a protease gene family in Fasciola hepatica using the polymerase chain reaction (PCR). Schweizer Archiv für Tierheilkunde 138, 125–132.

Hirazawa, N., Umeda, N., Hatanaka, A. and Kuroda, A. (2006). Characterization of serine proteases in the monogenean Neobenedenia girel-lae. Aquaculture 255, 188–195.

Hodová, I., Matějusová, I. and Gelnar, M. (2010). The surface topog-raphy of Eudiplozoon nipponicum (Monogenea) developmental stages parasitizing carp (Cyprinus carpio L.). Central European Journal of Biology 5, 702–709.

Horn, M., Nussbaumerová, M., Šanda, M., Kovářová, Z., Srba, J., Franta, Z., Sojka, D., Bogyo, M., Caffrey, C. R., Kopáček, P. and Mareš, M. (2009). Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics. Chemistry and Biology 16, 1053–1063.

Chan, V. J., Selzer, P. M., McKerrow, J. H. and Sakanari, J. A. (1999). Expression and alteration of the S2 subsite of the Leishmania major cathe-psin B-like cysteine protease. Biochemical Journal 340, 113–117.

Chauhan, S. S., Ray, D., Kane, S. E., Willingham, M. C. and Gottesman, M. M. (1998). Involvement of carboxy–terminal aminoacids in secretion of human lysosomal protease cathepsin L. Biochemistry 37, 8584–8594.

Choe, Y., Leonetti, F., Greenbaum, D. C., Lecaille, F., Bogyo, M., Brömme, D., Ellman, J. A. and Craik, C. S. (2006). Substrate profiling of cysteine proteases using a combinatorial peptide library identifies function-ally unique specificities. Journal of Biological Chemistry 281, 12824–12832.

Jennings, J. B. (1959). Studies on digestion in the monogenetic trematode Polystoma integerrimum. Journal of Helminthology 33, 197–204.

Jerala, R., Zerovnik, E., Kidric, J. and Turk, V. (1998). pH-induced conformational transitions of the propeptide of human cathepsin L. A role for a molten globule state in zymogen activation. Journal of Biological Chemistry 273, 11498–11504.

Karrer, K. M., Peiffer, S. L. and DiTomas, M. E. (1993). Two distinct gene subfamilies within the family of cysteine protease genes. Proceedings of the National Academy of Sciences of the United States of America 90, 3063–3067.

Kawatsu, H. (1978). Studies on the anemia of fish - IX. Hypochromic microcytic anemia of crusian carp caused by infestation with a trematode, Diplozoon nipponicum. Bulletin of the Japanese Society of Scientific Fisheries 44, 1315–1319.

Konstanzová, V., Koubková, B., Kašný, M., Ilgová, J., Dzika, E. and Gelnar, M. (2015). Ultrastructure of the digestive tract of Paradiplozoon homoion (Monogenea). Parasitology Research 114, 1485–1494.

Li, A. H., Moon, S.-U., Park, Y.-K., Na, B.-K., Hwang, M.-G., Oh, C.-M., Cho, S.-H., Kong, Y., Kim, T.-S. and Chung, P.-R. (2006). Identification and characterization of a cathepsin L-like cysteine protease from Taenia solium metacestode. Veterinary Parasitology 141, 251–259. Llewellyn, J. (1954). Observations on the food and the gut pigment of the Polyopisthocotylea (Trematoda: Monogenea). Parasitology 44, 428–37.

Lopez-Ordoñez, T., Rodriguez, M. H. and Hernández, F. D. (2001). Characterization of a cDNA encoding a cathepsin L-like protein of Rhodnius prolixus. Insect Molecular Biology 10, 505–511.

Ni, X., Canuel, M. and Morales, C. R. (2006). The sorting and traffick-ing of lysosomal proteins. Histology and Histopathology 21, 899–913.

Oliver, E. M., Skuce, P. J., McNair, C. M. and Knox, D. P. (2006). Identification and characterization of an asparaginyl proteinase (legumain) from the parasitic nematode, Haemonchus contortus. Parasitology 133, 237–244.

Rao, Y. Z. and Yang, T. B. (2007). cDNA cloning, mRNA expression and recombinant expression of a cathepsin L-like cysteine protease from Neobenedenia melleni (Monogenea: Capsalidae). Aquaculture 269, 41–53.

Renard, G., Garcia, J. F., Cardoso, F. C., Richter, M. F., Sakanari, J. A., Ozaki, L. S., Termignoni, C. and Masuda, A. (2000). Cloning and functional expression of a Boophilus microplus cathepsin L-like enzyme. Insect Biochemistry and Molecular Biology 30, 1017–1026.

Robinson, M. W., Dalton, J. P. and Donnelly, S. (2008). Helminth pathogen cathepsin proteases: it's a family affair. Trends in Biochemical Sciences 3, 601–608.

Sajid, M. and McKerrow, J. H. (2002). Cysteine proteases of parasitic organisms. Molecular and Biochemical Parasitology 120, 1–21.

Sajid, M., McKerrow, J. H., Hansell, E., Mathieu, M. A., Lucas, K. D., Hsieh, I., Greenbaum, D., Bogyo, M., Salter, J. P., Lim, K. C., Franklin, C., Kim, J.-H. and Caffrey, C. R. (2003). Functional expres-sion and characterization of Schistosoma mansoni cathepsin B and its

zransactivation by an endogenous asparaginyl endopeptidase. Molecular and Biochemical Parasitology 131, 65–75.

Smyth, J. D. and Halton, D. W. (1983). The Physiology of Trematodes. Cambridge University Press, Cambridge, UK.

Sojka, D., Hajdušek, O., Dvořák, J., Sajid, M., Franta, Z., Schneider, E. L., Craik, C. S., Vancová, M., Burešová, V., Bogyo, M., Sexton, K. B., McKerrow, J. H., Caffrey, C. R. and Kopáček, P. (2007). IrAE: an asparaginyl endopeptidase (legumain) in the gut of the hard tick Ixodes ricinus. International Journal for Parasitology 37, 713–724.

Sojka, D., Franta, Z., Horn, M., Hajdušek, O., Caffrey, C. R., Mareš, M. and Kopáček, P. (2008). Profiling of proteolytic enzymes in the gut of the tick Ixodes ricinus reveals an evolutionarily conserved network of aspartic and cysteine peptidases. Parasites & Vectors 1, 7.

Sojka, D., Franta, Z., Frantová, H., Bartošová, P., Horn, M., Váchová, J., O'Donoghue, A. J., Eroy-Reveles, A. A., Craik, C. S., Knudsen, G. M., Caffrey, C. R., McKerrow, J. H., Mareš, M. and Kopáček, P. (2012). Characterization of gut-associated cathepsin D hemo-globinase from tick Ixodes ricinus (IrCD1). Journal of Biological Chemistry 287, 21152–21163.

Sonenshine, D. E. (1991). Biology of Ticks Volume 1. Oxford University Press, New York.

Steverding, D. (2011). The cathepsin B-selective inhibitors CA-074 and CA-074Me inactivate cathepsin L under reducing conditions. Open Enzyme Inhibition Journal 4, 11–16.

Steverding, D., Sexton, D. W., Wang, X., Gehrke, S. S., Wagner, G. K. and Caffrey, C. R. (2012). Trypanosoma brucei: chemical evidence that cathepsin L is essential for survival and a relevant drug target. International Journal for Parasitology 42, 481–488.

Tinsley, R. C. (1973). Ultrastructural studies on the form and function of the gastrodermis of Protopolystoma xenopi (Monogenoidea: Polyopisthocotylea). Biological Bulletin 144, 541–555.

Turk, B., Turk, D. and Turk, V. (2000). Lysosomal cysteine proteases: more than scavengers. Biochimica et Biophysica Acta 1477, 98–111.

Valigurová, A., Hodová, I., Sonnek, R., Koubková, B. and Gelnar, M. (2011). Eudiplozoon nipponicum in focus: monogenean exhibiting a highly specialized adaptation for ectoparasitic lifestyle. Parasitology Research 108, 383–394.

Verity, C. K., Loukas, A., McManus, D. P. and Brindley, P. J. (2001). Schistosoma japonicum cathepsin D aspartic protease cleaves human IgG and other serum components. Parasitology 122, 415–421.

Vernet, T., Berti, P. J., de Montigny, C., Musil, R., Tessier, D. C., Ménard, R., Magny, M. C., Storer, A. C. and Thomas, D. Y. (1995).

Processing of the papain precursor. The ionization state of a conserved amino acid motif within the Pro region participates in the regulation of intra-molecular processing. Journal of Biological Chemistry 270, 10838–10846.

Williamson, A. L., Brindley, P. J., Knox, D. P., Hotez, P. J. and Loukas, A. (2003a). Digestive proteases of blood-feeding nematodes. Trends in Parasitology 19, 417–423.

Williamson, A. L., Brindley, P. J., Abbenante, G., Datu, B. J. D., Prociv, P., Berry, C., Girdwood, K., Pritchard, D. I., Fairlie, D. P., Hotez, P. J., Zhan, B. and Loukas, A. (2003b). Hookworm aspartic pro-tease, Na-APR-2, cleaves human hemoglobin and serum proteins in a host-specific fashion. Journal of Infectious Diseases 187, 484–494.

Williamson, A. L., Lecchi, P., Turk, B. E., Choe, Y., Hotez, P. J., McKerrow, J. H., Cantley, L. C., Sajid, M., Craik, C. S. and Loukas, A. (2004). A multi-enzyme cascade of hemoglobin proteolysis in the intestine of blood-feeding hookworms. Journal of Biological Chemistry 279, 35950–35957.

1.9. **Publikace č. 2**

První výsledky naší studie naznačily, že nejvyšší aktivita u dospělého monogenea *E. nipponicum* je přiřazena cysteinovým peptidázám z klanu CA a nejvíce zastoupeny jsou zde katepsiny L. Proto jsme se v této práci zaměřili na detailní analýzu transkriptomu dospělce se zaměřením na cysteinové peptidázy. Podařilo se nám identifikovat deset různých transkriptů kódujících katepsiny L a pouze jeden transkript kódující katepsin B. Bioinformatická analýza katepsinů L odhalila vysokou variabilitu v S2 kapse, která je zodpovědná za interakci se substrátem, a tedy naznačuje jejich široké využití v různých biologických procesech. Dva abundantní katepsiny L a jeden katepsin B byly vyprodukovány v rekombinantní formě, purifikovány a funkčně charakterizovány. Metodou RNA *in situ* hybridizace a imunohistochemií byly oba katepsiny L detekovány uvnitř trávicích buněk, ale i v lumen střeva. Tyto výsledky potvrzují jejich předpokládanou roli při trávení hostitelských krevních proteinů a zároveň odhalily potenciální extracelulární fázi trávení. Z výsledků vyplývá, že trávení krve u hematofágních monogeneí *E. nipponicum* je pravděpodobně více podobné trávení u fasciolidních motolic, kde hemoglobin je v lumen střeva štěpen na menší fragmenty, které jsou pak následně vstřebány do buněk gastrodermis. Hlavní roli při trávení hemoglobinu u *F. hepatica* hrají tři katepsiny L (CL1, CL3 a CL5) (Robinson *et al.*, 2008). Překvapivě katepsin B *E. nipponicum* schopný hydrolyzovat hemoglobin *in vitro* se na trávení krevních proteinů pravděpodobně nepodílí, díky své lokalizaci uvnitř vitelinních buněk.

Můj podíl na práci zahrnoval produkci a purifikaci rekombinantních katepsinů L, funkční charakterizace a jejich lokalizace ve tkáni dospělce *E. nipponicum* pomocí RNA *in situ* hybridizace a imunohistochemie. Dále jsem se podílela na bioinformatické anotaci enzymových struktur, interpretaci dat a sepisování publikace.

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.

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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

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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.
(Continued on next page)

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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.

Keywords: Cysteine peptidase, Protease, Cathepsin, S2 subsite, Haematophagy, Blood digestion, Monogenea, Diplozoidae, *Eudiplozoon nipponicum*, Fish parasite

Background

Blood-feeding monogeneans of the family Diplozoidae (Heteronchoinea) are ectoparasites that live on the gills of cyprinid fishes. One member of the family, *Eudiplozoon nipponicum*, is an important invasive species, first recorded in Europe in 1983 on farmed carp in France [1]. It is currently found throughout Europe and is a widespread representative of the helminth fauna of the common carp (*Cyprinus carpio*) in the Czech Republic [2]. The common carp is a fish of high economic importance in many Asian and European countries, with global aquaculture produc-tion yielding over 4 million tons in 2014 [3]. Since the parasite affects the health of farmed fish, although precise estimates are unknown, it is assumed it causes economic losses. Pathogenic effects of *E. nipponicum* are associated mainly with inducing hypochromic microcytic anaemia in the fish by a continuous intake of blood by worms attached to the carp gills [4].

Monogenea are a rather neglected group of Neodermata and only a handful of papers on their biochemistry and bioactive molecules have ever been published. Based on previous ultrastructural studies, it has been assumed that in diplozoid monogeneans, the digestion of blood, gathered by combined action of their powerful buccal suckers and muscular pharynx, takes place within the lysosomal cycle in the specialised cells of the intestinal epidermis [5–9], similarly as in blood-sucking mites such as ticks [10, 11]. Our previous study [12] has shown that the processing of blood in *E. nipponicum* relies on an evolutionarily con-served multi-enzyme network of cysteine and aspartic peptidases, similar to the proteolytic cascades of other blood-feeding helminths such as *Schistosoma*, *Fasciola*, *Ancylostoma*, etc. [13–16]. Among the endopeptidases *of E. nipponicum*, clan CA cysteine peptidase activities, including cathepsin L-like and cathepsin B-like activity, are dominant [12].

In helminths, cathepsins L and B play various roles. Due to their histolytic potency, they are involved in host invasion and tissue migration, but they also play a role in various pathological processes, immune evasion, and other parasite-host interactions, as well as in helminth reproduction, nutrient digestion, etc. [17–19]. In general, peptidases encoded in the helminth genomes are mostly temporarily expressed in the various life stages, thus reflecting the parasite's specific needs regarding digestion or other hydrolytic processes. For instance, a characterisation of several cathepsins L of the liver fluke *Fasciola hepatica* had shown that the infective larvae use cathepsin L3 to traverse the host's intestinal wall, while the fluke's migratory stages utilise cathepsin L2 to penetrate host liver tissue, and adults employ cathepsin L1 jointly with cathepsins L2 and L5 to digest host proteins [20]. The use of different peptidases with overlapping substrate specificities helps heteroxenous and tissue-migrating parasites to adapt to various environments and sources of nutrition within the hosts. On the other hand, little is known about the complex functioning of cysteine peptidases in monoxenous blood-feeding monogeneans that spend most of their life attached to a single type of host tissue, such as the gills.

In the present study, we focused on clan CA cysteine peptidases, namely cathepsins L and B, selected due to their abundance in the transcriptome of adult *E. nipponicum* worms. We have employed phylogenetic and bioinformatic analysis to investigate their relationship to other helminth peptidases. We have selected two of the most abundant cathepsins L and the only expressed cathepsin B, and produced these as functionally active recombinant forms using the *Pichia pastoris* expression system. A biochemical and functional characterisation of the recombinant enzymes was performed in order to understand their specificity and substrate preference. Furthermore, we have developed specific antibodies and RNA probes to localise proteins/transcripts within the worms' bodies by immunofluorescence assay and RNA in situ hybridisation technique. Our work thus presents the first detailed func-tional characterisation of monogenean peptidases.

Methods

Parasite material

Adult worms of *E. nipponicum* were collected from the gills of infected carps (*C. carpio*) freshly slaughtered in a commercial facility of the fishery Rybářství Třeboň, Plc., Třeboň basin, South Bohemia, Czech Republic. A soluble worm extract, parasite RNA, and first-strand cDNA were

obtained as previously described [12]. For RNA in situ hybridisation, the worms were flat-fixed between microscopic slides in Bouin's solution (Sigma-Aldrich, Darmstadt, Germany) at RT for 1 h, then transferred into fresh fixative and incubated at 4 °C overnight. For immunohistochemistry, 4% paraformaldehyde in PBS was employed as a fixative. Fixed worms were dehydrated with increasing concentrations of ethyl alcohol (70–100%), cleared with xylene, and embedded in paraffin.

Sequence analyses of cathepsin L-like and B-like peptidases of *E. nipponicum*

Transcripts from the transcriptome of adult *E. nipponicum* worms were assembled within a parallel project and de-posited in the NCBI GenBank database under accession number GFYM00000000. They were annotated by search-ing for the closest homologues. We have used BLASTp and BLASTn algorithms (E-value 1e-5) to mine the fol-lowing public sequence databases: NCBI non-redundant protein database [21], MEROPS database of peptidases and their inhibitors [22], UniProtKB/UniRef100 database and UniProtKB/TrEMBL only used for searching for protein sequences related to phylum Platyhelminthes (Taxon ID: 6157) [23], UniProtKB/Swiss-Prot [24], RCSB PDB [25] and DDBJ [26]. Sequences encoding cathepsin L-like and B-like peptidases of *E. nipponicum* were identified and their amino acid sequences (catalytic domains) aligned with sequences of formerly recognised as *E. nipponicum* cathepsins L, EnCL1 (GenBank: KP793605) and EnCL3 (GenBank: KP793606), *F. hepatica* cathepsin L1 (GenBank: AAT76664.1), *F. hepatica* cathepsin L3 (GenBank: CAC12807.1) and with *E. nipponicum* cathepsin B, EnCB (GenBank: MF346929), *Schistosoma mansoni* cathepsins B1 (GenBank: CAD44624.1) and B2 (GenBank: XP_018651608.1), respectively, using Clustal Omega tool (http://www.ebi.ac.uk/Tools/msa/clustalo/) [27]. The relative abundance of cathepsin L transcripts, which reflects the rate of transcription of the corresponding genes, was predicted by back mapping of raw Illumina reads to the assembled transcripts using Kallisto v. 0.43.0 [28]. The presence of a signal sequence in amino acid sequences of the enzymes was predicted by SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/) [29]. A theoretical position of the proregion and composition of the enzymes' S2 subsites of the active site were determined by multiple sequence alignment (see above). Molecular mass and theoretical pI of the deduced proteins were determined by Compute pI/Mw software available at the ExPASy web portal (https://web.expa-sy.org/compute_pi/) [30]. Potential N-glycosylation and O-glycosylation sites were identified using an online tool at NetNGlyc 1.0 and NetOGlyc 4.0 Servers (http:// www.cbs.dtu.dk/services/NetNGlyc [30] and http://www. cbs.dtu.dk/services/NetOGlyc [31]).

Eudiplozoon nipponicum cathepsin L1 and L2 amino acid sequences were used as queries for BLAST searches in the NCBI non-redundant protein database [32] and the Wormbase database of platyhelminths [33]. Recovered sequences were further analysed using CLANS software [34] and a cluster of 515 sequences containing *E. nipponicum* cathepsins was selected. Redundant sequences were filtered at 95% level of identity using the CD-HIT software [35]. Divergent and incomplete sequences were manually removed, which resulted in a dataset of 288 sequences. These were aligned using the MAFFT [36] and the align-ment was trimmed using BMGE (239 sites) [37]. We inferred a maximum-likelihood tree using the best-fit model (LG + C60 + G), and tested the topology with 10,000 ultrafast bootstraps in IQ-TREE [38]. Bayesian inference of phylogeny was run under the C60 site-heterogeneous mixture model using Phylobayes.

Expression of recombinant cathepsins L1, L3 and B in *Pichia pastoris* and their purification

Two abundant EnCLs (EnCL1 and EnCL3) and the only EnCB were selected for a functional expression. Gene-specific primers (Additional file 1) and the first-strand cDNA of adult *E. nipponicum* were used to amplify the yrEnCL1 (GenBank: KP793605), yrEnCL3 (GenBank: KP793606), and yrEnCB (GenBank: MF346929) genes. The amplified products were ligated into a *P. pastoris* expression vector pPICZα B as described previously [39], and the constructs were verified by Sanger sequencing. Protein expression in *P. pastoris* X-33 was carried out following a protocol described previously [40] and accord-ing to the manual of the Easy Select Pichia Expression Kit (Thermo Fisher Scientific, Waltham, Massachusetts). The yeast medium was centrifuged and the supernatant filtered (0.22 μm) and concentrated on AmiconUltra-15 filters 10,000 MWCO (Millipore, Darmstadt, Germany). The enzymes were affinity purified via their His-tag using Ni-chelating chromatography (HisTrap™ FF crude, GE Healthcare, Little Chalfont, United Kingdom) and a stepwise elution by imidazole (0.05–0.5 M). Yeast-origin hyperglycosylation of EnCB was removed by endoglycosidase F1 (Calbiochem, Darmstadt, Germany). Purification was completed by cation exchange FPLC on Mono S™ 5/50 GL column (GE Healthcare) using 20 mM MES buffer pH 6.0 and gradient elution by NaCl (0–1 M).

Expression of recombinant cathepsin L1 in *Escherichia coli* and its purification

The EnCL1 gene (GenBank: KP793605) was amplified using first-strand cDNA of adult *E. nipponicum* and specific primers (Additional file 2). The amplified product was ligated into the *E. coli* expression vector pet28a + (Novagen, Darmstadt, Germany) and verified by DNA sequencing.

EnCL1 was expressed *in E. coli* BL21 Star™ (DE3) (Invitrogen, Carlsbad, California) according to pET System Manual (Novagen). Harvested cells were lysed in 20 mM Tris-HCl pH 8/ 0.3 M NaCl/1% lauryl sarcosine/10 mM imidazole by sonication, and centrifuged at 10,000× g for 10 min. Inclusion bodies were solubilised with 6 M guanidine hydrochloride as described elsewhere [41]. The solubilised mixture was filtered (0.65 μm), passed over the column containing Ni-NTA beads (Qiagen, Hilden, Germany), and eluted by imidazole (0.05–0.5 M). Chromatographic fractions were analysed by SDS-PAGE and the identity of suspected protein bands was verified by mass spectrometry as described in [12].

Processing the yrEnCL3 pro-enzyme

Auto-processing of pro-yrEnCL3 was tested by incubation of the recombinant protein in 50 mM/100 mM CPB, with 2 mM DTT at pH values of 4, 5 and 6. Aliquots of 20 μl were taken from reactions at specific time intervals (15, 30, 60 and 120 min) at 37 °C, whereby incubations were stopped by adding 10 μM E-64. Processing of the enzyme to its mature form was monitored by SDS-PAGE, Western blotting and Edman degradation of N-terminus and subsequently confirmed by fluorometry with the corresponding synthetic peptide substrates (see below). Processing of yrEnCL3 was also monitored by affinity labelling using 20 μM fluorescent probe BODIPYgreen-DCG-04 [42] and 2.5 μg of the enzyme (30 min incubation at pH 5) as described previously [40]. Specificity of the probe towards the active site of the peptidase was verified by preincubation with the cysteine peptidase-specific inhibitor E-64 (10 μM) for 5 min.

Peptidase activity assays

Peptidase activities of recombinant cathepsins were measured with fluorogenic aminomethylcoumarin peptide substrates (Bachem) as described previously [12]. Substrates tested with yrEnCL1 and yrEnCL3 included Z-Phe-Arg-AMC (FR), Z-Leu-Arg-AMC (LR), Z-Arg-Arg-AMC (RR), Z-Pro-Arg-AMC (PR) and Z-Gly-Pro-Arg-AMC (GPR). The yrEnCB was tested with FR and RR substrates only. The purified rEnCLs (15 nM) and rEnCB (30 nM) were dissolved in 100 μl of 50 mM/100 mM CPB supplemented with 2 mM DTT, in pH range of 3–8. The reactions were initiated by adding the individual substrates (final concentration 50 μM) in 100 μl of the same buffer at 28 °C. The release of free AMC was measured at excitation and emission wavelengths of 355 and 460 nm, respectively, in Infinite M200 fluorometer (TECAN, Männedorf, Switzerland).

For inhibition assays, three inhibitors were used (final concentration 10 μM, Sigma-Aldrich): E-64 (L-trans-epoxysuccinyl-leucylamido [4-guanidino] butane), which is a general cysteine peptidase inhibitor, iCL (Arg-Lys-Leu-Leu-Trp-NH2), a reversible inhibitor of cathepsin L; and CA-074 (N-[L-3-trans-propylcarbamoyloxirane-2-carbonyl]-Ile-Pro-OH), which is a selective inhibitor of cathepsin B. Inhibitors were mixed with enzyme samples and incubated at 28 °C for 15 min prior to adding the substrates. Remaining hydrolysis rates were measured with the FR substrate (cathepsins L and B), with LR (cathepsins L), or with RR (cathepsin B) as described above.

The assay of exopeptidase activity of activated yrEnCB (75 nM) was carried out with benzoyl-glycinyl-histidinyl-leucine (Bz-Gly-His-Leu; final conc. 2 mm) as a substrate, using a modified protocol [43]. The activity was measured in 50 mM/100 mM CPB pH 4–6 containing 2 mM DTT and 0.05 mg/ml fluorescamine.

Hydrolysis of macromolecular substrates by recombinant cathepsins L and B

Processed yrEnCL1, yrEnCL3 and yrEnCB (2.5 μg each) were incubated at 37 °C with 50 μg of macromolecular substrates (bovine haemoglobin and albumin, human collagen I, IgG and fibrinogen; Sigma-Aldrich) dissolved in 100 μl of 50 mM/100 mM CPB with 2 mM DTT in pH 4.5–6. Aliquots (10 μl) for SDS-PAGE analysis in 12% gels were taken at various intervals (0, 30, 60 and 120 min and 16 h).

RNA *in situ* hybridisation

EnCL1/EnCL3 gene-specific primers (Additional file 3) and first-strand cDNA of adult *E. nipponicum* were used for the PCR. The amplified products were ligated into pGEM®-T Easy vector (Promega, Madison, Wisconsin) and verified by DNA sequencing. The constructs were linearised and used as a template for RNA probes. Both sense and anti-sense RNA probes were synthesised in vitro (Dig RNA Labelling Kit (SP6/T7); Roche, Basel, Switzerland). *In situ* hybridisation was performed using a modified protocol [44]. Briefly, the sections of adult worms on slides were incubated for 19 h at 41 °C (EnCL1) and at 37 °C (EnCL3) with specific RNA probes diluted 1:100 in a hybridisation mixture (5× SSC, 1× PBS, 50% deionised formamide, 1% Tween-20, 10% dextran sulphate Mw 500×, 1 mg/ml Torula yeast RNA). Detection was achieved with alkaline phosphatase-conjugated anti-digoxigenin antibodies (1:500, Roche) and Fast Red TR substrate (Sigma-Aldrich). Negative controls were incubated under the same conditions but with an anti-sense probe or without a probe. Specific strand RT-PCR was used for the detection of natural anti-sense transcripts occurring in the monogenean cells [45].

Production of peptidase-specific polyclonal antibodies

For the production of monospecific polyclonal anti-EnCL3 and anti-EnCB antibodies, ICR/CD1 mice (ENVIGO,

Huntingdon, United Kingdom) were injected subcutaneously twice, at a 14-day interval, with 30 μg of purified pro-yrEnCL3 or deglycosylated yrEnCB in sterile saline and TitermaxGold adjuvant (Sigma-Aldrich). The mice were boosted by an intramuscular injection of purified antigens (15 μg) in sterile saline after another two weeks. Anti-EnCL1 antibodies were produced in mice injected intraperitoneally with c.50 μg of brEnCL1 cut as a band from a 12% gel after SDS-PAGE. The strip of the gel containing the antigen was repeatedly and thoroughly washed in 50% methanol/10% acetic acid for several hours to remove SDS, washed several times in sterile saline, and homogenised in sterile saline using a motorised pellet pestle. Two antigen deliveries were performed at an interval of 14 days. All mice were bled under deep ketamine/xylazine anaesthesia 14 days after the last injection and sera were collected by centrifugation. Control sera were taken from the same mice prior to immunisation.

Western and affinity blotting

Specificity of the anti-cathepsin antibodies produced in mice was verified on PVDF membrane immunoblots of soluble worm extracts previously separated by SDS-PAGE in 12% gels (20 μg/well). The experiment was performed according to a modified protocol [40]. The membranes were first incubated for 1 h with mouse anti-EnCL3/anti-EnCL1/anti-EnCB /control sera diluted 1:100 in PBS-T and then for 1 h with horseradish peroxidase-labelled goat anti-mouse IgG (Sigma-Aldrich) diluted 1:5000 in PBS-T. Finally, the membranes were developed by the Opti-4CN Substrate Kit (Bio-Rad, Hercules, California).

Purified recombinant proteins (1 μg/well) were resolved by SDS-PAGE in 12% gels and either stained by CBB or trans-blotted onto a PVDF membrane. His-tagged enzymes were detected on blots either with a mouse biotinylated monoclonal Anti-polyhistidine antibody (Sigma-Aldrich), alternatively with 5 nM iBody4, which is a biotinylated copolymer containing nitrilotriacetic acid-bound nickel cations [46]. In both cases, detection was finalized using peroxidase-labelled streptavidin (Sigma-Aldrich) and the Opti-4CN™ Substrate kit.

Immunohistochemistry

Deparaffined and rehydrated 5 μm sections of worms on slides were heated 3 × 3 min in 0.01 M citrate buffer pH 6 containing 0.05% Tween 20 in a microwave oven (500 W) and then allowed to cool for 20 min. Subsequently, the sections were blocked and immunostained according to a modified Immunocytochemistry and Immunofluorescence Protocol (Abcam). After an overnight incubation with sera (1:50), the slides were placed in a dark chamber for 1 h at RT with Goat Anti-Mouse IgG H&L-Alexa Fluor® 568 (Ex: 578 nm/Em: 603 nm, Abcam, Cambridge, United Kingdom) diluted 1:200 in PBS-TX100 with 1% BSA. Finally, the sections were mounted in Vectashield with DAPI (Vector laboratories, Burlingame, California). Immunofluorescence was observed and photographed under fluorescence or confocal microscope.

Results

Bioinformatic and phylogenetic analyses of the primary structures of the enzymes

Ten unique transcripts encoding different cathepsin L-like sequences were discovered in the transcriptome of adult *E. nipponicum*. Of the predicted CL amino acid sequences, seven represented complete sequences of mature (processed) enzymes, with six encoding the complete sequences of zymogens including the pro-sequences. Two of the predicted complete zymogen sequences were identical with the sequence of EnCL1 (GenBank: KP793605) and EnCL3 (GenBank: KP793606) obtained previously by reverse transcription of parasite mRNA using degenerate primers, followed by RACE-PCR [12]. The complete pro-enzyme molecules were assigned EnCL1-EnCL6. The remaining four incomplete sequences shared a relatively high similarity with EnCL6: the five close orthologs were therefore assigned EnCL6a-EnCL6e. The alignment of the predicted amino acid sequences of mature EnCLs with cathepsins L1 (GenBank: AAT76664.1) and L3 (GenBank: CAC12807.1) of *F. hepatica* is presented in Additional file 4, including a mutual comparison of the amino acids of the S2 pocket, which determines the substrate-binding specificity of the enzymes. The alignment of all complete/incomplete amino acid sequences of ECLs is included in Additional file 5.

Interestingly, EnCL2 displays a substitution of the active site cysteine (Cys25) to serine (Ser25). In terms of amino acid constitutions of the S2 pocket (Additional files 4, 5 and 6), the EnCLs can be divided in 3 groups. One is formed by CL1, CL3, CL4 and CL5, which share the Leu67, Ala133, Leu157 motif (papain numbering used throughout this column), with a slightly hydrophobic Ala being at the bottom of the S2 pocket. The second includes EnCL6a, EnCL6b and EnCL6e, where Ala133 is replaced by the amphiphilic Gly133 (for EnCL6c and EnCL6d data were not available.). The third group contains only EnCL2, in which the S2 pocket differs substantially from that of the other EnCLs: Ala133 is replaced by the more hydrophobic Val133 at the bottom, and moreover, the two leucines are replaced by Trp67 and Phe157, which could make the S2 pocket highly hydrophobic. The most variable element in the S2 pocket is the amino acid position 205, where hydrophobic residues occur in CL3, CL4 and CL5, and uncharged hydrophilic residues in CL1 and CL6a. In the case of CL2, residue 205 is a positively charged Arg, whereas in CL6e, it is the negatively charged aspartate.

Some of the EnCLs include potential N-glycosylation sites: CL2 has one and CL4 has two in the mature

enzyme, others occur in the pro-sequences of CL2, CL4 and CL5. Several O-glycosylation sites have been predicted in all but one (EnCL6b) cathepsin L sequences. Most, however, are located in the pro-domains; CL5 has three, while all the other enzymes except for CL6d have one in the mature part (Table 1, Additional file 5).

The rate of transcription of the particular EnCLs was predicted by a reverse mapping of raw reads towards the selected transcripts. EnCL1 is the most abundant and EnCL3 is the sixth most abundant cathepsin L in *E. nipponicum* adults (Fig. 1).

Phylogenetic analysis has shown that *E. nipponicum* cathepsins L separate into two clusters: one contains CL1, CL3 and CL5, and the other includes CL2, CL4 and CL6a. The two clusters belong to separate clades, whereby each of the clusters also includes different cathepsins L of free-living rhabditophorans and CLs of digeneans and cestodes (Fig. 2, Additional file 7). It can thus be concluded that *E. nipponicum* cathepsins L are polyphyletic.

The cluster containing EnCL1 and EnCL3, for which the function in blood digestion was confirmed in this study, occurs within the same clade as the CL of another haematophagous monogenean, *Protopolystoma xenopodis*. The various CLs of the mucophagous *Gyrodactylus salaris* fall into both clades where the two clusters of EnCLs are located. Interesting is the highly supported position of myxosporean CLs within the neodermatan clade which contains also EnCL2, EnCL4 and EnCL6a.

In stark contrast to the numerous EnCLs, only one transcript encoding EnCB was found in the *E. nipponicum* transcriptome; it contained the whole sequence of the pro-enzyme. Its closest relative found in the databases is cathepsin B2 of *Schistosoma mansoni* (GenBank: XP_018651608.1) (see the alignment in Additional file 8).

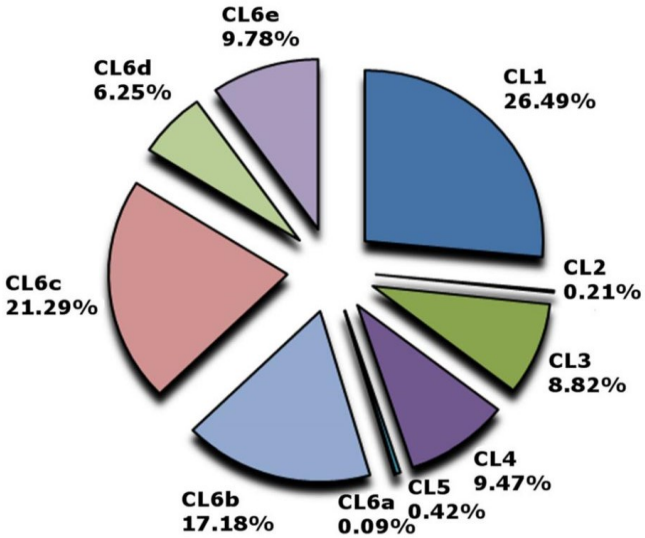

Fig. 1 Relative transcription rate of *E. nipponicum* cathepsins L. The relative transcription rate predicted by a back mapping of raw Illumina reads to the assembled transcripts was calculated as the percentage of all cathepsin L transcripts

Its primary structure contains the typical motif of an occluding loop, which is responsible for peptidyl dipeptidase activity of cathepsins B. One potential N-glycosylation site is positioned on the occluding loop. Seven O-glycosylation motifs were predicted in the molecule, five of them located within the pro-domain. EnCB also contains a modified 'haemoglobinase motif' (213)YWLIANSW–EWGD(226) in the asparagine active site region, which is ascribed to haemoglobinolytic cathepsins B of helminth blood-feeders [47]. The predicted/computed basic features of all EnCLs and EnCB are summarised in Table 1.

Table 1 Predicted/computed sequence-derived features of *E. nipponicum* cathepsins L and B

	Transcript_ID	Bases/AA	Completeness	SP	pI proenzyme/ mature enzyme	MW proenzyme/ mature enzyme (kDa)	N-/O-glyc sites
CL1	E_nip_trans_58808_m.372114	954/317	yes	no	5.86/6.08	34.9/24.36	0/7
CL2	E_nip_trans_70234_m.461805	1029/342	yes	yes	7.23/4.87	37/24.9	3/2
CL3	E_nip_trans_02967_m.11341	1107/368	yes	yes	4.80/4.15	38/24.1	0/5
CL4	E_nip_trans_06099_m.25531	1062/353	yes	yes	5.59/4.94	37.6/24.2	3/8
CL5	E_nip_trans_65378_m.396731	1071/356	yes	no	5.62/5.02	39.5/24	1/8
CL6a	E_nip_trans_15113_m.115989	1059/352	yes	yes	5.55/4.8	37.7/24.4	0/2
CL6b	E_nip_trans_04751_m.20488	519/173	iN	?	?/?	?/?	0/0
CL6c	E_nip_trans_04670_m.20209	642/214	iC	yes	?/?	?/?	0/4
CL6d	E_nip_trans_55822_m.362291	705/235	iC	yes	?/?	?/?	0/1
CL6e	E_nip_trans_60687_m.380367	768/255	iN	?	?/4.58	?/24.47	0/2
CB	E_nip_trans_02724_m.9562	1149/382	yes	yes	7.21/5.55	39.9/28.5	1/7

Abbreviations: AA Amino acids, SP Signal peptide, N-/O-glyc sites Number of potential N-glycosylation/O-glycosylation sites, iN Incomplete N-terminus, iC Incomplete C-terminus, ? data not available due to an incomplete sequence

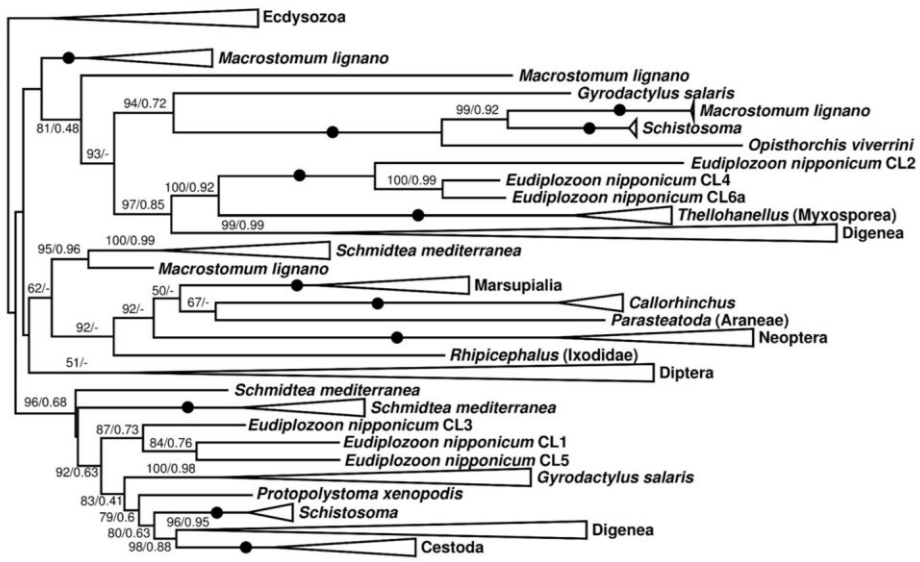

Fig. 2 Collapsed phylogram showing relationships between *E. nipponicum* cathepsins L and cathepsins L of other selected organisms. Collapsed unrooted maximum-likelihood tree of selected *E. nipponicum* cathepsins L inferred using the best-fit model (LG + C60 + G). Leaves of related organisms are collapsed. Ultrafast bootstrap supports and posterior probabilities are shown where ultrafast bootstrap is ≥ 50 or posterior probability ≥ 0.9. Maximum support (100/1.0) is indicated by a black circle

Functional expression and purification of recombinant cathepsins L1, L3 and B

EnCL1, EnCL3 and EnCB were expressed as pro-enzymes in yeasts. Since yrEnCL1 and yrEnCB had undergone self processing already in yeast cultivation medium, a high activity of both was confirmed in the presence of appropriate fluorogenic peptide substrates. The yrEnCL1 thus migrated in electrophoretic gels as a ~28 kDa dominant double band and reacted on the blot with anti-His-Tag mouse antibody and iBody4 at the same size (Fig. 3). The enzyme was partially purified by Ni-NTA affinity chromatography, but attempts to purify it to homogeneity by ion-exchange chromatography failed due to its poor stability. Activity and inhibition assays were therefore performed with only partially purified enzyme. Additionally, for the production of anti-EnCL1 antibodies it was necessary to use the inactive recombinant form produced in *E. coli* (brEnCL1).

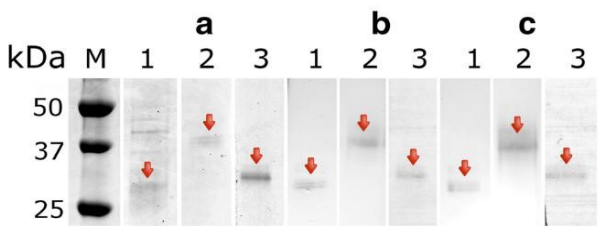

Fig. 3 SDS-PAGE and affinity−/immunoblots of yrEnCL1, yrEnCL3 and yrEnCB after purification. a 12% gel stained with Coomassie Brilliant Blue. b affinity blot labelling by iBody4. c immunoblot detection by anti-His-Tag antibody. Lane 1: yrEnCL1 (28 kDa partially processed form); Lane 2: yrEnCL3 (38 kDa pro-enzyme); Lane 3: yrEnCB (29 kDa deglycosylated processed enzyme). Arrows point to the enzyme bands

The affinity purified unprocessed pro-yrEnCL3 migrated in the gel as a double band at ~38 kDa, and reacted with the anti-His-Tag antibody and iBody4 at the same size (Fig. 3). Autocatalytic processing of pro-yrEnCL3 was most effective at pH 4, and resulted in a ~28 kDa band in the gel. Processing of the zymogen was less effective at pH 5, while at pH 6 it did not take place even after prolonged incubation. Affinity probe BODIPYgreen-DCG-04 bound strictly to the mature enzyme, while in the pro-enzyme no labelling was observed (Fig. 4). Edman degradation revealed LPTDVD sequence at the N-terminus of the processed EnCL3. Since the processed enzyme had limited stability, the purified pro-enzyme was used for the immunisation of mice.

YrEnCB pre-purified on a Ni-NTA column migrated as a broad band around 50 kDa. After enzymatic deglycosylation and subsequent purification by ion-exchange chromatography, it migrated as a ~29 kDa band in the gels, which correlated with the results of Western and affinity blotting (anti-His-Tag antibody, iBody4) (Fig. 3).

Activity and inhibition assays

Both EnCLs efficiently hydrolysed the FR substrate, and to a lesser extent the LR. No activity towards RR, PR and GPR was detected. The optima for hydrolysis of both substrates were pH 5.5 for yrEnCL1 and pH 6 for yrEnCL3. The preferred substrate for yrEnCB was the

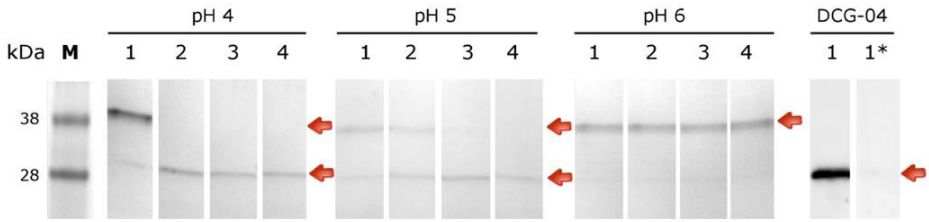

Fig. 4 SDS-PAGE and active site labelling of purified pro-yrEnCL3 after auto-activation at various pH. The 38 kDa zymogen was autocatalytically processed to a 28 kDa form during incubation in 50 mM/100 mM CPB containing 2 mM DTT at 37 °C. After incubation at various pH values, the protein was run in 12% polyacrylamide gels and stained with Coomassie Brilliant Blue (pH 4, pH 5 and pH 6). Lane 1: 15 min incubation; Lane 2: 30 min; Lane 3: 60 min; Lane 4120 min. DCG-04, a 12% gel showing active site labelling of the processed enzyme with fluorescent affinity probe BODIPY green DCG-04. Lane 1: labelling by the affinity probe after 30 min incubation at pH 5; 1*, binding of the affinity probe was blocked by E-64 inhibitor. The 38 kDa zymogen and the 28 kDa processed enzyme are indicated by arrows

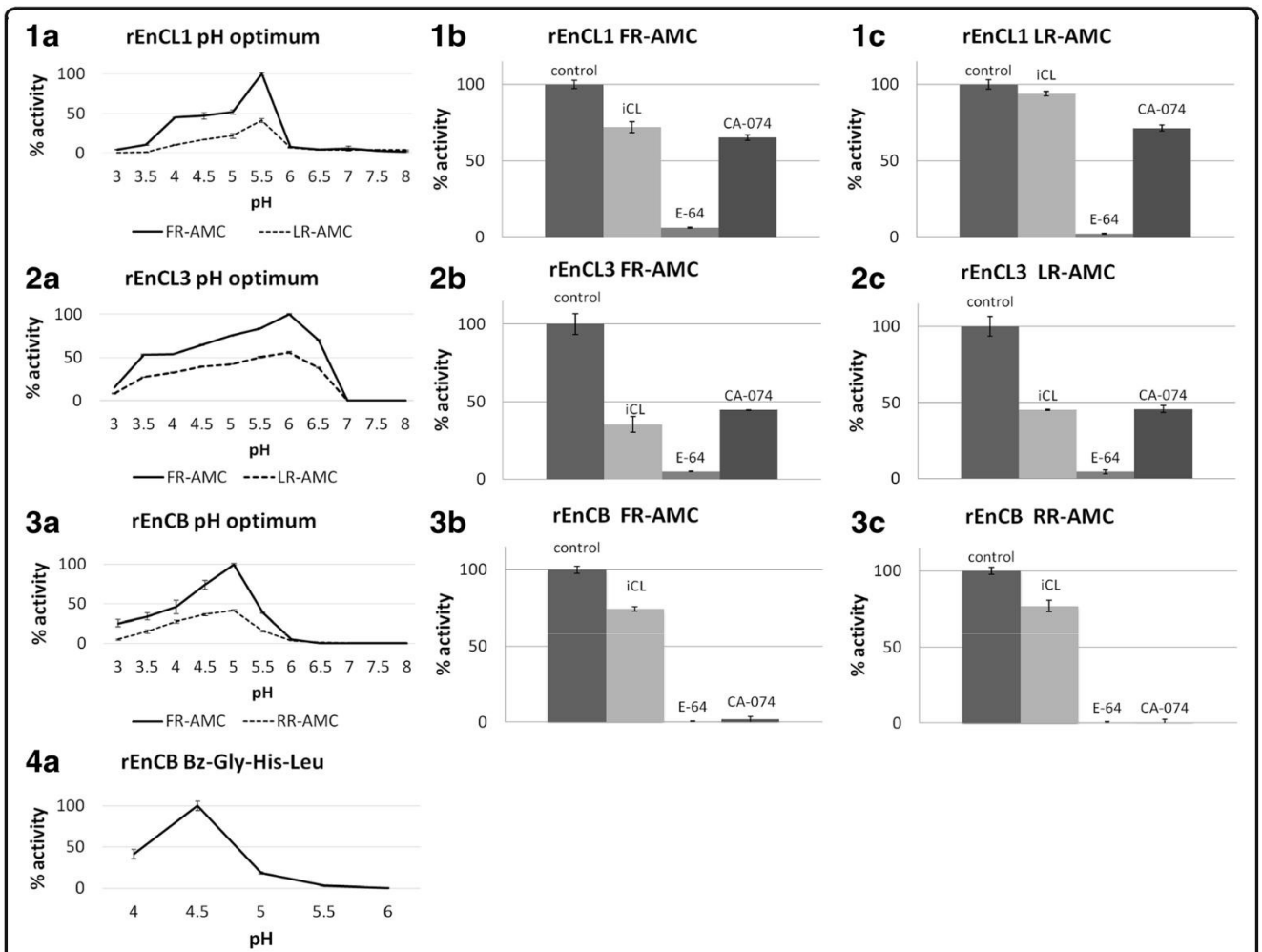

Fig. 5 pH optima of rEnCLs and rEnCB activities, and the effect of inhibitors on enzyme activities. Activity assays were performed in 50 mM/100 mM CPB with 2 mM DTT at pH 3–8. Inhibition assays were run at the pH optima of the particular enzymes. The values are expressed as the percentage of maximum activity in the sample without inhibitor. 1, yrEnCL1; 2, yrEnCL3; 3, yrEnCB; 4, yrEnCB. a pH optima of endopeptidase (1–3) and exopeptidase (4) activities. b inhibition assays with FR substrate. c inhibition assays with LR substrate (1–2) and RR substrate (3). The inhibitors (10 μM) are indicated in the graphs above the columns: E-64, general irreversible inhibitor of clan CA cysteine peptidases; iCL, reversible peptide inhibitor of cathepsin L; CA-074, irreversible inhibitor of cathepsin B

FR, while RR was cleaved less efficiently at the optimum of pH 5 (Fig. 5).

Activity of all three enzymes was effectively inhibited by 10 μM E-64 (the general inhibitor of clan CA family C1 cysteine peptidases) in the presence of either substrate. The cathepsin B-selective inhibitor CA-074 had completely suppressed the activity of yrEnCB at 10 μM and diminished the activity of yrEnCL1 and yrEnCL3 to ~70% and 45–65%, respectively, depending on the substrate. The reversible peptide inhibitor of cathepsin L (10 μM, iCL) partially inhibited all three cathepsins but was most effective in the case of yrEnCL3 with both substrates (c.40% remaining activity). The inhibition rate of yrEnCL1 was comparable with the effect on yrEnCB (c.90–70% remaining activity) (Fig. 5).

The exopeptidase (dipeptidyl peptidase) activity of yrEnCB peaked at pH 4.5, then rapidly dropping to c.40% at pH 4, and to less than 20% at pH 5 (Fig. 5).

Hydrolysis of macromolecular substrates by recombinant cathepsins L and B

Both EnCLs and EnCB efficiently degraded selected protein substrates (bovine haemoglobin and albumin, human collagen type I and fibrinogen) at pH 4.5. EnCLs readily hydrolysed also human IgG, which was poorly cleaved by yrEnCB at pH range 4.5–6.0 (Fig. 6). While the action of yrEnCL1 did not vary substantially between pH 4.5–6.0, yrEnCL3 did not act on the substrates (except fibrinogen) at pH 5.0 or higher (not shown). At pH, hydrolysis of all the macromolecular substrates by yrEnCB failed (not shown).

Western blotting and immunohistochemistry

Specificity of mouse anti-EnCL1, anti-EnCL3, and anti-EnCB antibodies was verified on immunoblots of the soluble worm extracts. The detected bands appeared at ~24 kDa (EnCL1), ~28 kDa (EnCL3) and ~29 kDa (EnCB). Reactions of the particular sera with the appro-priate enzyme bands were highly specific (Fig. 7), so they could be used for immunohistochemistry. Control preimmune sera did not exhibit any reaction.

The reactions of specific antibodies with histological sections of the worms showed the presence of both EnCL1 and EnCL3 in the lumen of the gut (Fig. 8). No reaction was observed in the haematin digestive cells or outside the digestive system.

Anti-yrEnCB antibodies reacted with vesicular structures in the vitelline cells of the adult parasites (Fig. 8). All control sera displayed no reaction.

RNA *in situ* hybridisation

In situ hybridisation with specific antisense-RNA probes showed a localisation of EnCL1 and EnCL3 transcripts specifically in the haematin (digestive) cells of the worms' gastrodermis (Fig. 9). No reactivity was found in other parts of the parasite body, including tegument, vitelline cells and the initial part of the digestive tract. The same pattern of reactivity was obtained when the sense-RNA probes were used. This was not a non-specific reaction, as demonstrated by the strand-specific RT-PCR which confirmed the presence of anti-sense transcripts in *E. nipponicum*. In the negative controls (without any probe), no reaction was observed.

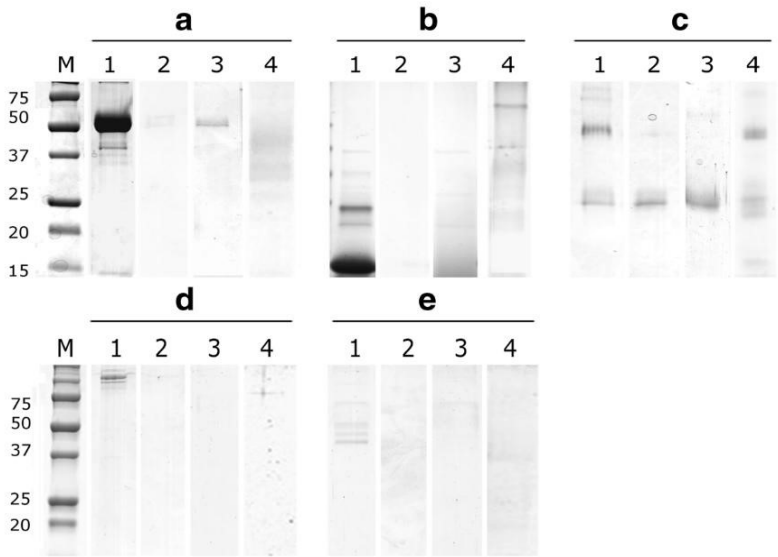

Fig. 6 SDS-PAGE of protein substrates degraded by recombinant EnCL1, EnCL3 and EnCB. Prior to electrophoresis in 12% polyacrylamide gel, the enzymes were incubated overnight with various protein substrates in 50 mM/100 mM CPB pH 4.5 containing 2 mM DTT. a Bovine serum albumin. b Bovine haemoglobin. c Human IgG. d Human type I collagen. e Human fibrinogen. Lane 1: controls (substrates without enzyme); Lane 2: yrEnCL1; Lane 3: yrEnCL3; 4: yrEnCB. Gels were stained with Coomassie Brilliant Blue

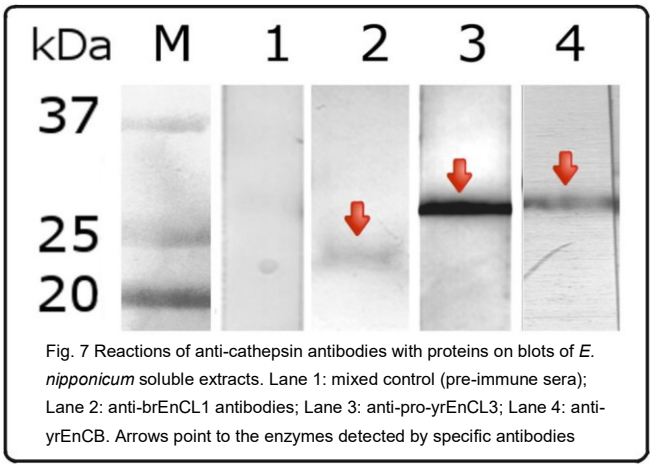

Fig. 7 Reactions of anti-cathepsin antibodies with proteins on blots of *E. nipponicum* soluble extracts. Lane 1: mixed control (pre-immune sera); Lane 2: anti-brEnCL1 antibodies; Lane 3: anti-pro-yrEnCL3; Lane 4: anti-yrEnCB. Arrows point to the enzymes detected by specific antibodies

Discussion

In a previous study [12], we proposed that cathepsin L-like peptidases, in cooperation with cathepsins D and B, play a leading role in the digestion of haemoglobin in the sanguinivorous monogenean *E. nipponicum*. Although the presence of cathepsins L in soluble extracts of the worms was suggested by several methods in that study, the large number of different cathepsin L transcripts (10 in total) found in the transcriptome of the adult life stage was not anticipated. In other blood-feeding helminths, such as *Fasciola hepatica*, multiple cathepsins L are involved in feeding and other important tasks, such as tissue migration, immune evasion, and possibly egg production and metacercarial excystment [48, 49]. Small changes (even single amino acid substitutions) in the primary structure, but especially in the S2 subsite of the active site of those cathepsin L types/isoforms, alter the substrate specificity [50–52]. The production of peptidases with overlapping

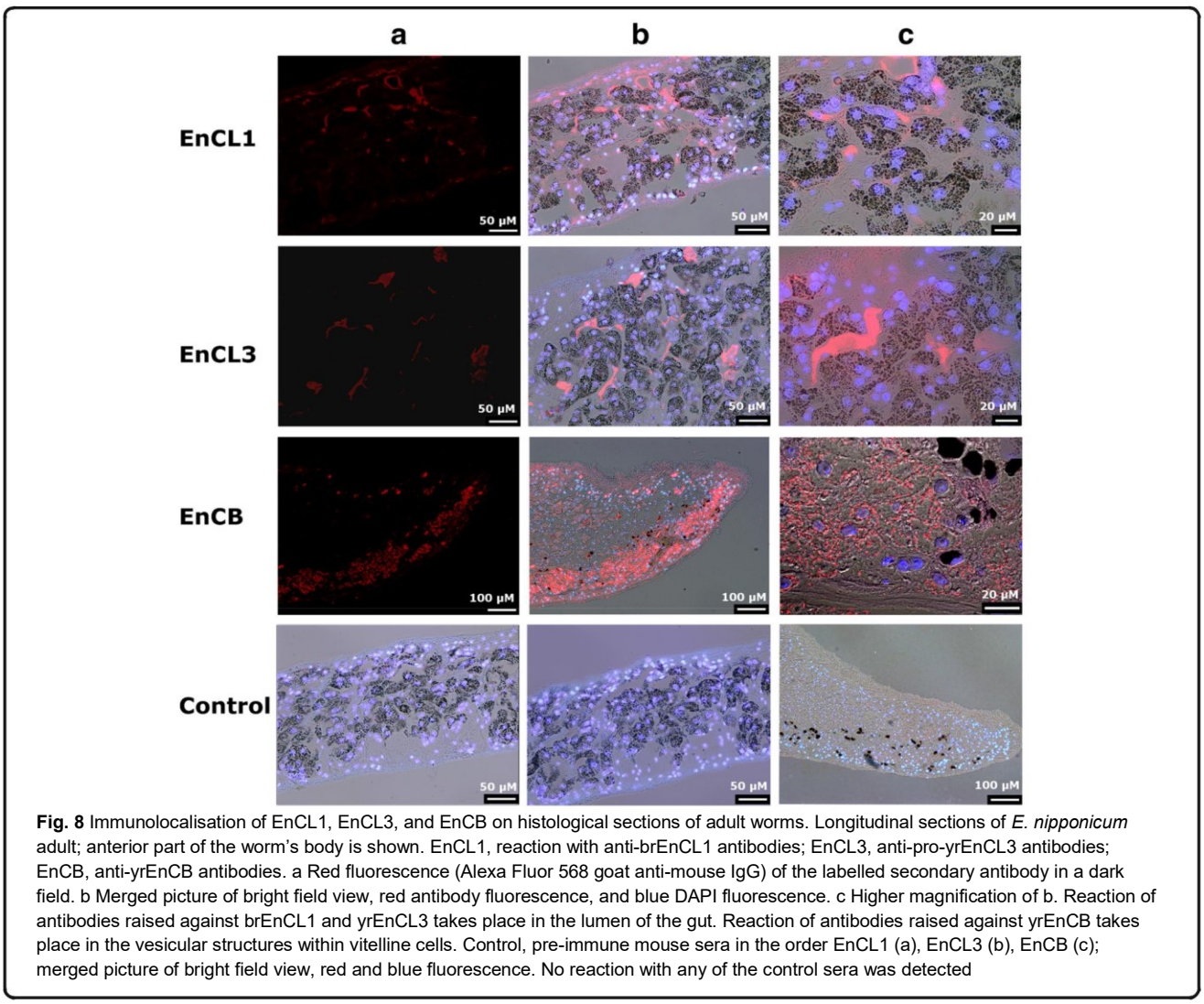

Fig. 8 Immunolocalisation of EnCL1, EnCL3, and EnCB on histological sections of adult worms. Longitudinal sections of *E. nipponicum* adult; anterior part of the worm's body is shown. EnCL1, reaction with anti-brEnCL1 antibodies; EnCL3, anti-pro-yrEnCL3 antibodies; EnCB, anti-yrEnCB antibodies. a Red fluorescence (Alexa Fluor 568 goat anti-mouse IgG) of the labelled secondary antibody in a dark field. b Merged picture of bright field view, red antibody fluorescence, and blue DAPI fluorescence. c Higher magnification of b. Reaction of antibodies raised against brEnCL1 and yrEnCL3 takes place in the lumen of the gut. Reaction of antibodies raised against yrEnCB takes place in the vesicular structures within vitelline cells. Control, pre-immune mouse sera in the order EnCL1 (a), EnCL3 (b), EnCB (c); merged picture of bright field view, red and blue fluorescence. No reaction with any of the control sera was detected

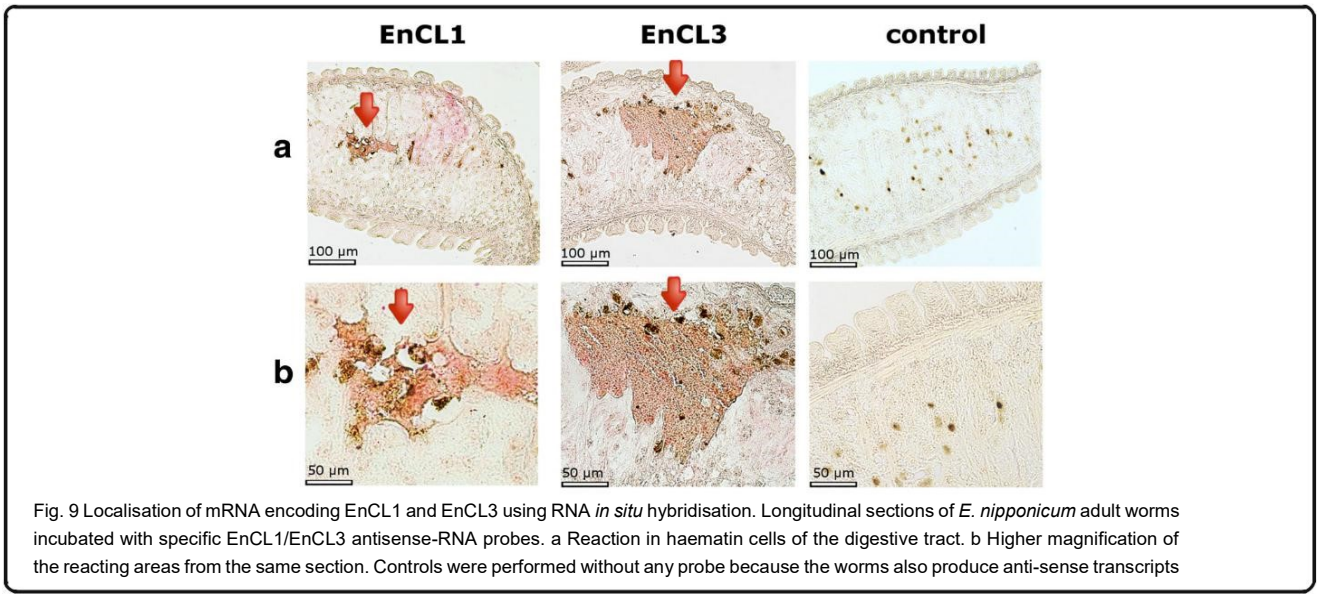

Fig. 9 Localisation of mRNA encoding EnCL1 and EnCL3 using RNA *in situ* hybridisation. Longitudinal sections of *E. nipponicum* adult worms incubated with specific EnCL1/EnCL3 antisense-RNA probes. a Reaction in haematin cells of the digestive tract. b Higher magnification of the reacting areas from the same section. Controls were performed without any probe because the worms also produce anti-sense transcripts

specificities enables the parasite to degrade host-derived macromolecules more efficiently.

All but one of the EnCLs characterised in this study possess a typical catalytic triad of cysteine peptidase active site (Cys/His/Asn); the exception is EnCL2, in which the active site cysteine (Cys25) is replaced by serine (Ser25). However, as described for congopain (cathepsin L-like peptidase) in *Trypanosoma congolense*, Cys-to-Ser mutation need not necessarily affect the proteolytic ability of an enzyme [53]. It has been also suggested that Cys-to-Ser mutation in the cysteine peptidases of parasites might be a way to moderate oxidation of the active site in case parasites have to deal with an oxygen-rich environment, such as blood or external environment [17]. In addition to this substitution in the active site, EnCL2 has in its S2 pocket Trp at position 67 (papain numbering used throughout the Discussion), which makes it a potential collagenase/elastase. Presence of the bulky aromatic residues Tyr67/Trp67 is typical of *F. hepatica* cathepsins L2 and L3, and of human cathepsin K, which all efficiently cleave collagen and elastin of the connective tissues due to their ability to accommodate Pro residues at P2 position in the substrates via a stabilising interaction with Tyr67/Trp67 [50–52, 54, 55]. However, the functionality and possible role of EnCL2 has not yet been studied. Its expression rate in adult worms is very low, which indicates that the enzyme is predominantly employed by another life stage (the larva: oncomiracidium; or the post-oncomiracidial stage: diporpa), or in some less prominent processes unconnected with blood digestion.

Since the composition of the S2 pocket is the key determinant of substrate specificity of papain-like cysteine peptidases [56], the amino acid sequences of EnCLs were aligned (Additional files 4, 5 and 6) and their S2 residues (positions 67, 133, 157, 205) compared. The comparison of various EnCLs revealed the existence of three groups differing in the polarity and charge of the S2 pocket. In EnCL2, the S2 pocket is strongly hydrophobic and carries a positive charge. EnCL1, EnCL3, EnCL4 and EnCL5 have a moderately hydrophobic S2 pocket with a neutral charge. The least hydrophobic S2 pocket is found in the closely related EnCL6 orthologs; it has a neutral charge in EnCL6a and 6b, and a negative charge in EnCL6e (for EnCL6c and 6d the complete data are not available). The presence of a negatively charged Asp205 in the S2 pocket may endow EnCL6e with the ability to accommodate a positively charged Arg at P2 position of the substrate, which would give this cathepsin L ortholog a cathepsin B-like substrate specificity. Additionally, the group of EnCL6 orthologs is atypical among cathepsins L by having an amphipathic Gly133 in the S2 pocket. This feature is, however, typical of cathepsins S, and Gly133 can also be found in a few other, yet unclassified, clan CA family C1 peptidases, such as the plerocercoid growth factor of the larval stage of the tapeworm *Spirometra* (Genbank: BAB62718.1) (see the alignment in Additional file 6). Nonetheless, specificity of the S2 pocket in papain-like peptidases is largely determined by the residue 205. Cathepsins S contain at this position a large aromatic non-polar Phe, which implies a short and more restricted pocket. Cathepsins L, on the other hand, usually include at this position branched aliphatic residues, which enables the accommodation of larger aromatic residues [57]. This was not observed in EnCL6a, which contains a polar Ser205. It thus seems that diversity in the composition of S2 subsites of *E. nipponicum* cathepsins L gives the parasite a wide array of efficient proteolytic tools with diverse affinities to potential P2 positions of protein substrates.

Phylogenetic analysis seems to support a supposition, inferred from the structural data and relative rates of expression, that in *E. nipponicum*, cathepsins L may be involved in various biological processes. The two functionally characterised enzymes, EnCL1 and EnCL3, which seem to play a central role in blood digestion by the parasite, form a monophyletic group with EnCL5. The other monophyletic clade formed by EnCL2, EnCL4 and EnCL6 is relatively distant from the first group, thus suggesting involvement in other processes or, in the case of the least proportionally represented transcripts such as EnCL2 and EnCL6a, a more sub-stantial expression of these orthologs in other life stages. The presence of myxosporean-like cathepsins L within the otherwise purely neodermatan clade may lead to a speculation about a lateral gene transfer, since myxosporeans share their fish hosts with monogeneans. Investigation of this possibility would, however, require a thorough analysis that is beyond the scope of the present study. Moreover, it should also be noted that the evolution of genes need not follow the branching structure of species-level evolutionary trees due to mechanisms such as gene duplication and independent gene loss. Particularly, genes of parasites are prone to phylogenetic artefacts caused by attraction between long branches, an effect we tried to mitigate by using site-heterogeneous mixture models [58].

Surprisingly, only one transcript coding for cathepsin B was found in the transcriptome of adult *E. nipponicum*. Amino acid sequence of EnCB had shown that this enzyme is a typical representative of cathepsins B, including its active site, composition of the S2 subsite, and the occluding loop responsible for exopeptidase activity. It also contains a 'haemoglobinase motif' assigned to helminth cathepsins B with substrate specificity for haemoglobin [47]. In the case of EnCB, this motif is slightly modified in its C-terminal region, where the original sequence described for helminth haemoglobinases 'DWGE' is replaced by 'EWGD' (Additional file 8). Nevertheless, as we experimentally verified, these purely conservative amino acid substitutions did not affect the ability of EnCB to cleave haemoglobin. Similar substitutions in the motif were described for schistosome cathepsins B2 (*S. mansoni* and *Trichobilharzia regenti*) that were found only outside the parasites' digestive system; it is assumed that their functions do not include the digestion of haemoglobin [59, 60]. In contrast to EnCB, *T. regenti* CB2 degraded haemoglobin at a negligible rate.

In the process of heterologous functional expression in *P. pastoris*, YrEnCL1 had undergone auto-activation in the cultivation medium by cleaving-off a significant proportion of its pro-sequence and migrated on SDS-PAGE as a 28 kDa band (the value thus differed from the expected ~35 kDa and ~24 kDa of the zymogen and fully processed enzyme, respectively). Other, less abundant bands also occasionally appeared in the gel; mass spectrometry had confirmed they were fragments of yrEnCL1, generated probably by auto-degradation (data not shown). This is consistent with the very limited stability of the partially processed enzyme observed during purification. Mono-specific anti-yrEnCL1 antibodies did, however, react with a ~24 kDa band on blots of soluble worm extracts, indicating a full processing of EnCL1 under natural conditions. The yrEnCL3, on the other hand, was obtained as a relatively stable zymogen. It was capable of autocatalytic activation under acidic conditions, but the size of the resulting product, ~28 kDa, was also somewhat higher than the predicted mass of ~24.1 kDa of the mature enzyme. Nevertheless, Edman degradation confirmed an amino acid sequence at the N-terminus of the processed yrENCL3 which had been predicted by bioinformatical methods, thus indicating that the processing was complete. Monospecific anti-yrEnCL3 antibodies reacted similarly with a ~28 kDa band on blots of soluble worm extracts. Given that the EnCL3 does not possess any N-glycosylation motifs, these differences cannot be explained by either natural glycosylation of the enzyme or by N-hyperglycosylation from *P. pastoris* in the case of the recombinant enzyme. It could be speculated that the observed shift in Mw of the enzyme was caused by disruption of disulphide bonds stabilizing cathepsin L structure, which resulted in mobility change during electrophoresis [60, 61]. Although EnCL3 possesses one potential O-glycosylation site in the mature part, the prediction software on the NetOglyc server produces predictions of mucin type GalNAc O-glycosylation sites in mammalian proteins, and the situation may be different in invertebrates. Moreover, we are not aware of any descriptions in literature of a cathepsin L with experimentally confirmed O-glycosylation.

S2 subsite specificity of yrEnCL1 and yrEnCL3 was tested over a range of pH with a set of oligopeptide synthetic substrates, varying in their Phe, Leu, Arg, and Pro at P2 position. As predicted from the composition of the S2 pockets, the enzymes were not able to cleave substrates containing Pro or Arg in this position. Consistently with most papain-like peptidases, both enzymes preferred at this position the bulky hydrophobic aromatic Phe and the branched aliphatic Leu, in order Phe > Leu [56]. The enzymes did, however, differ in their pH profiles of activity towards substrates, so that whereas yrEnCL1 was active only in a narrow pH range with a pH optimum at 5.5, yrEnCL3 had a broader pH activity profile with an optimum at pH 6. The slightly acidic pH optima correspond to those of cathepsin L-like peptidases of other blood-feeding parasites [61–64].

As expected, activity of both of the recombinant EnCLs was inhibited by the general papain-like cysteine

peptidase inhibitor E-64. Surprisingly, however, the iCL, a highly potent amphiphilic reversible peptide inhibitor of human cathepsin L [65], had but a little effect on the enzymes' activity, although it has Leu at the P2 position. One can thus hypothesise that a lower affinity to Trp at P1 position, found also in, e.g. cathepsin L of *Leishmania mexicana* [56], might account for this result. Remarkably, CA-074 (a specific inhibitor of cathepsin B) also partially reduced the activity of both cathepsins L. It has been reported elsewhere [66, 67], however, that CA-074 can decrease the activity of cathepsins L under reducing conditions and that could also explains our results.

Activity profiles, pH optimum, specificity to peptide substrates, inhibition by CA-074 and exopeptidase activity clearly show that EnCB is a typical member of cathepsins B group. The pro-enzyme had undergone auto-activation either in the yeast medium or during purification, and enzymatic deglycosylation demonstrated hyperglycosylation of the recombinant enzyme by yeast, which corre-sponds to the presence of an N-glycosylation motif in the EnCB molecule. Anti-yrEnCB monospecific antibodies detected native EnCB on blots of soluble worm extracts at ~29 kDa, which is adequate to the expected size of the processed enzyme which was probably slightly glycosylated naturally.

Both recombinant EnCL1 and EnCL3 efficiently degraded various blood proteins and type I collagen of the connective tissue. Unlike synthetic peptide substrates, macromolecular substrates (except for fibrinogen) were cleaved only at pH 4.5 by yrEnCL3, while yrEnCL1 hydrolysed all macro-molecular substrates at pH 4.5–6.0. Activity at low pH values is in accordance with the site of expression of both genes encoding the enzymes that was localised by in situ hybridisation in the haematin (digestive) cells, and with the presumed intracellular digestion of haemoglobin in the endolysosomal vesicles where pH below 4.5 would be expected [68]. Both enzymes, however, were also immunolocalised in the lumen of the parasite's gut. For other blood-feeding helminths, it has been estimated that the pH in the gut lumen is only slightly acidic (c.5.5–6.5) [43, 69, 70], and digestion therefore takes place in a local microenvironment between the lamellae of the digestive cells where the pH is more acidic (~4.5), as documented for *F. hepatica* [71]. Haematin cells of another studied blood-feeding diplozoid monogenean, *Paradiplozoon homoion*, also contain numerous lamellae on the side facing the lumen of the gut. Host erythrocytes are lysed in proximity of the haematin cells and released haemoglobin is taken in by lamellae of the cells via pino-cytosis [9]. These facts, together with presence of EnCL1 and EnCL3 in excretory/secretory products of the worms [12], and with the results of immunolocalisation, imply that in diplozoid monogeneans extracellular, luminal diges-tion of blood can take place. Moreover, if the enzymes are released from the gut lumen into host circulation, they could potentially participate in some host-parasite interactions by affecting, e.g. fibrinolysis (they cleaved fibrinogen in vitro) or immune response (degradation of immunoglobulins).

Surprisingly, both EnCLs hydrolysed collagen type I. This ability was attributed in human body primarily to family C1 lysosomal cysteine peptidase cathepsin K. Because of acceptance of Pro in the P2 position, cathepsin K can completely degrade collagen by cleaving within the repeated Gly-Pro-Xaa motifs that occur in the triple helix of collagen [54]. The ability to accommodate substrates with P2 proline has also been observed in some cathepsins L of *F. hepatica*, namely FhCL2, FhCL3 and FhCL5 [50, 55, 72]. On the other hand, neither yrEnCL1 nor yrEnCL3 cleaved synthetic substrates GPR and PR. So, though both enzymes degraded native collagen, they probably do not possess intrahelical activity and this likely makes them less effective collagenases. Such a phenomenon has been described in human cathepsin L and FhCL1 [51, 54].

During the localisation of expression of EnCL1 and EnCL3 by RNA in situ hybridisation, sense-RNA probes used as controls had shown similar reaction as antisense probes. It is known from the literature that over 30% of genes transcribed in humans also have antisense transcription [73], and antisense transcripts have been described for genes in a diverse group of eukaryotes [74–76]. The use of a specific strand RT-PCR has revealed that the EnCL1 and EnCL3 encoding genes also have naturally present antisense transcripts. Since the cathepsin L-like peptidases play important roles during the life of the monogeneans, antisense transcripts may be associated with the regulation of gene transcription [77].

It has originally been thought that EnCB is also involved in the proteolytic cascade of blood digestion in *E. nipponicum* [12]. In haematophagous parasites, many cathepsins B, together with other peptidases of the digestive cascades, function as important enzymes with a mixed mode of action, as endopeptidases that cleave proteins into smaller fragments and as exopeptidases (peptidyldipep-tidases, carboxypeptidases) that remove dipeptides from the C-termini of those protein fragments due to the presence of the occluding loop [78]. Immunofluorescence with monospecific anti-yrEnCB antibodies clearly showed the localisation of EnCB in vesicular structures in vitelline cells, but its presence in the digestive tract has not been detected at all. We could thus speculate on its function in vitellogenesis. The presence of cysteine peptidases in the reproductive system has been detected also in other helminths [79–82], but their role in worms' reproduction is still speculative. The lack of exopeptidase activity in the gut due to the absence of cathepsin B may be compensated by the activity of cathepsins C; at least three different transcripts of these

dipeptidyl peptidases (aminopeptidases) were found in *E. nipponicum* transcriptome (not shown).

Conclusions

To our knowledge, this work represents the first comprehensive exploration and functional annotation of a group of peptidases from a monogenean transcriptome. It also describes the very first functional characterisation of recombinant cysteine peptidases of monogenean origin. The lack of experimental data from other monogeneans, particularly those of a high economic importance (such as *Gyrodactylus salaris*, *Benedeni*a spp.), unfortunately precludes the possibility of drawing any comparisons. We have shown that the haematophagous monogenean *E. nipponicum* possesses a wide range of endopeptidases of cathepsin L type, most of which are highly expressed in adult worms. Some of these enzymes possess structural features rather unusual in cathepsins L. It holds true especially for the composition of S2 subsite of the active site, which is among the studied enzymes rather variable and includes even substitutions typical more of cathepsins B and cathepsins S. This expands our understanding of the structural diversity of cathepsins L in general. In contrast to the rich world of cathepsins L, the presence of just one cathepsin B in the worms was somewhat surprising. Despite its ability to hydrolyse haemoglobin *in vitro*, EnCB, due to its localisation in the vitelline cells of the parasite, does not seem to be involved in digestion. On the other hand, functional characterisation and localisation has revealed that EnCL1 and EnCL3 are important in the digestion of blood. Despite previous claims, it seems that the digestive process takes place not only within the intracellular lysosomal cycle, but at least in part also outside the gastrodermis, in the lumen of the gut. From there, the EnCLs may be eventually released into the blood circulation of the host and participate in elicitation of immune response and/or immunomodulation. Genomic data, currently available only for the mucophagous polyonchoinean *G. salaris* and the haematophagous heteronchoinean *Protopolystoma xenopodis*, and transcriptomic data from the mucophagous polyonchoinean *Neobenedenia melleni*, open a possibility for future comparative biochemical research of the two trophic strategies characteristic of the monogeneans. For example, preliminary data explorations show that mucus feeders may employ elastase-like serine peptidases that have not been found in blood-feeders (Mikeš, unpublished). Further research is likely to provide data interesting from an evolutionary point of view and information essential to understanding parasite-host interactions on a molecular level in this group of parasites, which would be of interest especially in context of pathogenesis and fish immunity. Although parasite peptidases have often been seen as promising targets for the development of vaccines or new inhibitor-based remedies, in the case of monogeneans we are somewhat sceptical regarding this possibility. The main reasons why we do not believe this is a promising direction are the likely costs linked to a complicated handling of large numbers of individual fish hosts in aquaculture, difficulties with administering a correct dosage of medica-tion (peptidase inhibitors) in an aquatic environment (assuming that a peroral formula would even be available) and the necessity of repeated administering in order to reach a lethal effect on the parasites. At the moment, most methods applied against monogeneans rely on mass treatment of farmed fish by readily available, low-cost chemical biocides, such as hydrogen peroxide, or antihelminthics, such as praziquantel [83].

Additional files

Additional file 1: Primers for the expression of yrEnCL1, yrEnCL3, and yrEnCB in *P. pastoris*. (PDF 239 kb)

Additional file 2: Primers for the expression of brEnCL1 in *E. coli.* (PDF 83 kb)

Additional file 3: Specific EnCL1/EnCL3 primers for the synthesis of probes for RNA in situ hybridisation. (PDF 84 kb)

Additional file 4: Amino acid sequence alignment of *E. nipponicum* cathepsins L with cathepsins L of *F. hepatica*. Signal peptides and pro-sequences were omitted. Numbers at the end of the lines show amino acid numbering of particular mature parts of the enzymes. Catalytic triad of the active site (C, H, N) is marked by triangles. Conserved motifs around active site residues are shaded in grey. Residues within the S2 subsite of the active site involved in determining the substrate specificity are shaded in black and indicated with numbers (papain numbering). Tripeptides of potential N-glycosylation sites are boxed. Predicted O-gly-cosylated residues are marked by grey squares. The alignment was made using the sequences of *F. hepatica* mature cathepsins L1 (GenBank: AAT76664.1) and L3 (GenBank: CAC12807.1). (PDF 1949 kb)

Additional file 5: Multiple alignment of all the complete/incomplete amino acid sequences of *E. nipponicum* cathepsins L inferred from the transcriptome of adult worms. Signal peptides are shaded in dark grey, position of the pro-region cleavage site is marked by arrows. ERFNIN- and GNFD-like motifs are underlined and indicated by underlined headings. The catalytic triad of the active site (C, H, N) is marked by triangles. Conserved motifs around active site residues are shaded in light grey. Residues within the S2 subsite of the active site involved in determining the substrate specificity are shaded in black and indicated with numbers (papain numbering). Tripeptides of potential N-glycosylation sites are boxed. Predicted O-glycosylated residues are marked by grey squares. (PDF 5783 kb)

Additional file 6: Composition of S2 subsites of the active sites shown in the alignment of parts of amino acid sequences of *E. nipponicum* cathepsin L orthologs 6a, 6b and 6e with human cathepsin S and with plerocercoid growth factor of Spirometra tapeworm. The catalytic triad of the active site (C, H, N) is marked by triangles. Conserved motifs around active site residues are shaded in grey. Residues within the S2 subsite of the active site involved in determining the substrate specificity are shaded in black and indicated with numbers (papain numbering). Abbreviations: PGF, *Spirometra erinaceieuropaei* plerocercoid growth factor (Genbank: BAB62718.1); HsCS, human cathepsin S (Genbank: AAC37592). (PDF 1406 kb)

Additional file 7: Phylogram showing relationships between *E. nipponicum* cathepsins L and cathepsins L of other selected organisms. Unrooted maximum-likelihood tree of selected *E. nipponicum* cathepsins L inferred using the best-fit model (LG + C60 + G). Ultrafast bootstrap supports and posterior probabilities are shown. The leaf descriptions contain the sequence ID, genus and taxonomic placement. (PDF 4260 kb)

Additional file 8: Amino acid sequence alignment of *E. nipponicum* cathepsin B with cathepsins B of *S. mansoni*. Whole zymogens including a signal peptide were aligned. Numbers at the end of the lines show amino acid numbering of the mature parts of particular enzymes. Position of the pro-region cleavage site is marked by an arrow. The catalytic triad of the active site (C, H, N) is marked by triangles. Conserved motifs around active site residues are shaded in grey. Tripeptides of potential N-glycosylation sites are boxed. Predicted O-glycosylated residues are marked by grey squares. The occluding loop typical of cathepsins B is underlined. The 'haemoglobinase motif' ascribed to cathepsins B with an assumed function in haemoglobinolysis in blood-feeding helminths, is shaded in black (it is modified in SmCB2 and EnCB). Residues within the S2 subsite of the active site involved in determining the substrate specificity are marked by a black dot and indicated with number (papain numbering). The alignment was made using sequences of *Schistosoma mansoni* cathepsins B1 (GenBank: CAD44624.1) and B2 (GenBank: XP_018651608.1). (PDF 1923 kb)

Abbreviations

AMC: 7-amino-4-methylcoumarin; BSA: Bovine serum albumin; Bz-Gly-His-Leu: Benzoyl-glycinyl-histidinyl-leucine; CA-074: N-[L-3-trans-propylcarbamoyloxirane-2-carbonyl]-Ile-Pro-OH; CBB: Coomassie Brilliant Blue; CPB: Citrate/phosphate buffer; DCG-04: fluorescent labelled probe, derivate of inhibitor E-64; DTT: Dithiothreitol; E-64: (L-trans-epoxysuccinyl–leucylamido [4-guanidino] butane); EnCB: Eudiplozoon nipponicum cathepsin B; EnCL: Eudiplozoon nipponicum cathepsin L; ESP: Excretory secretory products; FPLC: Fast protein liquid chromatography; FR: Substrate for serine and cysteine peptidases Z-Phe-Arg-AMC; GPR: Substrate for serine peptidases Z-Gly-Pro-Arg-AMC; His-tag: polyhistidine-tag (6xHis); ICL: Reversible inhibitor of cathepsin L(Arg-Lys-Leu-Leu-Trp-NH2); LR: Substrate for serine and cysteine peptidases Z-Leu-Arg-AMC; MES: (2-N-morpholino) ethanesulfonic acid; Ni-NTA: Nickel-nitrilotriacetic acid; PBS- TX100: Phosphate buffered saline containing 0.3% Triton X100; PBS: Phosphate buffered saline; PBS-T: Phosphate-buffered saline containing 0.05% Tween 20; PR: Substrate for serine peptidases Z-Pro-Arg-AMC; PVDF: Polyvinylidene difluoride; RACE-PCR: Rapid amplification of cDNA ends; RR: Substrate for cathepsin B Z-Arg-Arg-AMC; RT: Room temperature; RT-PCR: Reverse transcription-PCR; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; SSC: Saline-sodium citrate buffer

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Availability of data and materials

Transcripts from the assembled transcriptome of adult *E. nipponicum* worms have been deposited at DDBJ/EMBL/GenBank under the accession GFYM00000000. The version described in this paper is the first version, GFYM01000000.

Authors' contributions

LJ produced recombinant cathepsins L, performed their functional characterisation, *in situ* hybridisation and immunohistochemistry, participated in the bioinformatic annotation of enzyme structures, data interpretation, and in manuscript writing. HD produced the recombinant cathepsin B, performed its functional characterisation and immunohistochemistry, participated in the bioinformatic annotation of enzyme structure, data interpretation, and in manuscript writing. JD participated in the expression of recombinant cathepsins L and their biochemical characterisation. MK took part in designing the study and in data interpretation. LU was involved in designing of RNA probes and in the process of *in situ* hybridization. JV assembled the transcriptome, mined the sequence data and evaluated expression rates of the enzymes. VZ performed the phylogenetic analyses. LM conducted the work, prepared the study design, and participated in the purification of recombinant enzymes, bioinformatic annotation, interpretation of data, and in the production of the final draft of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The maintenance and care of experimental animals was carried out in accordance with European Directive 2010/63/EU, and according to Czech law (246/1992 and 359/2012) for biomedical research involving animals. Experiments were performed with a legal consent of the relevant institutional animal research ethics committees, namely the Professional Ethics Committee of the Faculty of Science, Charles University in Prague and the Branch for Research and Development of the Ministry of Education, Youth and Sports of the Czech Republic. The animal facility, its equipment, animal welfare and accompanying services, including maintenance of experimental animals, have been approved by the Branch of Animal Commodities of the Ministry of Agriculture of the Czech Republic (approval no. 13060/2014-MZE-17214).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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References

1. Denis A, Gabron C, Lambert A. Presence en France du deux parasites d'origine Est-asiatique*: Diplozoon nipponicum* Goto 1891 et Bothriocephalus acheilognathi Yamaguti, 1934 (Cestoda) chez Cyprinus carpio (Teleostei, Cyprinidae). Bull Fr Piscic. 1983;289:128–34.
2. Matějusová I, Koubková B, D' Amelio S, Cunningham CO. Genetic characterization of six species of diplozoids (Monogenea; Diplozoidae). Parasitology. 2001;123:465–74.
3. Fisheries and Aquaculture Department. About us - Fisheries and Aquaculture Department.. http://www.fao.org/fishery/about/en Updated 17 March 2017.
4. Kawatsu H. Studies on the anemia of fish-IX, hypochromic microcytic anemia of crucian carp caused by infestation with a trematode, *Diplozoon nipponicum*. Bull Japan Soc Sci fish. 1978;44:1315–9.

5 Smyth JD, Halton DW. The Monogenean - functional morphology. In: Smyth JD, Halton DW, editors. The Physiology of Trematodes. Cambridge: Cambridge University Press; 1983. p. 250–88.

6 Smyth JD, Halton DW. The Monogenean – physiology. In: Smyth JD, HaltonDW, editors. The Physiology of Trematodes. Cambridge: Cambridge University Press; 1983. p. 289–320.

7 Hodová I, Matějusová I, Gelnar M. The surface topography of *Eudiplozoon nipponicum* (Monogenea) developmental stages parasitizing carp (Cyprinus carpio L.). Cent Eur J Biol. 2010;5:702–9.

8 Valigurová A, Hodová I, Sonnek R, Koubková B, Gelnar M. *Eudiplozoon nipponicum* in focus: monogenean exhibiting a highly specialized adaptation for ectoparasitic lifestyle. Parasitol Res. 2011;108:383–94.

9 Konstanzová V, Koubková B, Kašný M, Ilgová J, Dzika E, Gelnar M. Ultrastructure of the digestive tract of *Paradiplozoon homoion* (Monogenea). Parasitol Res. 2015;114:1485–94.

10 Sonenshine D. Biology of ticks. Volume 1. New York: Oxford University Press; 1991.

11 Sojka D, Franta Z, Horn M, Caffrey CR, Mareš M, Kopáček P. New insights into the machinery of blood digestion by ticks. Trends Parasitol. 2013;29:276–85.

12 Jedličková L, Dvořáková H, Kašný M, Ilgová J, Potěšil D, Zdráhal Z, et al. Major acid endopeptidases of the blood-feeding monogenean *Eudiplozoon nipponicum* (Heteronchoinea: Diplozoidae). Parasitology. 2016;143:494–506.

13 Sajid M, JH MK. Cysteine proteases of parasitic organisms. Mol Biochem Parasitol. 2002;120:1–21.

14 Williamson AL, Brindley PJ, Knox DP, Hotez PJ, Loukas A. Digestive proteases of blood-feeding nematodes. Trends Parasitol. 2003;19:417–23.

15 Williamson AL, Lecchi P, Turk BE, Choe Y, Hotez PJ, JH MK, et al. A multi-enzyme cascade of hemoglobin proteolysis in the intestine of blood-feeding hookworms. J Biol Chem. 2004;279:35950–7.

16 Delcroix M, Sajid M, Caffrey CR, Lim KC, Dvořák J, Hsieh I, et al. A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. J Biol Chem. 2006;281:39316–29.

17 Atkinson H, Babbitt P, Sajid M. The global cysteine peptidase landscape in parasites. Trends Parasitol. 2009;25:573–81.

18 Kašný M, Mikeš L, Hampl V, Dvořák J, Caffrey CR, Dalton JP, et al. Peptidases of trematodes. Adv Parasitol. 2009;69:205–97.

19 Robinson MW, Dalton JP. Cysteine proteases of pathogenic organisms (Vol. 712). Boston: Springer; 2011.

20 McVeigh P, Maule AG, Dalton JP, Robinson MW. *Fasciola hepatica* virulence-associated cysteine peptidases: a systems biology perspective. Microbes Infect. 2012;14:301–10.

21 NCBI Resource Coordinators NR. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 2016;44:D7–19.

22 Rawlings ND, Barrett AJ, Finn R. Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res. 2016; 44:D343–50.

23 The UniProt Consortium. UniProt: the universal protein knowledgebase. Nucleic Acids Res. 2017;45:D158–69.

24 Boutet E, Lieberherr D, Tognolli M, Schneider M, Bansal P, Bridge AJ, et al. UniProtKB/Swiss-Prot, the manually annotated section of the UniProt KnowledgeBase: how to use the entry view. Methods Mol Biol. 2016;1374:23–54.

25 Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, et al. The protein data bank. Nucleic Acids Res. 2000;28:235–42.

26 Mashima J, Kodama Y, Kosuge T, Fujisawa T, Katayama T, Nagasaki H, et al. DNA data bank of Japan (DDBJ) progress report. Nucleic Acids Res. 2016;44: D51–7.

27 Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal omega. Mol Syst Biol. 2011;7:539.

28 Bray NL, Pimentel H, Melsted P, Pachter L. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol. 2016;34:525–7.

29 Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011;8:785–6.

30 Artimo P, Jonnalagedda M, Arnold K, Baratin D, Csardi G, de Castro E, et al. ExPASy: SIB bioinformatics resource portal. Nucleic Acids Res. 2012;40:W597–603.

31 Steentoft C, Vakhrushev SY, Joshi HJ, Kong Y, Vester-Christensen MB, KT-BG S, et al. Precision mapping of the human O-GalNAc glycoproteome through Simple Cell technology. EMBO J. 2013;32:1478–88.

32 Pruitt KD, Tatusova T, Maglott DR. NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res. 2007;35:D61–5.

33 Howe KL, Bolt BJ, Shafie M, Kersey P, Berriman M. WormBase ParaSite - a comprehensive resource for helminth genomics. Mol Biochem Parasitol. 2017;215:2–10.

34 Frickey T, Lupas A. CLANS: a Java application for visualizing protein families based on pairwise similarity. Bioinformatics. 2004;20:3702–4.

35 Li W, Godzik A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 2006;22:1658–9.

36 Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol Biol Evol. 2013; 30:772–80.

37 Criscuolo A, Gribaldo S. BMGE (block mapping and gathering with entropy): a new software for selection of phylogenetic informative regions from multiple sequence alignments. BMC Evol Biol. 2010;10:210.

38 Nguyen L-T, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 2015;32:268–74.

39 Caffrey CR, Salter JP, Lucas KD, Khiem D, Hsieh I, Lim K-C, et al. SmCB2, a novel tegumental cathepsin B from adult *Schistosoma mansoni*. Mol Biochem Parasitol. 2002;121:49–61.

40 Dvořák J, Delcroix M, Rossi A, Vopálenský V, Pospíšek M, Šedinová M, et al. Multiple cathepsin B isoforms in schistosomula of *Trichobilharzia regenti*: identification, characterisation and putative role in migration and nutrition. Int J Parasitol. 2005;35:895–910.

41 Wang B-L, Xu Y, Wu C-Q, Xu Y-M, Wang H-H. Cloning, expression, and refolding of a secretory protein ESAT-6 of *Mycobacterium tuberculosis*. Protein Expr Purif. 2005;39:184–8.

42 Greenbaum D, Baruch A, Hayrapetian L, Darula Z, Burlingame A, Medzihradszky KF, et al. Chemical approaches for functionally probing the proteome. Mol Cell Proteomics. 2002;1:60–8.

43 Sajid M, JH MK, Hansell E, Mathieu MA, Lucas KD, Hsieh I, et al. Functional expression and characterization of *Schistosoma mansoni* cathepsin B and its trans-activation by an endogenous asparaginyl endopeptidase. Mol Biochem Parasitol. 2003;131:65–75.

44 Quack T, Knobloch J, Beckmann S, Vicogne J, Dissous C, Grevelding CG. The formin-homology protein SmDia interacts with the Src Kinase SmTK and the GTPase SmRho1 in the gonads of *Schistosoma mansoni*. PLoS One. 2009;4: e6998.

45 ECH H, Donaldson ME, Saville BJ. Detection of antisense RNA transcripts by strand-specific RT-PCR. Methods Mol Biol. 2010;630:125–38.

46 Šácha P, Knedlík T, Schimer J, Tykvart J, Parolek J, Navrátil V, et al. iBodies: modular synthetic antibody mimetics based on hydrophilic polymers decorated with functional moieties. Angew Chem Int Ed Engl. 2016;55:2356–60.

47 Baig S, Damian RT, Peterson DS. A novel cathepsin B active site motif is shared by helminth bloodfeeders. Exp Parasitol. 2002;101:83–9.

48 Tort J, Brindley PJ, Knox D, Wolfe KH, Dalton JP. Proteinases and associated genes of parasitic helminths. Adv Parasitol. 1999;43:161–266.

49 Dalton JP, Caffrey CR, Sajid M, Stack C, Donnelly S, Loukas A, et al. Proteases in trematode biology. In: Maule A, Marks N, editors. Parasitic flatworms: molecular biology, biochemistry, immunology and physiology. Cambridge: CABI; 2006. p. 348–68.

50 Smooker PM, Whisstock JC, Irving JA, Siyaguna S, Spithill TW, Pike RN. A single amino acid substitution affects substrate specificity in cysteine proteinases from *Fasciola hepatica*. Protein Sci. 2000;9:2567–72.

51 Stack CM, Caffrey CR, Donnelly SM, Seshaadri A, Lowther J, Tort JF, et al. Structural and functional relationships in the virulence-associated cathepsin L proteases of the parasitic liver fluke, *Fasciola hepatica*. J Biol Chem. 2008; 283:9896–908.

52 Norbury LJ, Hung A, Beckham S, Pike RN, Spithill TW, Craik CS, et al. Analysis of *Fasciola* cathepsin L5 by S2 subsite substitutions and determination of the P1-P4 specificity reveals an unusual preference. Biochimie. 2012;94:1119–27.

53 Pillay D, Boulangé AF, THT C. Expression, purification and characterisation of two variant cysteine peptidases from *Trypanosoma congolense* with active site substitutions. Protein Expr Purif. 2010;74:264–71.

54 Lecaille F, Choe Y, Brandt W, Li Z, Craik CS, Brömme D. Selective inhibition of the collagenolytic activity of human cathepsin K by altering its S2 subsite specificity. Biochemistry. 2002;41:8447–54.

55 Corvo I, Cancela M, Cappetta M, Pi-Denis N, Tort JF, Roche L. The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen. Mol Biochem Parasitol. 2009;167:41–7.

56 Choe Y, Leonetti F, Greenbaum DC, Lecaille F, Bogyo M, Brömme D, et al. Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. J Biol Chem. 2006;281:12824–32.

57 Brömmes D, Bonneaug PR, Lachance P, Storer AC. Engineering the S2 subsite specificity of human cathepsin S to a cathepsin L-and cathepsin B-like specificity. J Biol Chem. 1994;269:30238–42.

58 Lartillot N, Brinkmann H, Philippe H. Suppression of long-branch attraction artefacts in the animal phylogeny using a site-heterogeneous model. BMC Evol Biol. 2007;7:S4.

59 Dolečková K, Kašný M, Mikeš L, Cartwright J, Jedelský P, Schneider EL, et al. The functional expression and characterisation of a cysteine peptidase from the invasive stage of the neuropathogenic schistosome *Trichobilharzia regent*i. Int J Parasitol. 2009;39:201–11.

60 McGrath ME. The lysosomal cysteine proteases. Annu Rev Biophys Biomol Struct. 1999;28:181–204.

61 Mikeš L, Man P. Purification and characterization of a saccharide-binding protein from penetration glands of *Diplostomum pseudospathaceum* - a bifunctional molecule with cysteine protease activity. Parasitology. 2003;127: 69–77.

62 Brady CP, Brindley PJ, Dowd AJ, Dalton JP. *Schistosoma mansoni:* differential expression of cathepsins L1 and L2 suggests discrete biological functions for each enzyme. Exp Parasitol. 2000;94:75–83.

63 Dvořák J, Mashiyama ST, Sajid M, Braschi S, Delcroix M, Schneider EL, et al. SmCL3, a gastrodermal cysteine protease of the human blood fluke *Schistosoma mansoni*. PLoS Negl Trop Dis. 2009;3:e449.

64 Lowther J, Robinson MW, Donnelly SM, Xu W, Stack CM, Matthews JM, et al. The importance of pH in regulating the function of the *Fasciola hepatica* cathepsin L1 cysteine protease. PLoS Negl Trop Dis. 2009;3:e369.

65 Franta Z, Sojka D, Frantová H, Dvořák J, Horn M, Srba J, et al. IrCL1 - the haemoglobinolytic cathepsin L of the hard tick, Ixodes ricinus. Int J Parasitol. 2011;41:1253–62.

66 Brinker A, Weber E, Stoll D, Voigt J, Müller A, Sewald N, et al. Highly potent inhibitors of human cathepsin L identified by screening combinatorial pentapeptide amide collections. Eur J Biochem. 2000;267:5085–92.

67 Steverding D. The cathepsin B-selective inhibitors CA-074 and CA-074Me inactivate cathepsin L under reducing conditions. Open Enzym Inhib J. 2011;4:11–6.

68 Steverding D, Sexton DW, Wang X, Gehrke SS, Wagner GK, Caffrey CR. *Trypanosoma brucei*: chemical evidence that cathepsin L is essential for survival and a relevant drug target. Int J Parasitol. 2012;42:481–8.

69 Mindell JA. Lysosomal acidification mechanisms. Annu Rev Physiol. 2012;74: 69–86.

70 Chappell CL, Dresden MH. *Schistosoma mansoni*: Proteinase activity of “hemoglobinase” from the digestive tract of adult worms. Exp Parasitol. 1986;61:160–7.

71 Halton DW. Nutritional adaptations to parasitism within the Platyhelminthes. Int J Parasitol. 1997;27:693–704.

72 Dalton JP, Skelly P, Halton DW. Role of the tegument and gut in nutrient uptake by parasitic platyhelminths. Can J Zool. 2004;82:211–32.

73 Robinson MW, Corvo I, Jones PM, George AM, Padula MP, To J, et al. Collagenolytic activities of the major secreted cathepsin L peptidases involved in the virulence of the helminth pathogen, *Fasciola hepatica*. PLoS Negl Trop Dis. 2011;5:e1012.

74 Ozsolak F, Kapranov P, Foissac S, Kim SW, Fishilevich E, Monaghan AP, et al. Comprehensive polyadenylation site maps in yeast and human reveal pervasive alternative polyadenylation. Cell. 2010;143:1018–29.

75 Osato N, Yamada H, Satoh K, Ooka H, Yamamoto M, Suzuki K, et al. Antisense transcripts with rice full-length cDNAs. Genome Biol. 2003;5:R5.

76 Steigele S, Nieselt K. Open reading frames provide a rich pool of potential natural antisense transcripts in fungal genomes. Nucleic Acids Res. 2005;33: 5034–44.

77 Henke C, Strissel PL, Schubert M-T, Mitchell M, Stolt CC, Faschingbauer F, et al. Selective expression of sense and antisense transcripts of the sushi-ichi-related retrotransposon-derived family during mouse placentogenesis. Retrovirology. 2015;12:9.

78 Pelechano V, Steinmetz LM. Gene regulation by antisense transcription. Nat Rev Genet. 2013;14:880–93.

79 Illy C, Quraishi O, Wang J, Purisima E, Vernet T, Mort JS. Role of the occluding loop in cathepsin B activity. J Biol Chem. 1997;272:1197–202.

80 Michel A, Ghoneim H, Resto M, Klinkert MQ, Kunz W. Sequence, characterization and localization of a cysteine proteinase cathepsin L in **Schistosoma mansoni**. Mol Biochem Parasitol. 1995;73:7–18.

81 Britton C, Murray L. A cathepsin L protease essential for Caenorhabditis elegans embryogenesis is functionally conserved in parasitic nematodes. Mol Biochem Parasitol. 2002;122:21–33.

82 Pinlaor P, Kaewpitoon N, Laha T, Sripa B, Kaewkes S, Morales ME, et al. Cathepsin F cysteine protease of the human liver fluke. PLoS Negl Trop Dis. 2009;3:e398.

83 Ogawa K. Diseases of cultured marine fishes caused by Platyhelminthes (Monogenea, Digenea, Cestoda). Parasitology. 2015;142:178–95.

Additional files:

Additional file 1:

yrEnCL3Fwd	ATACTCGAG**AAAAGA**GCTGATCAAAATGAATCACCCTTCCAGA
yrEnCL3Rev	ATGCGGCCGC**TTA**ATGATGATGATGATGATGAACGAGAGGATATGAGG
yrEnCL1Fwd	ATACTCGAG**AAAAGA**ATGGCAGGTCAGGATCACTGGGCTTCATTCAAG
yrEnCL1Rev	ATGCGGCCGC**CTA***ATGATGATGATGATGATGATG*TACGAGTGGATAGCTTGC
yrEnCBFwd	ATACTCGAG**AAAAGA**CGAGTTTTCCACAGTCAG
yrEnCBRev	AATGCGGCCGCTTAC*ATGATGATGATGATGATG*ATAAGTTTCGGTATCCC

Primers for the expression of *yrEnCL1*, *yrEnCL3*, and *yrEnCB* in *P. pastoris.*

Additional file 2:

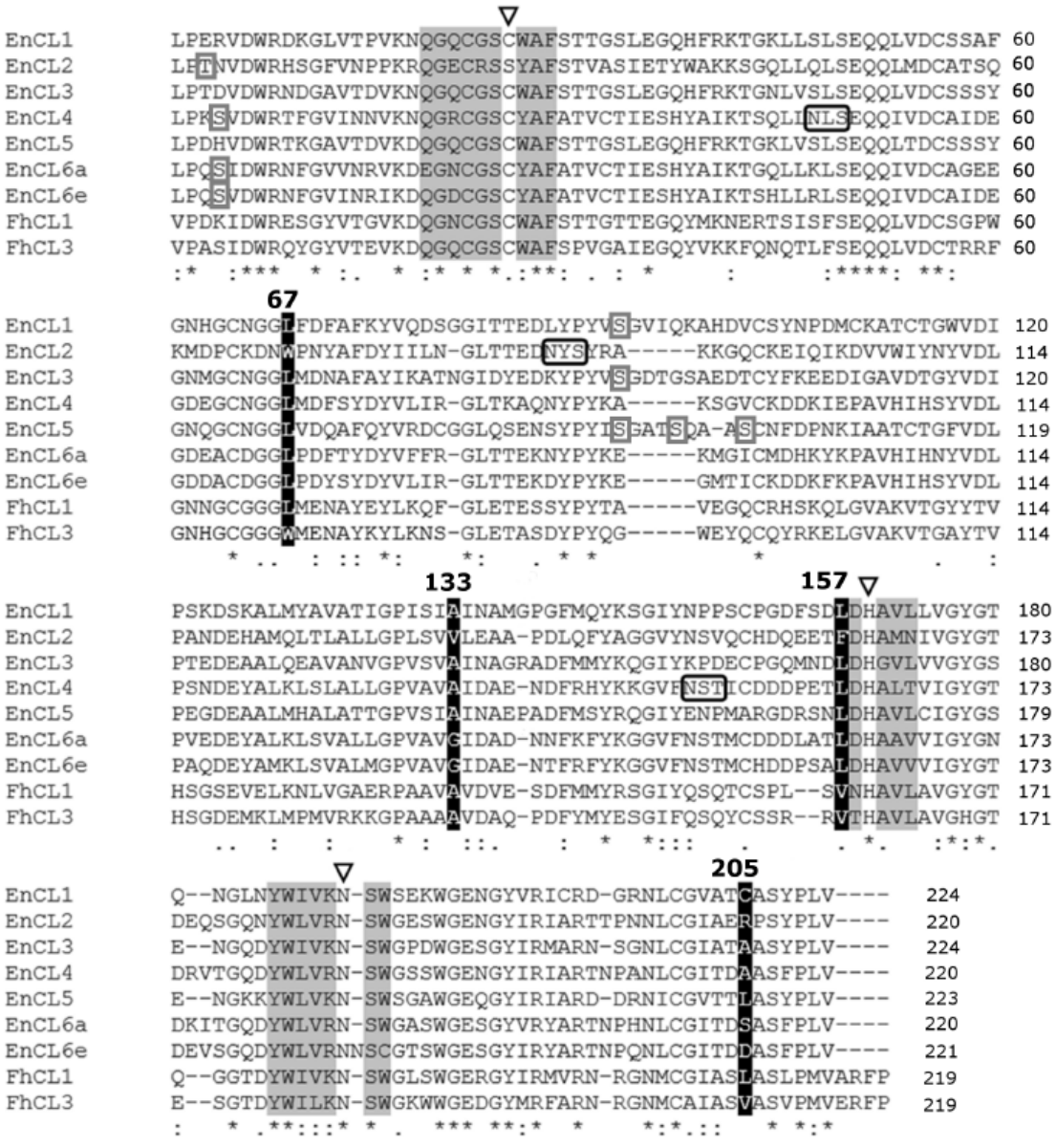
brCL1Fwd	CATAGAGCTCATGGCAGGTCAGGATCACTGGGCT
brCL1Rev	GATACTCGAGTACGAGTGGATAGCTTGCGCATGTTGCCAC

Primers for the expression of *yrEnCL1*, *yrEnCL3*, and *yrEnCB* in *P. pastoris.*

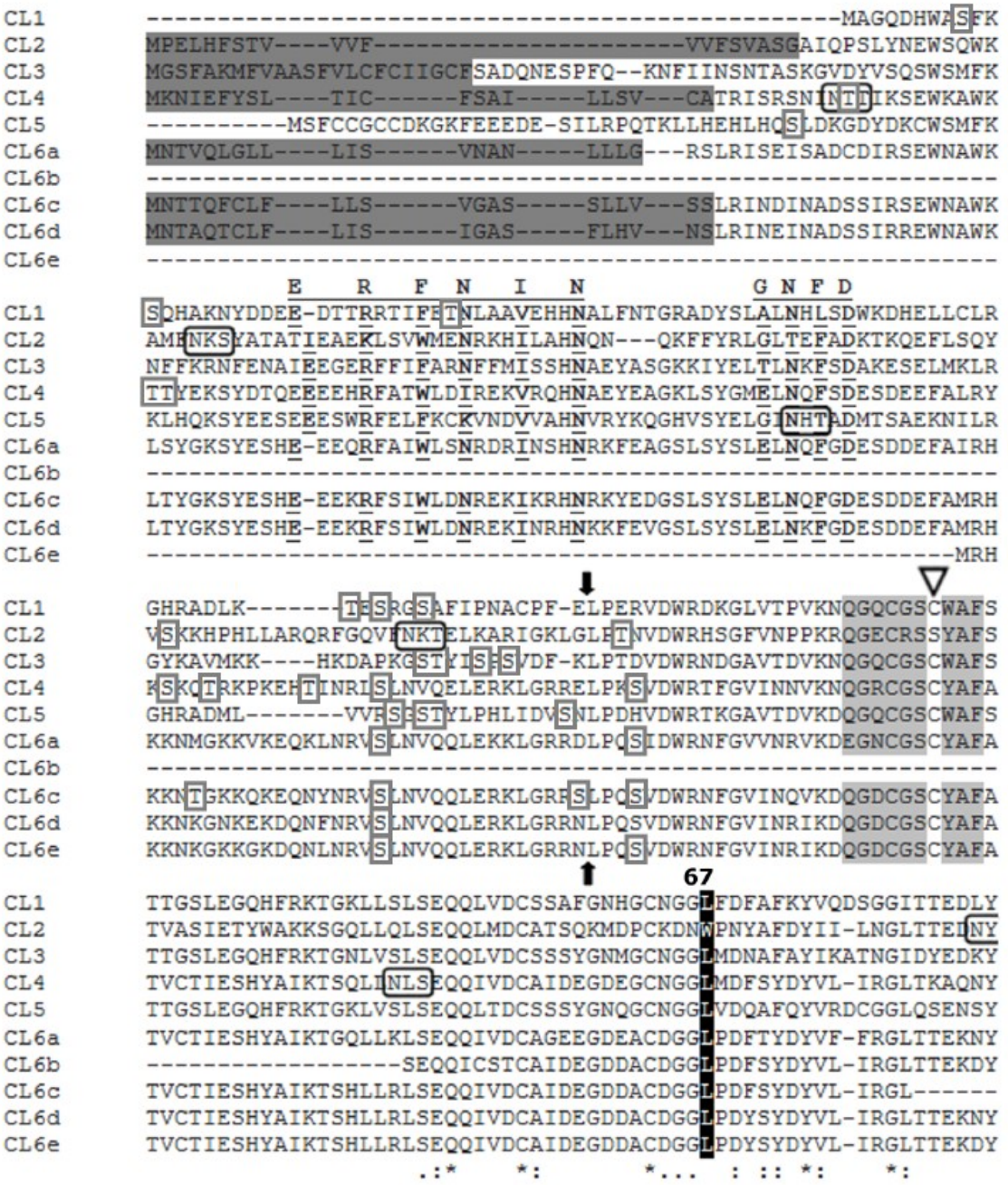
Additional file 3:

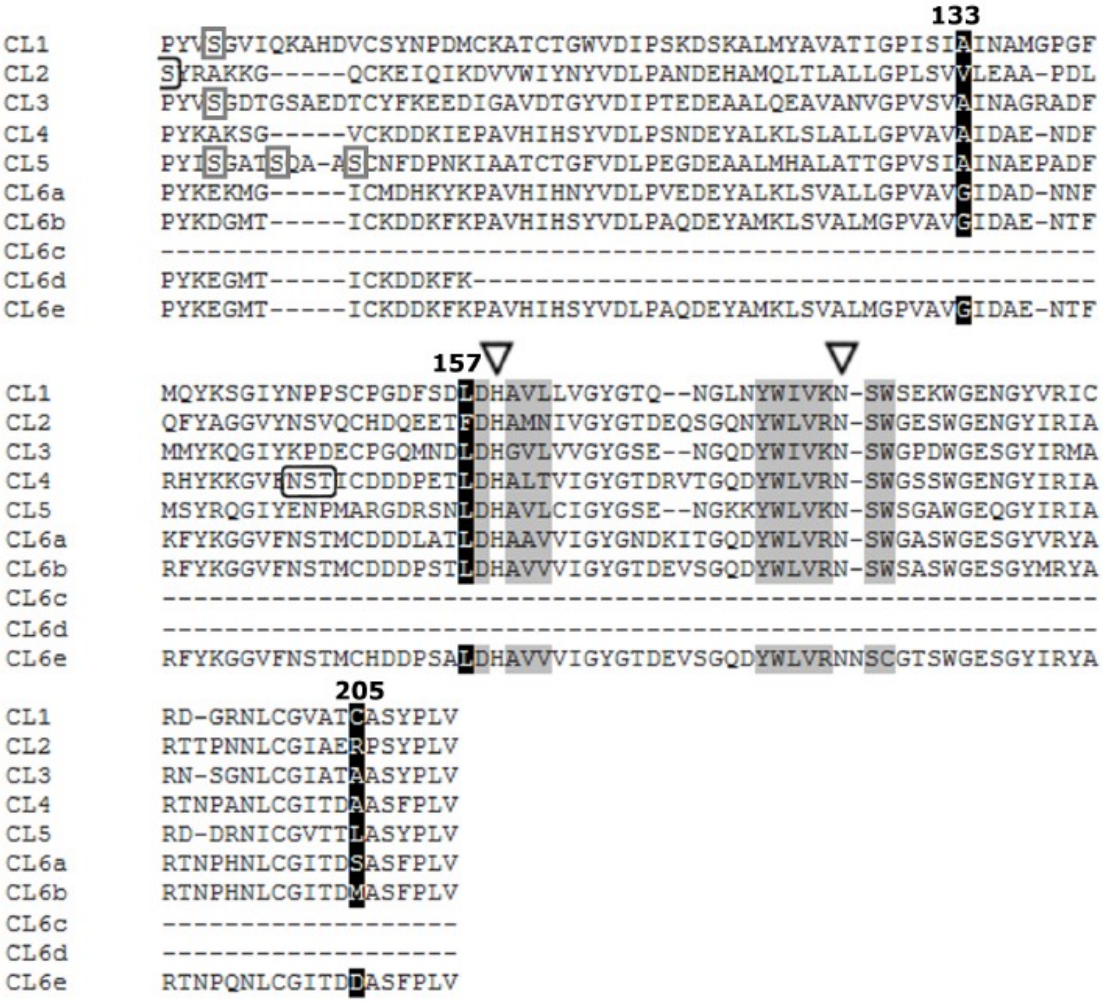
EnCL1Fwd	GAC ACA ACA CGA AGA ACA ATT TTT GAG
EnCL1Rev	AGT GCT GAC CTT CCA ATG AC
EnCL3Fwd	TTT GTA TCA TCG GCT GTT TCA GTG
EnCL3Rev	CTA CAT CTG TAG GCA ACT TGA AAT CT

Specific *EnCL1/EnCL3* primers for the synthesis of probes for RNA *in situ* hybridisation.

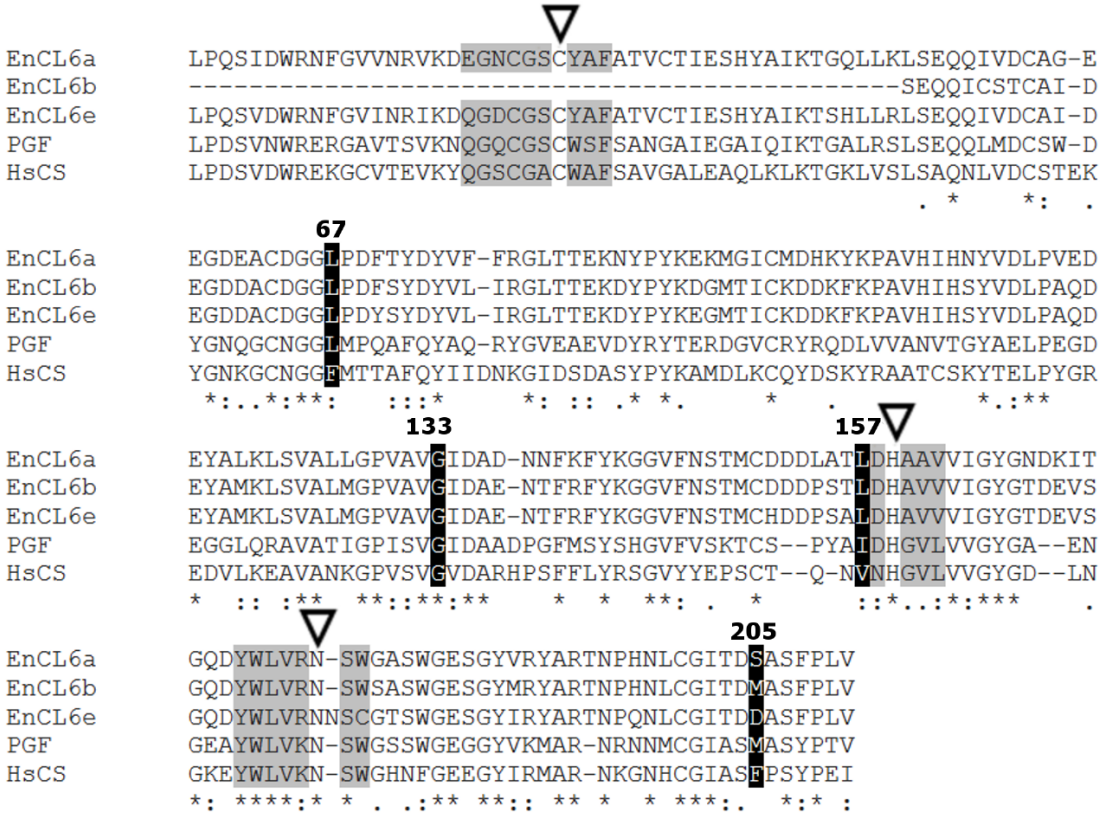


Additional file 4: Amino acid sequence alignment of *E. nipponicum* cathepsins L with cathepsins L of *F. hepatica*. Signal peptides and pro-sequences were omitted. Numbers at the end of the lines show amino acid numbering of particular mature parts of the enzymes. Catalytic triad of the active site (C, H, N) is marked by triangles. Conserved motifs around active site residues are shaded in grey. Residues within the S2 subsite of the active site involved in determining the substrate specificity are shaded in black and indicated with numbers (papain numbering). Tripeptides of potential N-glycosylation sites are boxed. Predicted O-glycosylated residues are marked by grey squares. The alignment was made using the sequences of *F. hepatica* mature cathepsins L1 (GenBank: AAT76664.1) and L3 (GenBank: CAC12807.1).

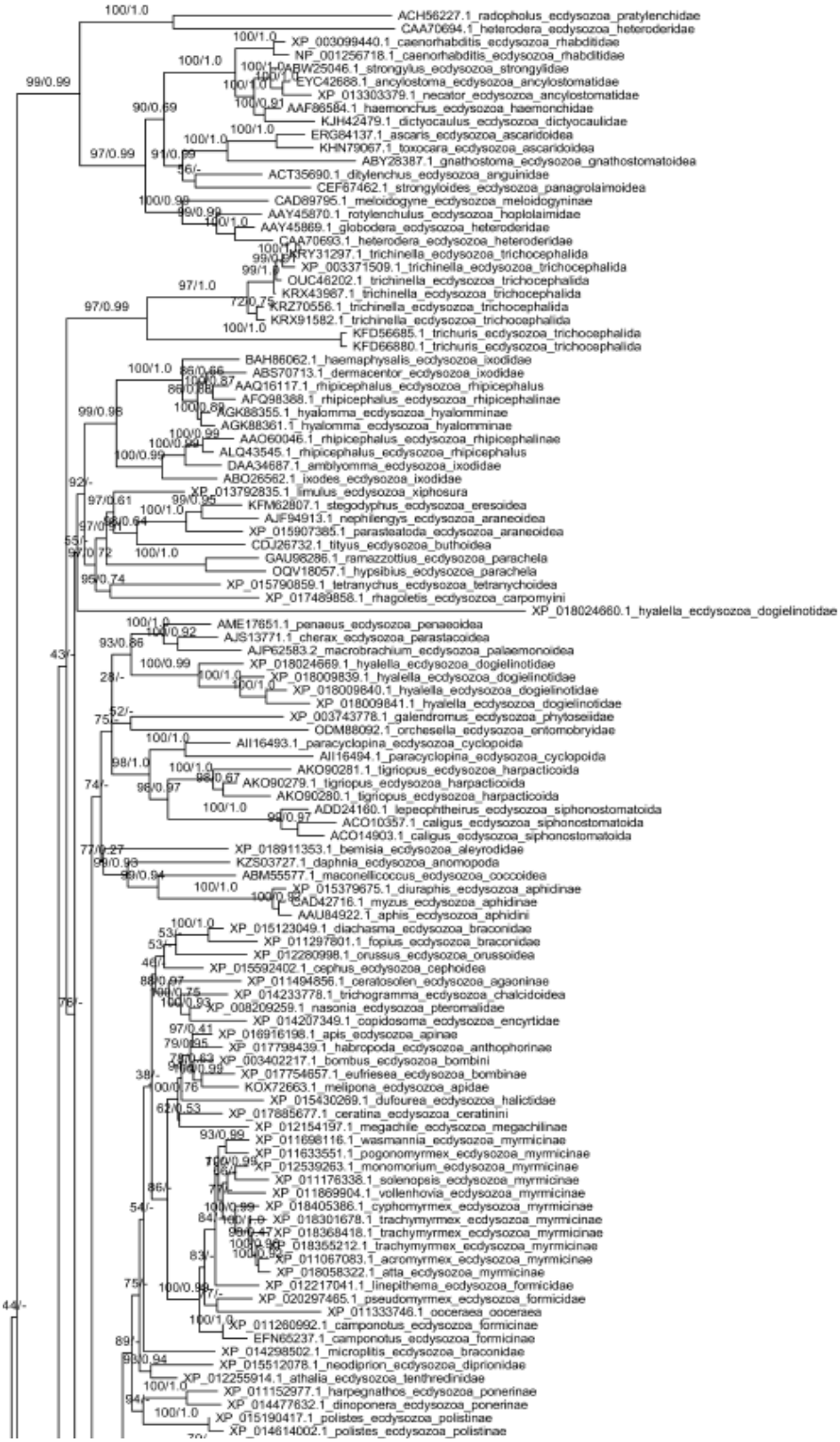

CL1
CL2
CL3
CL4
CL5
CL6a
CL6b
CL6c
CL6d
CL6e
67



Additional file 5: Multiple alignment of all the complete/incomplete amino acid sequences of *E. nipponicum* cathepsins L inferred from the transcriptome of adult worms. Signal peptides are shaded in dark grey, position of the pro-region cleavage site is marked by arrows. ERFNIN- and GNFD-like motifs are underlined and indicated by underlined headings. The catalytic triad of the active site (C, H, N) is marked by triangles. Conserved motifs around active site residues are shaded in light grey. Residues within the S2 subsite of the active site involved in determining the substrate specificity are shaded in black and indicated with numbers (papain numbering). Tripeptides of potential N-glycosylation sites are boxed. Predicted O-glycosylated residues are marked by grey squares.



Additional file 6: Composition of S2 subsites of the active sites shown in the alignment of parts of amino acid sequences of *E. nipponicum* cathepsin L orthologs 6a, 6b and 6e with human cathepsin S and with plerocercoid growth factor of *Spirometra* tapeworm. The catalytic triad of the active site (C, H, N) is marked by triangles. Conserved motifs around active site residues are shaded in grey. Residues within the S2 subsite of the active site involved in determining the substrate specificity are shaded in black and indicated with numbers (papain numbering). *Abbreviations*: PGF, *Spirometra erinaceieuropaei* plerocercoid growth factor (Genbank: BAB62718.1); *HsCS*, human cathepsin S (Genbank: AAC37592).


ACH56227.1_radopholus_ecdysozoa_pratylenchidae
CAA70694.1_heterodera_ecdysozoa_heteroderidae
XP_003099440.1_caenorhabditis_ecdysozoa_rhabditidae
NP_001256718.1_caenorhabditis_ecdysozoa_rhabditidae
DABW25046.1_strongylus_ecdysozoa_strongylidae
EYC42688.1_ancylostoma_ecdysozoa_ancylostomatidae
XP_013303379.1_necator_ecdysozoa_ancylostomatidae
AAF86584.1_haemonchus_ecdysozoa_haemonchidae
KJH42479.1_dictyocaulus_ecdysozoa_dictyocaulidae
ERG84137.1_ascaris_ecdysozoa_ascaridoidea
KHN79067.1_toxocara_ecdysozoa_ascaridoidea
ABY28387.1_gnathostoma_ecdysozoa_gnathostomatoidea
ACT35690.1_ditylenchus_ecdysozoa_anguinidae
CEF67462.1_strongyloides_ecdysozoa_panagrolaimoidea
CAD89795.1_meloidogyne_ecdysozoa_meloidogyninae
AAY45870.1_rotylenchulus_ecdysozoa_hoplolaimidae
AAY45869.1_globodera_ecdysozoa_heteroderidae
CAA70693.1_heterodera_ecdysozoa_heteroderidae
KRY31297.1_trichinella_ecdysozoa_trichocephalida
XP_003371509.1_trichinella_ecdysozoa_trichocephalida
OUC46202.1_trichinella_ecdysozoa_trichocephalida
KRX43987.1_trichinella_ecdysozoa_trichocephalida
KRZ70556.1_trichinella_ecdysozoa_trichocephalida
KRX91582.1_trichinella_ecdysozoa_trichocephalida
KFD56685.1_trichuris_ecdysozoa_trichocephalida
KFD66880.1_trichuris_ecdysozoa_trichocephalida
BAH86062.1_haemaphysalis_ecdysozoa_ixodidae
ABS70713.1_dermacentor_ecdysozoa_ixodidae
AAQ16117.1_rhipicephalus_ecdysozoa_rhipicephalus
AFQ98388.1_rhipicephalus_ecdysozoa_rhipicephalinae
AGK88355.1_hyalomma_ecdysozoa_hyalomminae
AGK88361.1_hyalomma_ecdysozoa_hyalomminae
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ALQ43545.1_rhipicephalus_ecdysozoa_rhipicephalus
DAA34687.1_amblyomma_ecdysozoa_ixodidae
ABO26562.1_ixodes_ecdysozoa_ixodidae
XP_013792835.1_limulus_ecdysozoa_xiphosura
KFM62807.1_stegodyphus_ecdysozoa_eresoidea
AJF94913.1_nephilengys_ecdysozoa_araneoidea
XP_015907385.1_parasteatoda_ecdysozoa_araneoidea
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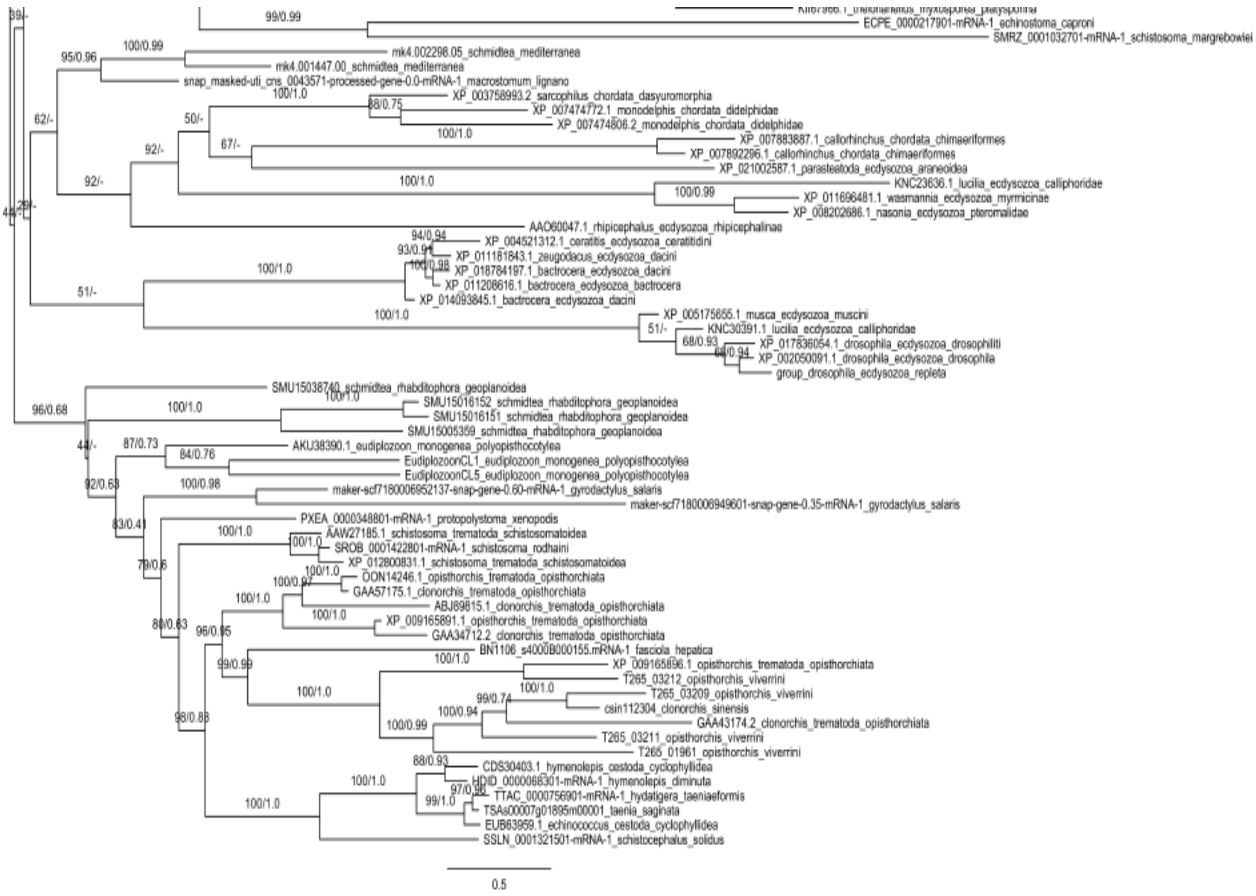

Additional file 7: Phylogram showing relationships between *E. nipponicum* cathepsins L and cathepsins L of other selected organisms. Unrooted maximum-likelihood tree of selected *E. nipponicum* cathepsins L inferred using the best-fit model (LG + C60 + G). Ultrafast bootstrap supports and posterior probabilities are shown. The leaf descriptions contain the sequence ID, genus and taxonomic placement.

Additional file 8: Phylogram showing relationships between *E. nipponicum* cathepsins L and cathepsins L of other selected organisms. Unrooted maximum-likelihood tree of selected *E. nipponicum* cathepsins L inferred using the best-fit model (LG + C60 + G). Ultrafast bootstrap supports and posterior probabilities are shown. The leaf descriptions contain the sequence ID, genus and taxonomic placement. Amino acid sequence alignment of *E. nipponicum* cathepsin B with cathepsins B of *S. mansoni*. Whole zymogens including a signal peptide were aligned. Numbers at the end of the lines show amino acid numbering of the mature parts of particular enzymes. Position of the pro-region cleavage site is marked by an arrow. The catalytic triad of the active site (C, H, N) is marked by triangles. Conserved motifs around active site residues are shaded in grey. Tripeptides of potential N-glycosylation sites are boxed. Predicted O-glycosylated residues are marked by grey squares. The occluding loop typical of cathepsins B is underlined. The 'haemoglobinase motif' ascribed to cathepsins B with an assumed function in haemoglobinolysis in blood-feeding helminths, is shaded in black (it is modified in *SmCB2* and *EnCB*). Residues within the S2 subsite of the active site involved in determining the substrate specificity are marked by a black dot and indicated with number (papain numbering). The alignment was made using sequences of *Schistosoma mansoni* cathepsins B1 (GenBank: CAD44624.1) and B2 (GenBank: XP_018651608.1).

1.10. **Publikace č. 3**

V této práci jsme se zaměřili na inhibitory Kunitzova typu, které byly nalezeny v transkriptomu dospělce u hematofágního monogenea *E. nipponicum*. EnKT1 byl vybrán kvůli své vysoké homologii s textilininem-1, antihemorhagickým faktorem z pakobry východní (*Pseudonaja textilis*), a byl vyprodukován v rekombinantní formě v bakteriálním systému, přečištěn a biochemicky charakterizován. V *in vitro* experimentech EnKT1 blokoval bovinní faktor Xa, který je zapojen do koagulační kaskády. Nicméně ještě lépe blokoval lidský plazmin a plazmový kallikrein. Pomocí metody tromboelastografie byla potvrzena jeho antikoagulační aktivita zprostředkovaná inhibicí faktoru Xa, ale nepodařilo se prokázat jeho antifibrinolytickou aktivitu. Na druhou stranu se podařilo prokázat, že EnKT1 je schopen blokovat rybí komplement *in vitro*. Imunolokalizace a RNA *in situ* hybridizace detekovaly EnKT1 uvnitř trávicích buněk ve střevě dospělce. Výsledky lokalizace spolu s přítomností signálních sekvencí napovídají, že dochází k jeho sekreci do střeva, kde pravděpodobně hraje významnou roli jako antikoagulant při blokaci hostitelské koagulační kaskády. Může hrát i významnou roli při blokaci hostitelského komplementu, tedy sloužit i jako ochrana gastrodermis parazita před lytickými účinky komplementu. V transkriptomu dospělců byly nalezeny i další transkripty kódující Kunitz inhibitory a v homogenátu dospělce byla detekována inhibiční aktivita vůči trombinu.

Můj podíl na práci zahrnoval produkci a purifikaci rekombinantního EnKT1 a jeho funkční charakterizaci a lokalizace pomocí metody RNA *in situ* hybridizace ve tkáni dospělého červa. Dále jsem se podílela na interpretaci dat a sepisování publikace.

A novel Kunitz protein with proposed dual function from *Eudiplozoon nipponicum* (Monogenea) impairs haemostasis and action of complement *in vitro*.

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A novel Kunitz protein with proposed dual function from *Eudiplozoon nipponicum* (Monogenea) impairs haemostasis and action of complement *in vitro*

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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 textilis*. 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.

1. Introduction

Blood-feeding parasites need to control haemostasis of host blood for the completion of feeding. For this purpose they produce antihaemostatic molecules including anticoagulants, vasodilators and anti-platelet factors. Parasite anticoagulants often act as inhibitors of serine peptidases involved in the coagulation cascade, particularly Factor X (FX), Factor VII (FVII), and thrombin (Francischetti et al., 2002; Hovius et al., 2008; Tsujimoto et al., 2012; Ranasinghe et al., 2015c). These inhibitors largely belong to annexin, serpin and Kunitz type protein families (Francischetti et al., 2002; van Genderen et al., 2008; Huntington, 2013).

Kunitz inhibitors (KIs) act against serine and in rare cases even cysteine and aspartic peptidase activities (Oliva et al., 2010; Smith et al., 2016). They are usually small proteins of approximately 6 - 8 kDa and their common structural feature is a reactive peptidase-binding loop containing six cysteine residues that create three disulfide bonds responsible for the stabilization of proper protein folding. The amino acid positions P1 and P1' of the reactive

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site are located in the centre of the loop, where P1 is the important determinant of the specificity towards serine peptidases, although P6-P5' residues may be involved in interactions with peptidase exosites (Laskowski and Kato, 1980; Krowarsch et al., 1999; Ranasinghe and McManus, 2013). KIs regulate various biological processes in organisms such as cell proliferation, cuticle formation and tissue remodelling, ion channel blocking, coagulation, fibrinolysis, inflammation, etc. (Lee et al., 2010; Shigetomi et al., 2010; Ranasinghe and McManus, 2013). They also function as important bioactive compounds in venoms of sea anemones, spiders, scorpions, snakes, etc. (Yuan et al., 2008; Flight et al., 2009; Millers et al., 2009; Peigneur et al., 2011; Zhao et al., 2011). Based on their function, KIs have been recently categorized into five groups: body trypsin inhibitors, venom chymotrypsin inhibitors, venom trypsin inhibitors, dual-function toxins and K^+ channel blockers (Yuan et al., 2008).

Several KIs have also been found in endoparasitic helminths. Their predicted or extrapolated functions include protection against host digestive enzymes (Milstone et al., 2000; Fló et al., 2017), regulation of endogenous cysteine peptidases (Smith et al., 2016), immune evasion and immunomodulation (Milstone et al., 2000; Falcón et al., 2014; Ranasinghe et al., 2015a, b, c; Fló et al., 2017).

In this paper we present a thorough characterisation of the first Kunitz type inhibitor from the Class Monogenea, a group of ectoparasitic helminths that have been neglected in terms of biochemical and molecular research, despite the fact that several species cause major devastation in fish aquaculture. We employed the advantage of having high quality transcriptomic data from the adults of *Eudiplozoon nipponicum* (Heteronchoinea: Diplozoidae), a cosmopolitan haematophagous parasite inhabiting the gills of a fish of high global economic importance, the common carp (*Cyprinus carpio*). The parasite has been considered an invasive species in Europe and other parts of the world outside Asia, inducing hypochromic microcytic anemia in the host (Kawatsu, 1978; Matějusová et al., 2001; Buchmann and Bresciani, 2006; Reed et al., 2009). We show that the Kunitz type inhibitor of *E. nipponicum*, named EnKT1, is a potent secretory anticoagulant and complement inhibitor, although its primary structure resembles textilinin-1, an anti-haemorrhagic factor from the venom of the Eastern brown snake, *Pseudonaja textilis* (Masci et al., 2000). The protein was produced in a recombinant form, functionally characterised and localised within the body of the worm. Thus, this study represents, to our knowledge, the first characterisation of a monogenean protein affecting the blood coagulation cascade and complement, while having features which also imply possible involvement in associated biological processes such as inflammation.

2. Materials and methods

2.1. Parasite material

Fresh living adult *E. nipponicum* worms were obtained from the gills of common carp (*Cyprinus carpio*) which originated from fish farm ponds in South Bohemia, Czech Republic. The worms were collected immediately after fish slaughter in a commercial facility of Rybářství Třeboň Holding, Plc., Czech Republic, and carefully washed in autoclaved tap water. Soluble protein extract of the worms (solPE) and excretory/secretory products (ESP) were pre-pared as described previously (Jedličková et al., 2016). Total RNA was isolated from the homogenate of 10 adult worms by using an High pure RNA isolation kit (Roche Diagnostics GmbH, Germany), the concentration was measured in a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). First strand cDNA was reverse-transcribed from 0.75 mg of total RNA by the use of a Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics) following the manufacturer's instructions. For RNA *in situ* hybridisation, the worms were fixed in Bouin's solution (Sigma-Aldrich, USA) at room temperature for 1 h and transferred to a fresh solution overnight (4 LC). Worms intended for immunohistochemistry were fixed in 4% paraformaldehyde. Fixed worms were dehydrated with increasing concentrations of ethyl alcohol (50-100%), cleared with xylene and embedded in paraffin (more details are in Jedličková et al., 2018).

2.2. Ethics statement

The protection, care and use of experimental animals were carried out in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and its appendix, European Directive 2010/63/ EU, and according to Czech law (246/1992 and 359/2012) for biomedical research including animals. Our experimental procedures conform with the legal consent of the Professional Ethics Committee of the Faculty of Science, Charles University, Prague, Czech Republic and the Branch for Research and Development of the Ministry of Education, Youth and Sports of the Czech Republic. The equipment, facility and welfare conditions of experimental animals were approved by the Branch of Animal Commodities of the Ministry of Agriculture of the Czech Republic (approval no. 13060/2014-MZE-17214). The absolute minimal number of animals (one mouse) necessary for the production of antibodies was used in this study. Healthy donors' blood was provided by three consenting co-authors of this paper. Blood from a FX-deficient patient was used on the occasion of a regular health check of that person at the Institute of Haematology and Blood Transfusion, Prague, under a written consent signed by the patient. All blood samples were collected by authorized staff at the abovementioned Institute.

2.3. Inhibitory activities of worm soluble extracts (solPE)

Inhibition assays were performed with three factors (serine peptidases) involved in blood coagulation and related processes, and their respective fluorogenic oligopeptide substrates: human thrombin (668 pM, Sigma-Aldrich: T6884) and Boc-Val-Pro-Arg-AMC (VPR), bovine activated Factor X (FXa) (945 pM, Sigma-Aldrich: F9302) and Boc-Ile-Glu-Gly-Arg-AMC (IEGR), human plasmin (735 pM, Sigma-Aldrich: 10602361001) and D-Ala-Leu-Lys-AMC (ALK). An aliquot of solPE (5 mg of total protein) was pre-incubated with each peptidase for 10 min at room temperature in 0.1 M Hepes, 0.3 M NaCl, 0.01 M $CaCl_2$, pH 8.0 in a final volume of 100 ml in 96-well black flat bottom plates (Nunc, Thermo Scientific). The reaction was started by adding 100 ml of 40 lM substrate in the same buffer. Controls did not contain solPE. The production of free fluorophore was measured in 2 min cycles at 28 LC for 30 min using an Infinite M200 ßuorimeter (TECAN, Austria) at 355 nm excitation and 460 nm emission wavelengths. All measurements were performed in triplicate and repeated twice. Values in graphs are expressed as means with S.D.

2.4. Sequence analysis of the EnKT1 gene and mass spectrometry

The sequence of a Kunitz type inhibitor (EnKT1) was mined from the Transcriptome Shotgun Assembly project of adult *E. nipponicum* deposited at DDBJ/EMBL/GenBank under the accession GFYM00000000. The version used in this paper is the first version, GFYM01000000. The completeness of the sequence was verified by PCR using isolated *E. nipponicum* mRNA as a starting material, and by Sanger sequencing (DNA Sequencing Laboratory, Faculty of Science, Charles University, Czech Republic). The

presence of a signal sequence was detected by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011). Prediction of the molecular weight and theoretical pI was performed by the Compute pI/Mw tool (Expasy, http://web.expasy.org/compute_pi/) (Artimo et al., 2012). Potential N-glycosylation sites were detected by the NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) (Artimo et al., 2012). A search for similar protein sequences was carried out with BLASTp (https://blast.ncbi.nlm.nih.gov/) against the non-redundant protein sequences database. Selected amino acid sequences of Kunitz proteins were aligned with the sequence of EnKT1 (GenBank accession number MF346930) using the Clustal Omega tool (Embl-Ebi, http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011).

LC-MS/MS analyses of *E. nipponicum* ESP were undertaken using a RSLCnano system connected to an Orbitrap Elite hybrid spectrometer (Thermo Fisher ScientiÞc, USA) as described previously (Ilgová et al., 2017).

2.5. Production of recombinant EnKT1 in *E. coli* and its purification

The sequence of the EnKT1 gene without the part coding the signal peptide was codon-optimized in silico for expression in a bacterial system, *E. coli* BL-21, using a Codon Optimization Tool (Integrated DNA Technologies, https://eu.idtdna.com/CodonOpt). The gene was commercially synthesized and inserted by restriction digestion into the vector pET22b+ using restriction sites for NdeI-XhoI enzymes (commissioned work performed by the company GenScript, USA). A 6x His-tag was incorporated on the C-terminus of EnKT1. The construct was transformed into BL21 Star™(DE3) *E. coli* (Invitrogen, USA). The expression of recombinant EnKT1 (rEnKT1) was performed according to the manufacturer's instruction in the pET System Manual (Novagen). Harvested cells were resuspended in 20 mM Tris-HCl, 0.3 M NaCl, 1% lauryl sar-cosine, 10 mM imidazole, pH 8.0. The solution was sonicated on ice (10 W, 3 30 s), centrifuged at 10,000g for 10 min at 4 LC, and filtered through a 0.22 µm filter. Recombinant EnKT1 was purified by Ni-chelating chromatography (HisTrap™ FF crude, GE Healthcare, USA) and eluted from the column by stepwise increasing concentrations of imidazole (0.04-0.5 M). The protein concentration was determined by a Quant-iT™ Protein Assay Kit (Thermo Fisher, USA). Fractions after chromatography were resolved by SDS-PAGE in 4-12% Mini-PROTEAN TGX™ gels (Bio-Rad, USA) stained by Coomassie Brilliant Blue R-250. The isolated band of corresponding size was excised from the gel, and the identity of the protein was confirmed by mass spectrometry (MALDI TOF/ TOF, Laboratory of Mass Spectrometry, Charles University).

2.6. EnKT1-specific polyclonal antibody

Monospecific polyclonal antibodies against rEnKT1 were produced in an ICR/CD1 mouse (ENVIGO, Italy) that was injected s.c. three times at intervals of 14 days with 30 mg of purified rEnKT1 in TitermaxGold adjuvant according to the manufacturer's protocol (Sigma-Aldrich). The mouse was bled under deep ketamine/xylazine anaesthesia 2 weeks after the last injection and serum was collected. Control serum was taken from the same mouse prior to immunization.

2.7. Detection of rEnKT1 on blots

The His-tagged rEnKT1was detected on blots using iBody4, a biotinylated copolymer containing nitrilotriacetic acid-bound nickel cations (Šácha et al., 2016). Purified rEnKT1 (2 mg) was run in a 4-12% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (PVDF, Bio-Rad). Monospecific polyclonal antibodies against rEnKT1 and control sera (both diluted 1:100) were veriÞed on immunoblots. The rEnKT1 (1 mg/well) and worm soluble protein extract (20 mg/well) were separated by SDS-PAGE in 4-12% gels. The blots were further processed according to previously published protocols (Jedličková et al., 2018).

2.8. Stability of the rEnKT1-trypsin complex

Trypsin (1 mg, Sigma-Aldrich 93614) was incubated in the presence of rEnKT1 (1 mg) in 0.1 M Hepes, 0.3 M NaCl, 10 mM $CaCl_2$, pH 8.0 for 15 min at room temperature (molar ratio trypsin: rEnKT1 1:2.2). Then, non-reducing or reducing (DTT) SDS-PAGE sample buffer was added to the aliquots of the mixture at room temperature. Alternatively, samples were boiled in reducing sample buffer. As controls, trypsin or rEnKT1 alone were treated under the same conditions. Samples were separated by SDS-PAGE at 80 V and gels were stained by Coomassie Brilliant Blue R-250.

2.9. Inhibitory properties of recombinant EnKT1

The ability of rEnKT1 to act as an inhibitor was tested using a panel of serine peptidases: thrombin (Sigma-Aldrich T6884), FXa (Sigma-Aldrich F9302), plasmin (Sigma-Aldrich 10602361001), plasma kallikrein (Sigma-Aldrich K2638), trypsin (Sigma-Aldrich 93614), activated Factor VII (FVIIa) (NovoSeven, Novo Nordisk). Additionally, purified recombinant cysteine peptidases (cathepsins from *E. nipponicum*) - EnCL1 (GenBank accession number KP793605), EnCL3 (GenBank accession number KP793606), and EnCB (GenBank accession number MF346929) - available in our laboratory were tested (Jedličková et al., 2018). The activities were measured with respective fluorogenic oligopeptide substrates for particular proteases and assay conditions for each tested peptidase/substrate as presented in Table 1. The peptidases were preincubated with different concentrations of rEnKT1 (0, 44, 220, 440, 1100 and 2200 nM were final concentrations after addition of substrate solution to the reaction mixture) for 15 min at room temperature in 96-well black flat bottom plates (Nunc, Thermo Scientific). Measurements were performed under the same conditions as described above. The IC_{50} values were calculated for each peptidase using non-linear regression in GraphPad Prism software version 7.02.

Table 1
Panel of serine and cysteine peptidases and conditions used to test the inhibitory specificity of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (rEnKT1).

Enzyme	Buffer	Substrate
E. nipponicum cathepsin L1 (3 nM)	0.1 M citrate/0.2 M phosphate pH 5 + 2 mM DTT	Z-FR-AMC
E. nipponicum cathepsin L3 (3 nM)	0.1 M citrate/0.2 M phosphate pH 5 + 2 mM DTT	Z-FR-AMC
E. nipponicum cathepsin B (3 nM)	0.1 M citrate/0.2 M phosphate pH 5 + 2 mM DTT	Z-FR-AMC
Trypsin (40 nM)	0.1 M Hepes, 0.3 M NaCl, 10 mM CaCl, pH 8	Z-LR-AMC
Plasmin (735 pM)	0.1 M Hepes, 0.3 M NaCl, 10 mM CaCl, pH 8	Z-ALK-AMC
Thrombin (668 pM)	0.1 M Hepes, 0.3 M NaCl, 10 mM CaCl, pH 8	Z-VPR-AMC
Factor Xa (945 pM)	0.1 M Hepes, 0.3 M NaCl, 10 mM CaCl, pH 8	Z-IEGR-AMC
Plasma kallikrein (3.5 nM)	0.1 M Hepes, 0.3 M NaCl, 10 mM CaCl, pH 8	Z-FR-AMC
Factor VII (4 nM)	0.1 M Hepes, 0.3 M NaCl, 10 mM CaCl, pH 8	Z-VPR-AMC

2.10. Thromboelastography

Rotational thromboelastography was performed using a ROTEM analyser (ROTEM delta, Tem International GmbH, Germany) at the Institute of Haematology and Blood Transfusion in Prague, Czech Republic. ROTEM measures the elastic properties of the whole blood clot during its formation and lysis through impedance. The reaction time, ie. clotting time (CT) is the time from start to the formation of the first detectable clot. The maximum clot firmness (MCF) is the point where the clot is at its strongest. ML is maximum lysis as a percentage of MCF. The ML60 value is the percentage of lysis measured 60 min after MCF is reached (Flight et al., 2005). CT was measured with star-tem (Tem Interna-tional GmbH, Germany) ROTEM system reagent for recalcifying citrated blood (native thromboelastography, NATEG). The measurements were performed with whole human blood in biological triplicates (three different healthy donors are co-authors of the manuscript). One additional measurement was done with whole blood of a donor with FX deficiency. ML60 and MCF were measured with ex-tem (Tem International GmbH) ROTEM system reagent containing Ca^{2+} and tissue factor (extrinsic thromboelastography, EXTEG), and with 14 nM (Þnal concentration) of tissue plasminogen activator added (tPA, Sigma-Aldrich); this concentration of tPA was chosen as it resulted in full clot lysis in 90 min in a normal plasma sample. Three different final concentrations of purified rEnKT1 and aprotinin (positive control, Sigma-Aldrich) used in the test were 200 nM, 2 mM and 4 mM. Each experiment was performed for at least 60 min after the MCF was reached.

2.11. The effect of rEnKT1 on fish complement

Since teleost complement readily lyzes erythrocytes from various mammals, and those from rabbits were found to be the best complement activators (Sunyer and Tort, 1995; Boshra et al., 2006), we used fish serum and rabbit erythrocytes to test the effect of rEnKT1 on the activity of fish complement *in vitro*. Fresh goldfish (*Carassius auratus*) blood serum was obtained from the Department of Zoology, Charles University, Prague. Rabbit erythrocytes in Alsever‘s solution were purchased from ITEST plus Ltd., Czech Republic. The erythrocytes were washed three times in an excess of PBS and finally diluted to a 2% suspension (v/v). The assay was performed in a 96-well round bottomed microtiter plate (Nunc, Den-mark). Each well contained 100 ml of 20% fish serum in PBS premixed with various amounts of purified rEnKT1 (0.08-4 mM concentration). After 10 min incubation at room temperature, 100 ml of erythrocyte suspension were added. Controls did not contain either rEnKT1 or fish serum, or both. Additional controls were performed with heat-inactivated serum (56 LC, 30 min) and with bovine serum albumin (BSA) used instead of rEnKT1 at equal concentrations. Since the undiluted goldfish serum lysed rabbit erythrocytes immediately after their addition to the reaction, and the lysis time was virtually non-measurable, we used only 20% working concentration (i.e. 10% final serum concentration), which had been empirically established as optimal. Reaction wells were observed individually under a stereomicroscope using oblique illumination and an aluminium pad, and the time needed for lysis of the erythrocytes was measured. When full lysis was achieved, the reaction mixture turned from opaque to transparent. The experiment was repeated three times.

2.12. RNA *in situ* hybridisation

The specific amplified PCR product was obtained by PCR using gene-specific EnKT1 primers covering the whole EnKT1 gene sequence (forward: 5‘ ATG GGC AGT AAG TTA ATC TTA TCT ATG G 3‘ and reverse: 5‘ TTAAACCACTGGACAGGTGACTC 3‘) and First-strand cDNA of adult *E. nipponicum*. The product was ligated into a pGEM -T Easy vector according to the manufacturer‘s instructions. The resulting circular construct containing an EnKT1 gene insert was transformed into *E. coli* (XL1-Blue strain), and a positive clone was verified by DNA sequencing. Verified constructs were linearized (SacI/NcoI restriction enzymes) and used for the synthesis of antisense and sense RNA probes labelled with digoxigenin for *in situ* hybridisation *in vitro* according to the manufacturer‘s instructions (Dig RNA Labelling Kit SP6/T7, Roche). Histological sections (5 µm thick) of adult *E. nipponicum* were deparafinated in xylene, rehydrated and treated by proteinase K (final concentration 0.75 mg/ml, 17 min, 37 LC). Hybridisation was performed with RNA probes for 19 h at 42 LC employing a modified protocol as described previously (Quack et al., 2009). After that, sections were stringently washed 4 x 15 min successively in 2 concentrated saline-sodium citrate buffer (SSC), 1 SSC, 0.5 SSC and 0.1 SSC. Final detection was performed with alkaline phosphatase-conjugated anti-digoxigenin antibodies (1:500, Roche 11093274910) and Fast Red TR (Sigma-Aldrich F4523). As negative controls, sections were incubated under the same conditions without any probe. Signals were detected and photographed using an Olympus BX 51 microscope equipped with a DP70 camera (Olympus Co., Japan). Strand-specific real-time PCR (RT-PCR) was performed to control the presence of naturally occurring antisense transcripts in *E. nipponicum* cells (Ho et al., 2010).

2.13. Immunohistochemistry

Histological sections of worms (5 mm each) were prepared and blocked as described in Jedličková et al. (2018). After overnight incubation with sera diluted 1:10 in blocking buffer, the slides were washed 3 15 min at room temperature in PBS-Tr (phos-phate buffered saline/0.25% Triton X-100), incubated for 1 h at 37 LC with horseradish peroxidase (HRP)-labeled rabbit-anti-mouse IgG secondary antibody (ThermoFisher) diluted 1:2000, and washed again 3 x 15 min in PBS-Tr. Signal amplification was performed using the Tyramide Signal Amplification Plus Cyanine 5 System (Perkin Elmer, USA) according to the manufacturer‘s protocol. Finally, the sections were mounted in ProLong Diamond Antifade Mountant (Invitrogen). Fluorescence was observed and photographed under the Olympus IX83 microscope equipped with a PCO edge 5.5 camera and a CoolLED pE-4000 LED illumination system. Cyanine 5 signal was detected using a 635 nm diode and an emission filter 700/75. Appropriate lighting settings were determined using control slides probed with pre-immune serum to define the background signal threshold. Image stacks of optical sections were processed with Fiji software.

3. Results

3.1. Inhibitory activities of soluble worm protein extracts (solPE)

Fluorometric analyses showed that the solPE decreased peptidolytic activities of plasmin by 85%, thrombin by 60%, and FXa by 20% at chosen concentrations (Fig. 1). Control fluorometric assays performed only with solPE and peptide substrates of the three peptidases mentioned above did not reveal any hydrolysis of the substrates by peptidases of worm origin.

3.2. Sequence analyses of EnKT1 gene/protein

The nucleotide sequence of EnKT1 has 369 nucleotides and corresponds to a translated protein of 122 amino acids, of which 26 residues represent a signal peptide (Fig. 2). The mature protein is composed of 96 amino acids with a theoretical Mw of

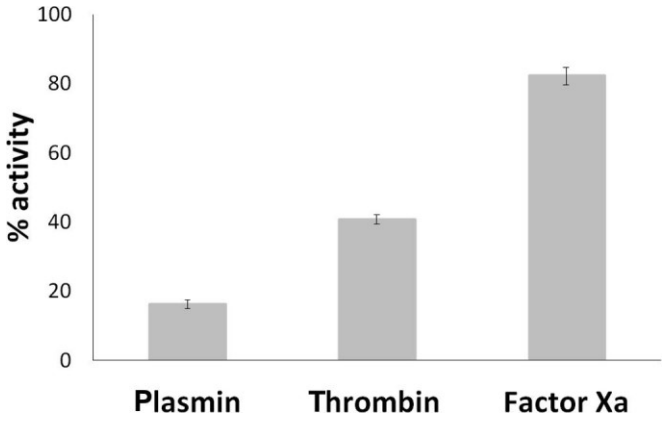

Fig. 1. Inhibitory effects of *Eudiplozoon nipponicum* soluble protein extract (solPE) on peptidases involved in haemostasis/fibrinolysis. Peptidases were preincubated with 5 mg of solPE. Activity was measured with appropriate substrates VPR-AMC (thrombin), IEGR-AMC (FXa) and ALK-AMC (plasmin). Bars represent S.D.

10.176 kDa, and pI 8.3. The Mw of the recombinant protein is slightly higher due to a 6x His-tag attached to the C-terminus. Putative N-glycosylation motifs are absent. EnKT1 possesses arginine residue in P1 (R_{81}) and alanine in $P1^0$ (A_{82}) positions. BlastP revealed that EnKT1 (GenBank accession number MF346930) has the highest sequence identity (69%) with textilinin-1 (GenBank accession number Q90WA1.1), a Kunitz type inhibitor isolated from the venom of the Eastern brown snake, *Pseudonaja textilis* (Flight et al., 2005). An alignment of amino acid sequences of the predicted inhibitory domain of EnKT1 with selected sequences of other Kunitz type domains from helminths, animal venoms, and with bovine aprotinin is presented in Supplementary Fig. S1. Comparison of the amino acid residues in the Kunitz type reactive loop of EnKT1, textilinin-1 and aprotinin, which represent important positions involved in interactions with the active site and exosites of serine peptidases, can be found in Table 2.

Two peptides matching parts of the amino acid sequence of EnKT1 were identified by mass spectrometry in *E. nipponicum* ESP (Fig. 2), indicating that the inhibitor may be secreted outside the worm's body.

3.3. Production of rEnKT1 in *E. coli*, its purification and detection

The recombinant EnKT1 expressed in *E. coli* was puriÞed to homogeneity by NiNTA affinity chromatography. It resolved in SDS-PAGE gels as a 12 kDa protein band in a reducing buffer. Its identity was verified by mass spectrometry. Transblotted protein (2 mg) was detected on PVDF membrane by iBODY4 binding to His-tag (Fig. 3). A reaction of mouse anti-rEnKT1 antibodies was confirmed on a western blot of rEnKT1 where the detected band occurred around 12 kDa. Control serum did not show any reaction, and therefore the sera were used for immunohistochemistry (Fig. 3). No reaction with antibodies was observed in the case of worm protein extract, solPE (not shown).

3.4. Stability of the rEnKT1-trypsin complex

Recombinant EnKT1 formed a complex with trypsin *in vitro*. A protein band around 38 kDa in the SDS-PAGE gel corresponded to the theoretical size of the complex (Fig. 4A,B). The trypsin-rEnKT1 complex was stable and unaffected by DTT and SDS in the reducing SDS-PAGE sample buffer (Fig. 4B) unless the sample was boiled (Fig. 4C).

3.5. Inhibition assays

rEnKT1 was tested for its inhibitory potential against a set of serine and cysteine peptidases. Inhibitory activities were demonstrated towards pancreatic trypsin, plasma kallikrein, plasmin and FXa with IC_{50} values indicated in Fig. 5. The rEnKT1 did not inhibit thrombin, FVIIa, and any of the tested recombinant cysteine peptidases - cathepsins B, L1 and L3 from *E. nipponicum*.

3.6. Thromboelastography

Thromboelastography was used for evaluation of the effect of rEnKT1 on fibrin clot formation and lysis in whole human blood. Purified rEnKT1 slowed the coagulation process in a concentration-dependent manner - the clotting time was prolonged by 60% in the presence of 4 mM inhibitor. In the case of blood from a patient with FX deficiency (<1% of normal plasma concentration), no effect on the time needed for fibrin clot formation was observed (Fig. 6). Particular CT data can be found in Supplementary Table S1.

No effect of rEnKT1 (0.2-4 mM) was recorded on ML60 in tPA - stimulated lysis of fibrin clot in whole blood in comparison with the positive control containing aprotinin at the same concentration, where inhibition of fibrinolysis was obvious at 2 mM (Table 3). On the other hand, we observed some inhibitory effect of rEnKT1 on MCF values; however, the significance of this data could not be evaluated statistically due to a low number of samples (Table 3).

3.7. Inhibition of fish complement-mediated cell lysis by rEnKT1

The lysis of rabbit erythrocytes in the presence of 10% fish serum was achieved within 3 min 55 s on average ±5 s. Inactivation of the serum by heat resulted in a loss of lytic activity. The final concentration of 0.04 mM rEnKT1 prolonged the time needed for complete lysis by 33% and 2 mM rEnKT1 by 145%, relevant to the control without rEnKT1 (Fig. 7). rEnKT1 alone had no effect on erythrocyte integrity even at a 20 mM concentration (this was verified also under a light microscope). BSA did not affect the lysis time in the presence of normal serum.

3.8. Localization of EnKT1 in adult worms

RNA *in situ* hybridisation with a specific antisense-RNA probe localised EnKT1 transcripts inside haematin (digestive) cells on his-tological sections of adult *E. nipponicum* worms (Fig. 8). No signals were found elsewhere, including parenchymal tissue, tegument, gonads or upper digestive tract. The same result was observed with

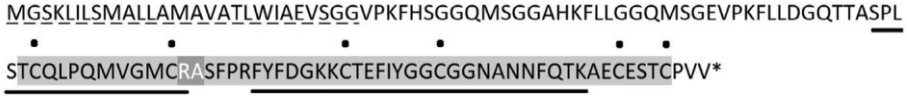

Fig. 2. Complete amino acid sequence of Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (EnKT1). Signal sequence on EnKT1 is marked by a dashed line. The predicted Kunitz domain is shaded in light grey. Cysteine residues stabilizing the Kunitz domain loop by forming disulfide bonds are marked by black dots. The P1 and $P1^0$ positions of the reactive site are indicated by white letters. Solid underlining indicates peptides identiÞed by mass spectrometry in excretory/secretory products of adult *E. nipponicum*.

Table 2
Primary structures of the P6-P5^0 region and P18^0 residue of the active loop of Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (EnKT1) textilinin-1, and aprotinin.

	P6	P5	P4	P3	P2	P1	P1^0	P2^0	P3^0	P4^0	P5^0	P18^0
EnKT1	M	V	G	M	C	R	A	S	F	P	R	F
Textilinin-1	D	T	G	P	C	R	V	R	F	P	S	F
Aprotinin	Y	T	G	P	C	K	A	R	I	I	R	F

Positions with non-conservative substitutions are printed in boldface type. The alignment was made with the use of partial sequences of EnKT1 (GenBank accession number MF346930), textilinin-1 (GenBank accession number Q90WA1.1), and aprotinin (GenBank accession number P00974.2)].

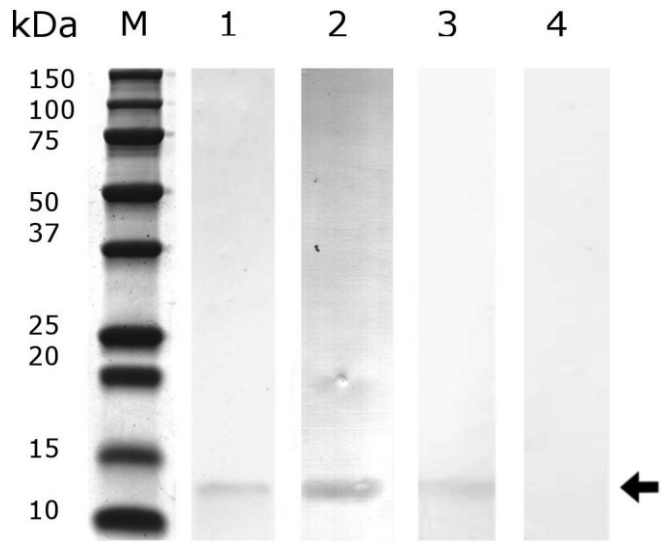

Fig. 3. Detection of the purified recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (rEnKT1) by SDS-PAGE and blotting. Lane 1, rEnKT1 in 4- 12% gel stained with Coomassie Brilliant Blue R-250. Lane 2, rEnKT1 band labelled by an iBody4 affinity probe on a blot. Lane 3, rEnKT1 band was detected by mouse anti-rEnKT1 antibodies on a blot. Lane 4, control blot with pre-immune serum.

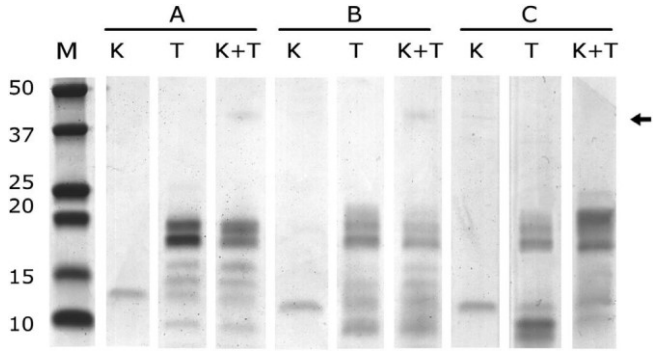

Fig. 4. Complex formation of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (rEnKT1) with trypsin. (A) Blots were run under non-reducing conditions. (B) Blots were run under reducing conditions. (C) Blots were run under reducing conditions with a boiled sample. The rEnKT1-trypsin complex is indicated by an arrow. Low MW bands in lanes with trypsin correspond to intermediate products of trypsin autohydrolysis. K, EnKT1 alone. T, trypsin alone. K + T, EnKT1 + trypsin.

a sense probe; however, strand-specific RT-PCR also revealed anti-sense transcription in *E. nipponicum*, which also was proven earlier in the case of some other transcripts (Jedličková et al., 2018). No reaction was observed in negative controls.

Immunolocalisation of EnKT1 with specific antibodies on histological sections of the adult worms showed the presence of EnKT1 only inside haematin digestive cells (Fig. 8). Pre-immune serum did not show any reaction.

4. Discussion

Haematophagous or blood/tissue-dwelling parasites employ protein inhibitors of haemocoagulation to enable their effective blood feeding or survival within the environment of the host. The targets of these inhibitors often include serine peptidases, predom-inantly thrombin (Factor IIa), FXa and FVIIa, due to their important position within the coagulation cascade (Francischetti et al., 2002; Hovius et al., 2008; Ranasinghe et al., 2015b,c). In this study, we focused on putative anticoagulants from neglected parasitic platy-helminths in terms of biochemical research, the blood-feeding diplozoid monogenean E. nipponicum. We combined biochemical and molecular approaches inclusive of transcriptomics in a search for effective anticoagulants. In the soluble extracts from adult worms (solPE) we detected activities that were able to block pep-tidolytic activities of three serine peptidases involved in haemosta-sis/Þbrinolysis in vertebrates Ð thrombin, FXa and plasmin. The results suggested that E. nipponicum may possess more than one serine peptidase inhibitor, a potential regulator of haemostasis, and so the worm extract seems to represent a complex sample con-taining a spectrum of inhibitors with different speciÞcities/func-tions. This indication can be supported by the presence of several Kunitz proteins, serpins and annexins in the transcriptome of adult worms (not shown). On the other hand, activities of the three tested heterologous peptidases might have been also affected to a certain degree by the presence of endogenous peptidases in solPE, although even prolonged incubation never resulted in a complete loss or a dramatic decrease in their activity.

Employing the transcriptomic data from adult *E. nipponicum*, we discovered a complete sequence of a Kunitz type protein, named EnKT1, including a signal peptide determining its secretory nature. A bioinformatic analysis showed it as an interesting protein due to a high sequence similarity with textilinin-1, an antihaemorrhagic factor from the venom of the Australian Eastern brown snake, *Pseudonaja textilis* (Masci et al., 2000). In addition, the sequence of EnKT1 has one Kunitz type domain and shows 41-60% overall identity with the most similar Kunitz proteins from other platyhelminths. The motif of the peptidase-interacting site within EnKT1 has Arg at the P1 position, suggesting that it is an inhibitor of trypsin-like peptidases. This was verified by fluorometric assays using synthetic peptidyl substrates and by the ability of rEnKT1 to form a stable complex with trypsin.

Single-domain Kunitz inhibitors (KIs) from blood-feeding ectoparasites may target a large spectrum of serine peptidases including procoagulant enzymes such as FXa and FVIIa, but usually do not act on thrombin (Factor IIa). On the other hand, several two-domain KIs from ticks may block the activity of this factor by a non-canonical mechanism of peptidase inhibition (Corral-Rodr'guez et al., 2009). To test the inhibitory specificity of rEnKT1, we used a panel of five serine peptidases involved in blood clotting, fibrinolysis and some related processes. As the coagulation mechanisms are highly conserved in vertebrates including teleost fishes (Tavares-Dias and Oliveira, 2009), we employed commercially available serine peptidases of human/bovine origin. In addition, we tested bovine trypsin and three recombinant cysteine peptidases from *E. nipponicum*. We had verified by a multiple alignment

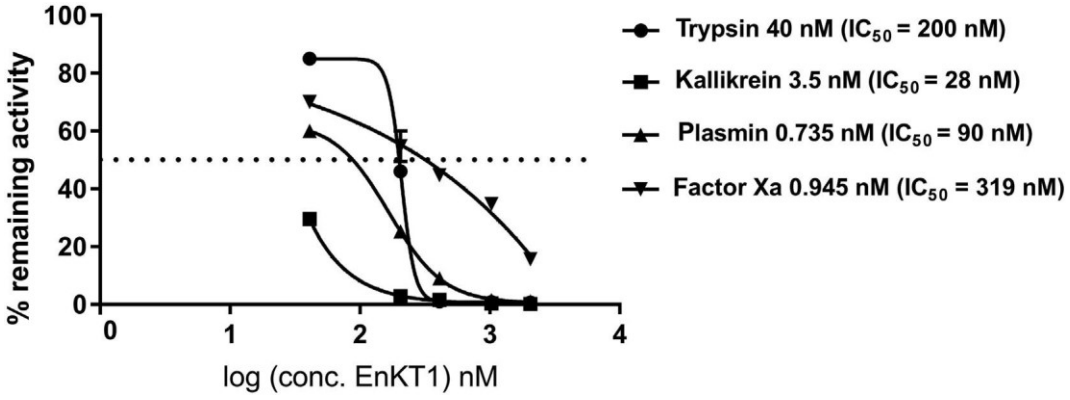

Fig. 5. Relative inhibition of trypsin, plasma kallikrein, plasmin and Factor Xa by recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (rEnKT1). The dotted line indicates 50% inhibition of peptidase activity (IC_{50}). Relative inhibition values are related to the enzymes' activities at 0 nM concentration of rEnKT1 inhibitor, which were always taken as 100%. The IC_{50} values were extrapolated by the software using non-linear regression including 0 nM concentration of rEnKT1 in the calculations. Bars represent S.D.

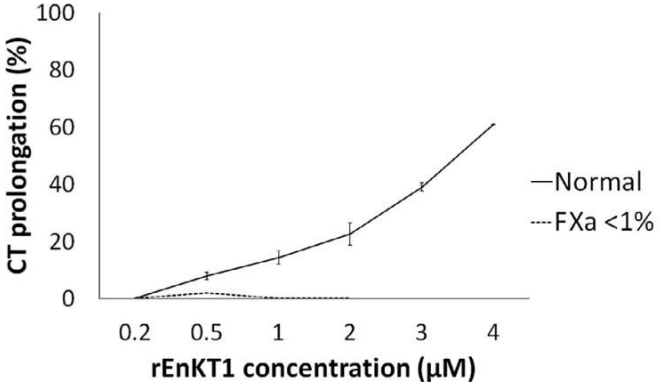

Fig. 6. The effect of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (rEnKT1) on formation of a fibrin clot in whole human blood. The black line shows relative prolongation of coagulation time in the presence of different concentrations of rEnKT1 in relation to the control. The dashed line shows no effect of rEnKT1 in the case of blood from a patient with Factor X deficiency (values related to the control from the same patient). Bars represent S.D. for three biological replicates. CT, clotting time (measured by rotational native thromboelastography).

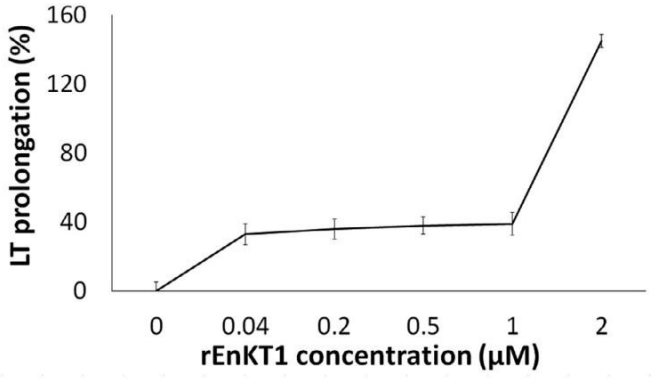

Fig. 7. Concentration-dependent effect of recombinant Kunitz-type inhibitor 1 from Eudiplozoon nipponicum (rEnKT1) on lysis of rabbit erythrocytes by goldÞsh complement. LT, lysis time (time needed for lysis of rabbit erythrocytes in 1% suspension, in the presence of 10% goldÞsh serum). Bars represent S.D.

Table 3
The effect of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (rEnKT1) and aprotinin on clot lysis and maximum clot firmness (MCF) values in whole human blood.

Experiment	ML60 (%)	MCF (mm)	
Control (without tPA)	8.8 ± 2.7	57	0
Control (with tPA)	97.7 ± 3.9	47	11.3
Aprotinin, 200 nM	99.7 ± 0.5	46	2.8
Aprotinin, 2 mM	18.7 ± 6.1	56	1.4
Aprotinin, 4 mM	14.3 ± 4.0	54.5 ± 2.1	
rEnKT1, 200 nM	100 ± 0.0	30 ± 7.0	
rEnKT1, 2 mM	99.6 ± 0.5	42.5 ± 2.1	
rEnKT1, 4 mM	99 ± 1.41	36	± 2.8

ML60, percent lysis of the fibrin clot measured 60 min after the maximum clot firmness was reached. tPA, tissue plasminogen activator.

that, despite the overall sequence heterogeneity, FX, plasmin and thrombin from *Homo sapiens*, *Bos taurus* and *Cyprinus carpio* share highly conserved sequence motifs around residues of the active sites and important substrate-binding sites, which are either identical among the species or include conservative amino acid substitutions (Supplementary Fig. S2).

Although the IC_{50} value of rEnKT1 was the lowest with plasma kallikrein (which is able to convert plasminogen to plasmin) and plasmin, i.e. peptidases involved in fibrinolysis (Plow et al., 1995), the functional tests with human blood confirmed that the inhibitor has no effect on the lysis of tPA-stimulated fibrin clots. On the other hand, the effect on prolongation of clotting time was significant, most likely due to the inhibition of FXa, albeit the IC_{50} of rEnKT1 with this enzyme was an order of magnitude higher than with kallikrein and plasmin. The effect on FXa was further confirmed by using blood from a FX-deficient patient, where rEnKT1 had no effect on the time needed for blood coagulation which is, indeed, much longer compared with healthy persons (Manikkan, 2012; Supplementary Table S1). Thus, we can consider EnKT1 a potential anticoagulant inhibiting FXa, which is a key factor situated in the pivotal position of the coagulation cascade in vertebrates including fish, where its function is to convert prothrombin to thrombin (Krishnaswamy, 2013).

Despite the overall amino acid sequence similarity of EnKT1 and textilinin-1, the two inhibitors possess certain non-conservative differences in the amino acid composition of the Kunitz domain active loop. It is known that in addition to P1-Arg, which is present in both, coagulation proteases also require specific interactions with other residues surrounding the scissile bonds in peptide substrates, in particular with those at the P3-$P3^0$ sites (Manithody et al., 2012). However, it is difficult to elucidate the role of individual amino acid residues within the active loop of EnKT1 in binding

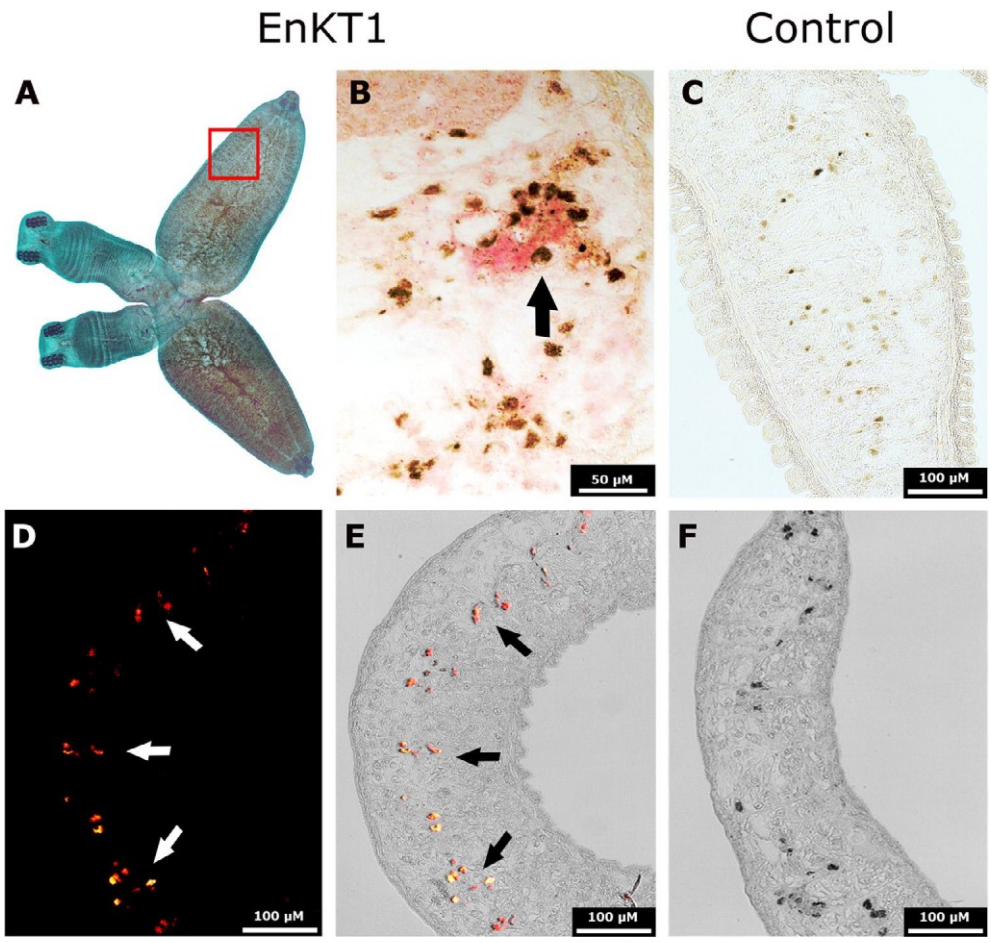

Fig. 8. Localisation of transcripts of Kunitz-type inhibitor 1 gene from *Eudiplozoon nipponicum* (Enkt1) and EnKT1 protein on histological sections of *E. nipponicum* adult worms. (A) Image of whole worms (pair) with an area marked by a square which indicates the approximate position within a worm's body corresponding to the body part in B. *In situ* RNA hybridisation: (B) reaction of an antisense RNA probe within haematin cells from the digestive tract; (C) negative control. Immunohistochemistry: (D) a reaction of anti-rEnKT1 antibodies within haematin cells (ßuorescence); (E) the same as D with merged ßuorescence + bright Þeld; (F) control with pre-immune serum. Arrows indicate localisation of EnKT1 transcripts/proteins inside haematin digestive cells.

to FXa without experiments employing mutant forms of the loop. Obvious divergences from the primary structure of textilinin-1 occur, especially in residues at positions P6 (hydrophobic versus polar negative), P5 (hydrophobic versus polar), $P2^0$ (polar versus polar positive), and $P5^0$ (polar positive versus polar) (see Table 2). So, it seems that some of these residues may support the ability of EnKT1 to effectively inhibit FXa, contrary to textilinin-1 and aprotinin which do not inhibit FXa (Flight et al., 2009) and are each more similar to the other in the primary structure of the Kunitz domain, than to EnKT1.

Plasma (pre)kallikrein has been for a long time believed to play a significant role in coagulation, as a part of the contact (plasma kallikrein-kinin) system that should activate the intrinsic coagulation pathway (Dennis et al., 1995; Wu, 2015). For example, the antithrombotic effect of *Schistosoma japonicum*/*Schistosoma mansoni* Kunitz proteins SjKI-1/SmKI-1 had been proposed to be based on the inhibition of the intrinsic pathway activation (Ranasinghe et al., 2015a,c). In contrast to these assumptions, recent research indicated that in humans the kallikrein-kinin contact system does not contribute to haemostasis *in vivo* (Schmaier, 2016). Moreover, it should be noted in the context of *E. nipponicum* biology as a fish parasite, that teleost fishes lack components of the plasma kallikrein-kinin system (Wong and Takei, 2013).

Plasmin, the enzyme ensuring proteolytic degradation of a fibrin clot, was inhibited by rEnKT1 in vitro with an IC_{50} in the nanomolar range. Surprisingly, no effect was observed on fibrinolysis *in vitro* functional tests, where rapid conversion of plasminogen to plasmin was facilitated by addition of tPA. While the reason is currently not obvious, it may be related to the concentrations of rEnKT1 used in the experiment which, however, were equal to the concentrations of aprotinin that significantly inhibited fibrinolysis. Perhaps the difference in anti-fibrinolytic action of these two inhibitors in the system may be caused by sequestration of rEnKT1 by FXa. Aprotinin, on the other hand, does not bind to FXa (Flight et al., 2009). Nevertheless, the results of thromboelastography make sense in terms of the parasite's need to keep the ingested blood in a fluid form. It was interesting to note the effect of rEnKT1 on MCF values in the EXTEG. A low MCF is generally indicative of decreased platelet function (or number), a low fibrinogen level or disorders of fibrin polymerization due to, e.g., decreased activity of factor XIII (transglutaminase stabilizing fibrin by polymerization). The lower extem MCF values in the presence of rEnKT1 could hypothetically indicate that EnKT1 might have an inhibitory effect on the function of platelets or on the enzymatic activity of factor XIII.

As verified by an *in vitro* test with rabbit erythrocytes and gold-fish serum, another function of EnKT1 may be the inhibition of the host complement cascade. Since FXa and plasmin can act as both C3 and C5 convertases (Amara et al., 2010), the inhibition of the two host peptidases may prevent complement activation inside

the parasites gut, and help in this way to avoid damage to the cells of gastrodermis. There is also a possibility that EnKT1 could interact with C1r/C1s/MASP serine peptidases involved in classical and lectin pathways of complement activation. Such an interaction was observed in the case of the human Kunitz-domain-based tissue factor pathway inhibitor (TFPI) that inhibits the lectin pathway of complement activation by direct binding to MASP-2 serine peptidase (Keizer et al., 2015). For EnKT1, however, this should be further tested experimentally. Despite of the mechanism of inhibition, the ability to impair host complement action would be of particular importance for a fish parasite, since a combination of high complement titers in teleosts (compared with mammals) and activation of the system over a wide range of temperatures, together with the diversity of some of its key components (C3 in particular), makes complement an extremely powerful innate defense system in fish (Boshra et al., 2006).

Transcripts of the EnKT1 gene were detected by RNA *in situ* hybridisation inside the digestive haematin cells. Furthermore, the same localisation was supported for the protein by immuno-histochemistry using anti-rEnKT1 antibodies. Since the EnKT1 transcript also encodes a signal sequence, the inhibitor is most likely secreted from these cells to the intestinal lumen of the worm. The important point is that we must think about the function of EnKT1 in the context of events that happen in the parasite‘s gut after ingestion of blood, rather than in the context of physiological processes in the host‘s circulation. In the digestive tract, EnKT1 could simultaneously interfere with haemocoagulation and with complement activation in ingested host blood. By prolonging the time needed for coagulation and complement-mediated cell lysis, EnKT1 might provide a longer time-frame for the parasite‘s digestive cysteine and aspartic peptidases to hydrolyze soluble proteins including clotting factors, fibrinogen and complement components in a blood meal (Jedličková et al., 2016, 2018). Nevertheless, EnKT1 was also detected in ESP of adult worms by mass spectrometry analyses. This implies that the secreted inhibitor could be delivered from the parasite‘s intestine to the feeding site (wound) on the host‘s gills and into the host‘s circulation, where it might interfere with coagulation and other physiological/immune processes based on the activities of serine peptidases such as complement-coordinated events in inflammation, and immunity in general (Markiewski and Lambris, 2007).

In conclusion, we note that despite the high negative economic impact of some monogenean species on fish aquaculture, no experimental data on the molecular basis of host-parasite interactions are available even for highly important pathogens such as *Gyrodactylus salaris* and *Benedenia* spp. Thus, the present study opens a fresh view on these interactions and deepens our knowledge of digestion-related events in sanguinivorous monogeneans. It also represents, to our knowledge, the first detection and biochemical/functional characterisation of a serine peptidase inhibitor from Monogenea. EnKT1, a member of Kunitz protein family, acts as a potent secretory anticoagulant targeting FXa of the coagulation cascade, as well as an inhibitor of complement-mediated cell lysis. The features of EnKT1, together with the detection of anti-thrombin activity in worm extracts, and with the presence of six other Kunitz proteins, three serpins (unpublished) and a cystatin (Ilgová et al., 2017) in the *E. nipponicum* transcriptome, suggest that blood-feeding monogeneans operate a sophisticated array of tools interfering with host physiology and immunity. Further research on structures and functions of these molecules might result in finding interesting parasite-derived peptidase inhibitors with a potential in human biomedicine, or in the development of vaccines targeting proteins which seem to be crucial for survival of monogeneans, the devastating pests of farmed fish.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.11.010.

References

Amara, U., Flierl, M.A., Rittirsch, D., Klos, A., Chen, H., Acker, B., Bruckner, U.B., Nilsson, B., Gebhard, F., Lambris, J.D., Huber-Lang, M., 2010. Molecular Intercommunication between the complement and coagulation systems. J. Immunol. 185, 5628Ð5636.

Artimo, P., Jonnalagedda, M., Arnold, K., Baratin, D., Csardi, G., de Castro, E., Duvaud, S., Flegel, V., Fortier, A., Gasteiger, E., Grosdidier, A., Hernandez, C., Ioannidis, V., Kuznetsov, D., Liechti, R., Moretti, S., Mostaguir, K., Redaschi, N., Rossier, G., Xenarios, I., Stockinger, H., 2012. ExPASy: SIB bioinformatics resource portal. Nucleic. Acids Res. 40, W597ÐW603.

Boshra, H., Li, J., Sunyer, J.O., 2006. Recent advances on the complement system of teleost fish. Fish Shellfish Immunol. 20, 239Ð262.

Buchmann, K., Bresciani, J., 2006. Monogenea Phylum Platyhelminthes. In: Woo, P.T. K. (Ed.), Fish Diseases and Disorders Volume 1. 2nd edition. CABI, Wallingford, pp. 297Ð344.

Corral-Rodríguez, M.ç., Macedo-Ribeiro, S., Barbosa Pereira, P.J., Fuentes-Prior, P., 2009. Tick-derived Kunitz-type inhibitors as antihemostatic factors. Insect Biochem. Mol. Biol. 39, 579Ð595.

Dennis, M.S., Herzka, A., Lazarus, R.A., 1995. Potent and selective kunitz domain inhibitors of plasma kallikrein designed by phage display. J. Biol. Chem. 207, 25411Ð25417.

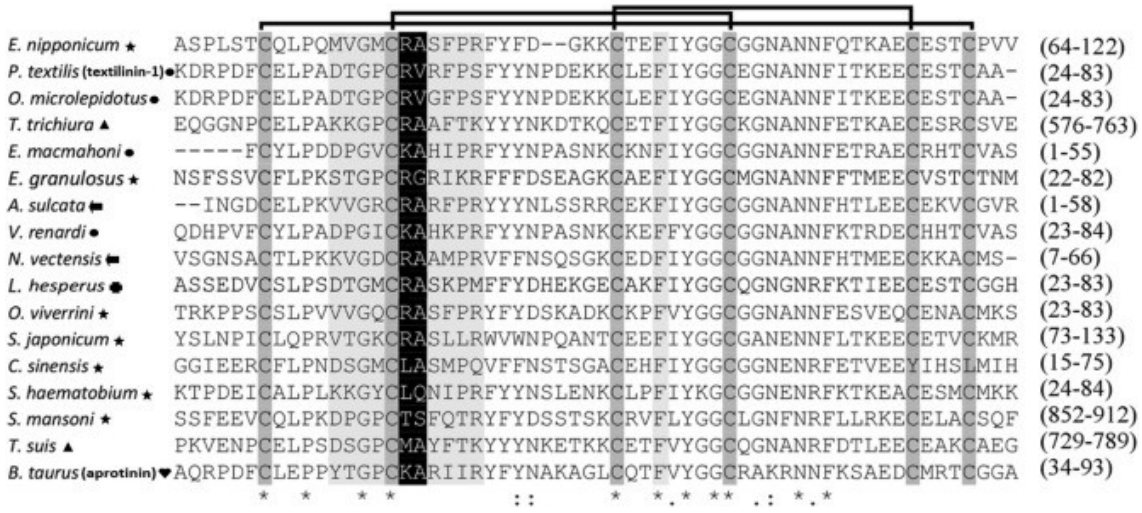
Falcón, C.R., Masih, D., Gatti, G., Sanchez, M.C., Motrán, C.C., Cervi, L., 2014. *Fasciola hepatica* Kunitz type molecule decreases dendritic cell activation and their ability to induce inflammatory responses. PLoS One 9, e114505.

Flight, S., Johnson, L., Trabi, M., Gaffney, P., Lavin, M., de Jersey, J., Masci, P., 2005. Comparison of textilinin-1 with aprotinin as serine protease inhibitors and as antifibrinolytic agents. Pathophysiol. Haemost. Thromb. 34, 188Ð193.

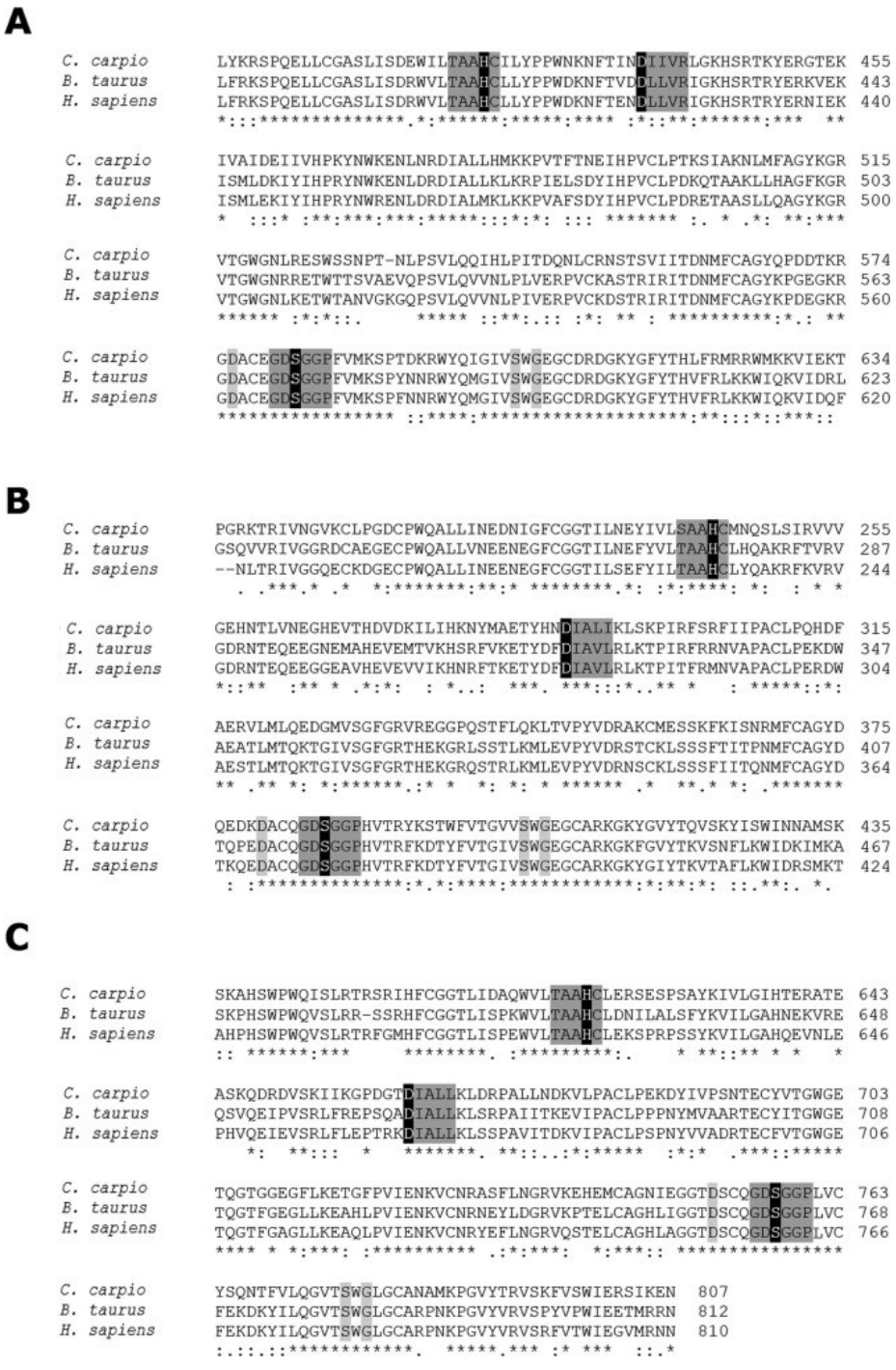
Flight, S.M., Johnson, L.A., Du, Q.S., Warner, R.L., Trabi, M., Gaffney, P.J., Lavin, M.F., de Jersey, J., Masci, P.P., 2009. Textilinin-1, an alternative anti-bleeding agent to

aprotinin: importance of plasmin inhibition in controlling blood loss. Br. J.
Haematol. 145, 207-211.
Fló, M., Margenat, M., Pellizza, L., Grana, M., Dur‡, N.R., Bá Ez, A., Salceda, E., Soto, E., Alvarez, B., Ferná Ndez, C., 2017. Functional diversity of secreted cestode Kunitz proteins: inhibition of serine peptidases and blockade of cation channels. PLoS Pathog. 13, 1-33.
Francischetti, I.M.B., Valenzuela, J.G., Andersen, J.F., Mather, T.N., Ribeiro, J.M.C., 2002. Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, *Ixodes scapularis*: identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. Blood 99, 3602-3612.
Ho, E.C.H., Donaldson, M.E., Saville, B.J., 2010. Detection of antisense RNA transcripts by strand-specific RT-PCR. Methods Mol. Biol. 630, 125-138.
Hovius, J.W.R., Levi, M., Fikrig, E., 2008. Salivating for knowledge: potential pharmacological agents in tick saliva. PLoS Med. 5, e43.
Huntington, J.A., 2013. Thrombin inhibition by the serpins. J. Thromb. Haemost. 11, 254Đ264.
Ilgová, J., Jedličková, L., Dvořáková, H., Benovics, M., Mikeš, L., Janda, L., Vorel, J., Roudnický, P., Potěšil, D., Zdráhal, Z., Gelnar, M., Kašný, M., 2017. A novel type i cystatin of parasite origin with atypical legumain-binding domain. Sci. Rep. 7, 17526.
Jedličková, L., Dvořáková, H., Kašný, M., Ilgová, J., Potěšil, D., Zdráhal, Z., Mikeš, L., 2016. Major acid endopeptidases of the blood-feeding monogenean *Eudiplozoon nipponicum* (Heteronchoinea: Diplozoidae). Parasitology 143, 494-506.
Jedličková , L., Dvořáková, H., Dvořák, J., Kašný, M., Ulrychová, L., Vorel, J., Žárský, V., Mikeš, L., 2018. 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. Parasit. Vectors. 11, 142.
Kawatsu, H., 1978. Studies on the anemia of Fish-IX, hypochromic microcytic anemia of crucian carp caused by infestation with a trematode, *Diplozoon nipponicum*. Bull. Japan Soc. Sci. Fish 44, 1315-1319.
Keizer, M.P., Pouw, R.B., Kamp, A.M., Patiwael, S., Marsman, G., Hart, M.H., Zeerleder, S., Kuijpers, T.W., Wouters, D., 2015. TFPI inhibits lectin pathway of complement activation by direct interaction with MASP-2. Eur. J. Immunol. 45, 544-550.
Krishnaswamy, S., 2013. The transition of prothrombin to thrombin. J. Thromb. Haemost. 11, 265-276.
Krowarsch, D., Dadlez, M., Buczek, O., Krokoszynska, I., Smalas, A.O., Otlewski, J., 1999. Interscaffolding additivity: binding of P1 variants of bovine pancreatic trypsin inhibitor to four serine proteases. J. Mol. Biol. 289, 175-186.
Laskowski, M., Kato, I., 1980. Protein inhibitors of proteinases. Annu. Rev. Biochem.
49, 593-626.
Lee, J.H., Kim, C.H., Shin, Y.P., Park, H.J., Park, S., Lee, H.M., Kim, B.S., Lee, I.H., 2010. Characterization of Kunitz-type protease inhibitor purified from hemolymph of *Galleria mellonella* larvae. Insect Biochem. Mol. Biol. 40, 873-882.
Manikkan, A.T., 2012. Factor X deÞciency: an uncommon presentation of AL amyloidosis. Ups. J. Med. Sci. 117, 457-459.
Manithody, C., Yang, L., Rezaie, A.R., 2012. IdentiÞcation of exosite residues of factor Xa involved in recognition of PAR-2 on endothelial cells. Biochemistry 51, 2551-2557.
Markiewski, M.M., Lambris, J.D., 2007. The role of complement in inflammatory diseases from behind the scenes into the spotlight. Am. J. Pathol. 171, 715-727.
Masci, P.P., Whitaker, A.N., Sparrow, L.G., de Jersey, J., Winzor, D.J., Watters, D.J., Lavin, M.F., Gaffney, P.J., 2000. Textilinins from *Pseudonaja textilis textilis*. Characterization of two plasmin inhibitors that reduce bleeding in an animal model. Blood Coagul Fibrinolysis 11, 385-393.
Matějusová, I., Koubková, B., D'Amelio, S., Cunningham, C.O., 2001. Genetic characterization of six species of diplozoids (Monogenea; Diplozoidae). Parasitology 123, 465-474.
Millers, E.-K.I., Trabi, M., Masci, P.P., Lavin, M.F., de Jersey, J., Guddat, L.W., 2009. Crystal structure of textilinin-1, a Kunitz-type serine protease inhibitor from the venom of the Australian common brown snake (Pseudonaja textilis). FEBS J. 276, 3163-3175.
Milstone, A.M., Harrison, L.M., Bungiro, R.D., Kuzmic, P., Cappello, M., 2000. A broad spectrum Kunitz type serine protease inhibitor secreted by the hookworm *Ancylostoma ceylanicum*. J. Biol. Chem. 275, 29391-29399.
Oliva, M.L.V., Silva, M.C.C., Sallai, R.C., Brito, M.V., Sampaio, M.U., 2010. A novel subclassification for Kunitz proteinase inhibitors from leguminous seeds. Biochimie 92, 1667Đ1673.
Peigneur, S., Billen, B., Derua, R., Waelkens, E., Debaveye, S., BŽress, L., Tytgat, J., 2011. A bifunctional sea anemone peptide with Kunitz type protease and potassium channel inhibiting properties. Biochem. Pharmacol. 82, 81-90.
Petersen, T.N., Brunak, S., von Heijne, G., Nielsen, H., 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nature Methods 8, 785-786.
Plow, E.F., Herren, T., Redlitz, A., Miles, L.A., Hoover-Plow, J.L., 1995. The cell biology of the plasminogen system. FASEB J. 9, 939-945.
Quack, T., Knobloch, J., Beckmann, S., Vicogne, J., Dissous, C., Grevelding, C.G., 2009. The formin-homology protein SmDia interacts with the Src kinase SmTK and the GTPase SmRho1 in the gonads of *Schistosoma mansoni*. PLoS One 4, e6998.
Ranasinghe, S., McManus, D.P., 2013. Structure and function of invertebrate Kunitz serine protease inhibitors. Dev. Comp. Immunol. 39, 219-227.
Ranasinghe, S.L., Fischer, K., Gobert, G.N., Mcmanus, D.P., 2015a. Functional expression of a novel Kunitz type protease inhibitor from the human blood fluke *Schistosoma mansoni*. Parasit. Vectors. 8, 1-10.
Ranasinghe, S.L., Fischer, K., Zhang, W., Gobert, G.N., McManus, D.P., 2015b. Cloning and characterization of two potent kunitz type protease inhibitors from *Echinococcus granulosus*. PLoS Negl. Trop. Dis. 9, e0004268.
Ranasinghe, S.L., Fischer, K., Gobert, G.N., McManus, D.P., 2015c. A novel coagulation inhibitor from *Schistosoma japonicum*. Parasitology 142, 1663-1672.
Reed, P., Francis-Floyd, R., Klinger, R. and Petty, D., 2009. Monogenean Parasites of Fish. University of Florida IFAS extension, UF/IFAS Fact Sheet FA28. http://Þsheries.tamu.edu/Þles/2013/09/Monogenean-Parasites-of-Fish.pdf.
Shigetomi, H., Onogi, A., Kajiwara, H., Yoshida, S., Furukawa, N., Haruta, S., Tanase, Y., Kanayama, S., Noguchi, T., Yamada, Y., Oi, H., Kobayashi, H., 2010. Anti-inflammatory actions of serine protease inhibitors containing the Kunitz domain. Inßamm. Res. 59, 679-687.
Schmaier, A.H., 2016. The contact activation andkallikrein/kinin systems: pathophysiologic and physiologic activities. J. Thromb. Haemost. 14, 28-39.
Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D., Higgins, D.G., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Mol. Syst. Biol. 7, 539.
Smith, D., Tikhonova, I.G., Jewhurst, H.L., Drysdale, O.C., Dvořák, J., Robinson, M.W., Cwiklinski, K., Dalton, J.P., 2016. Unexpected activity of a novel Kunitz-type Inhibitor: inhibition of cysteine proteases but not serine proteases. J. Biol. Chem. 291, 19220-19234.
Sunyer, J.O., Tort, L., 1995. Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. Vet. Immunol. Immunopathol. 45, 333-345.
Šácha, P., Knedlík, T., Schimer, J., Tykvart, J., Parolek, J., Navrátil, V., Dvořáková, P., Sedlák, F., Ulbrich, K., Strohalm, J., Majer, P., Šubr, V., Konvalinka, J., 2016. iBodies: modular synthetic antibody mimetics based on hydrophilic polymers decorated with functional moieties. Angew. Chem. Int. Ed. Engl. 55, 2356-2360.
Tavares-Dias, M., Oliveira, S.R., 2009. A review of the blood coagulation system of fish. Braz. J. Biosci. 7, 205-224.
Tsujimoto, H., Kotsyfakis, M., Francischetti, I.M.B., Eum, J.H., Strand, M.R., Champagne, D.E., 2012. Simukunin from the salivary glands of the black fly *Simulium vittatum* inhibits enzymes that regulate clotting and inflammatory responses. PloS One 7, e29964.
van Genderen, H.O., Kenis, H., Hofstra, L., Narula, J., Reutelingsperger, C.P.M., 2008. Extracellular annexin A5: functions of phosphatidylserine-binding and two-dimensional crystallization. Biochim. Biophys. Acta 1783, 953-963.
Wong, M.K.S., Takei, Y., 2013. Lack of plasma kallikrein-kinin system cascade in teleosts. PLoS One 8, e81057.
Wu, Y., 2015. Contact pathway of coagulation and inflammation. Thromb. J. 13, 1Đ9.
Yuan, C.-H., He, Q.-Y., Peng, K., Diao, J.-B., Jiang, L.-P., Tang, X., Liang, S.-P., 2008. Discovery of a distinct superfamily of Kunitz-type toxin (KTT) from tarantulas.PloS One 3, e3414.
Zhao, R., Dai, H., Qiu, S., Li, T., He, Y., Ma, Y., Chen, Z., Wu, Y., Li, W., Cao, Z., 2011. SdPI, the Þrst functionally characterized Kunitz-type trypsin inhibitor from scorpion venom. PloS One 6, e27548.

Additional files:



Supplementary fig. S1. Amino acid sequence alignment of predicted Kunitz domains of the Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (EnKT1) and other selected Kunitz proteins. The P1 and P1′ positions of the reactive sites are shaded in black. Cysteine residues stabilizing Kunitz domain loops by creating disulfide bonds are shaded in dark grey. Important residues of reactive sites at P6–P5′ positions and the P18′ position are shaded in light grey. Numbering represents true positions within the amino acid sequences of particular proteins. Organisms were divided into six groups according to their taxonomic classification and marked by symbols: parasitic platyhelminths, parasitic nematodes, snakes, anemones, spiders, bovines. Sequences used: *Pseudonaja textilis* (GenBank accession number Q90WA1.1), *Oxyuranus microlepidotus* (Q6ITB4.1), *Trichuris* trichiura (CDW57127.1), *Eristicophis macmahoni* (P24541.1), *Echinococcus* granulosus (EUB61666.1), *Anemonia sulcata* (Q9TWF8.1), *Vipera renardi* (AEI52542.1), *Nematostella vectensis* (XP_001621919.1), *Latrodectus hesperus* (ADV40356.1), *Opisthorchis* viverrini (OON23666.1), *Schistosoma* japonicum (CAX74161.1), *Clonorchis sinensis* (GAA54961.1), *Schistosoma haematobium* (XP_012800948.1), *Schistosoma mansoni* (XP_018648650.1), *Trichuris suis* (KHJ42567.1), *Bos taurus* (P00974.2).



Supplementary fig. S2. Alignment of partial amino acid sequences of enzymes involved in coagulation and fibrinolysis from carp, bovine and human. (**A**) Thrombin *Cyprinus carpio* (GenBank accession number KTF81317.1), *Bos taurus* (NP_776302.1), *Homo sapiens* (AAH51332.1), (**B**) Factor Xa – *Cyprinus carpio* (KTF93720.1), *Bos taurus* (NP_001073682.1), *Homo sapiens* (NP_001299603.1), (**C**) plasmin – *Cyprinus carpio* (KTF80813.1), *Bos taurus* (NP_776376.1), *Homo sapiens* (AAA36451.1). The catalytic triads of the active site are shaded in black. Conserved motifs around active site residues are shaded in dark grey. Residues of the substrate-binding sites involved in determination of substrate specificity are shaded in light grey.

Experimental blood	CT (s)					
	Control/0.2 µM r*En*KT1	Control/0.5 µM r*En*KT1	Control/1 µM r*En*KT1	Control/2 µM r*En*KT1	Control/3 µM r*En*KT1	Control/4 µM r*En*KT1
Healthy	832/826	879/752	752/847	701/868	706/989	722/1162
	900/901	793/871	783/925	737/879	707/976	735/1176
	973/966	851/913	926/1042	749/956	737/1009	706/1138
FXa (‹1%)	1021/1020	1021/1001	1066/1099	1082/1040	ND	ND

Supplementary Table S1. Particular values of coagulation times of recalcified citrated whole human blood in the presence of various concentrations of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (r*En*KT1). Measured with star-tem ROTEM reagent (NATEG) in three biological replicates (healthy donors) and in one patient with Factor X deficiency. CT is expressed in seconds. Each concentration of r*En*KT1 was associated with an appropriate control (PBS without r*En*KT1). ND, not done.

Závěr a diskuze

Tato dizertační práce představuje první rozsáhlejší průzkum a funkční popis bioaktivních molekul, které se podílejí na příjmu a trávení krve u krevsajících monogeneí. Z biochemického a molekulárního hlediska se jedná o opomíjenou skupinu ektoparazitických helmintů i přesto, že některé druhy způsobují velké škody v akvakulturách ryb. Důkazem toho může být, že v rámci monogeneí jsou dostupná genomická data pouze u dvou druhů, mukofága *Gyrodactylus salaris* a hematofága *Protopolystoma xenopodis*, a transkriptomová data pouze u mukofága *Neobenedenia melleni* a nyní též díky naší práci u hematofága *Eudiplozoon nipponicum*. Tato data nabízejí do budoucna možnost provedení srovnávací studie těchto dvou potravních strategií. Z předběžného průzkumu je např. patrné, že mukofágové mají elastáze podobné serinové peptidázy, které nebyly nalezeny u hematofágů (Mikeš, nepublikováno). Nicméně momentální nedostatek experimentálních dat u ostatních monogeneí vylučuje provést jakékoliv srovnání z biochemického hlediska. Informace jsou dostupné pouze z ultrastrukturálních a histochemických analýz. Proto tato práce představuje v podstatě pilotní studii.

Eudiplozoon nipponicum je ektoparazit, který se řadí mezi prvoústé živočichy s jedním příjímacím a vyvrhovacím otvorem. S hostitelem komunikuje pouze skrze ústní otvor. Nicméně doposud nebyly popsány žádné molekuly, které se těchto interakcí účastní. V předním střevě se nacházejí četné přídatné žlázky, ve kterých by mohly být obsaženy různé bioaktivní molekuly, které by se mohly podílet na interakcích s hostitelem, nebo na příjmu a zpracování krve (Smyth *et* Halton, 1983a; b). Další molekuly se mohou dostat do exkrečně sekrečních produktů parazita s nestrávenými zbytky potravy, které se uvolňují regurgitací ze střeva ven. Trávení proteinů je u krevsajících parazitů jedním z hlavních proteolytických procesů, který se může značně lišit u jednotlivých druhů parazitů zastoupením podílejících se peptidáz, ale i místem, kde probíhá. Např. u schistosom trávení probíhá extracelulárně v lumen střeva, kam jsou trávicí peptidázy sekretovány z gastrodermis (Caffrey *et al.,* 2004). U fasciol dochází ke kombinaci extracelulárního a intracelulárního trávení. Nejprve dochází k degradaci hemoglobinu v lumen střeva na menší fragmenty, které jsou pak vstřebány do buněk gastrodermis, kde trávení pokračuje (Robinson *et al.,* 2008). U klíšťat probíhá trávení hemoglobinu intracelulárně v lysozomálním cyklu uvnitř trávicích buněk (Sonenshine,

1991). U *E. nipponicum* se nám podařilo prokázat, že za hlavní proteolytickou aktivitu jsou zodpovědné cysteinové peptidázy, a to zejména katepsiny L. Nicméně z výsledků práce je patrné, že na degradaci hemoglobinu se podílejí jak cysteinové, tak aspartické peptidázy. Podobně je tomu i u mnoha dalších parazitů, jako je motolice *Schistosoma mansoni* (Caffrey *et al.,* 2004) nebo klíště *Ixodes ricinus* (Sojka *et al.,* 2013). U *E. nipponicum* aktivita katepsinů L převažovala nad aktivitou katepsinů D a následně i nad katepsiny B. Proto jsem se v této práci zaměřila na detailní charakteristiku katepsinů L. Pomocí degenerovaných primerů a PCR metod byly získány dvě kompletní sekvence katepsinů L *E. nipponicum* (EnCL1 a EnCL3), které byly vyprodukovány v rekombinantní formě. Katepsiny L prokázaly substrátové preference typické pro katepsiny L, lišily se pouze v pH profilech aktivity vůči fluorogenním substrátům. Byly schopné štěpit hemoglobin, albumin, kolagen, IgG a fibrinogen a oba katepsiny byly lokalizovány uvnitř hematinových (trávicích) buněk, ale i v lumen střeva. Na základě ultrastrukturních analýz z dostupné literatury se předpokládalo, že trávení krve probíhá intracelulárně v lysozomálním cyklu uvnitř trávicích buněk (Smyth *et* Halton, 1983) podobně jako je tomu u klíšťat (Sonenshine, 1991). Nicméně z výsledků této práce je patrné, že k první fázi trávení by mohlo pravděpodobně docházet extracelulárně v lumen střeva za přítomnosti sekretovaných peptidáz, podobně jako je tomu u motolice *Fasciola hepatica* (Robinson *et al.,* 2008).

Transkriptomová analýza dospělce krevsajícího monogenea *E. nipponicum* odhalila široké spektrum katepsinů L (10 transkriptů), pouze jeden katepsin B a tři katepsiny D. Jeden z katepsinů D obsahuje mutaci v aktivním místě, což způsobuje ztrátu jeho hydrolytické aktivity (Dvořáková, nepublikováno). Analýza transkriptomu odpovídá poměrově zastoupení enzymů u některých dalších krevsajících helmintů. Např. u *F. hepatica* bylo nalezeno 27 cysteinových peptidáz a pouze 1 aspartická peptidáza (katepsin D). U *Fasciola gigantica* bylo identifikováno 14 cysteinových peptidáz (Kašný *et al.,* 2009; Rawlings *et al.,* 2010) a u *Fascioloides magna* jich bylo zaznamenáno 9 (Cantacessi *et al.,* 2012). Produkce širokého spektra peptidáz s různými substrátovými preferencemi umožnuje parazitovi efektivněji degradovat hostitelské makromolekuly. Strukturní vlastnosti některých katepsinů L u *E. nipponicum* vykazovaly odlišnosti od typických katepsinů L. S2 kapsa je klíčový determinant substrátové specifity papain-like peptidáz (Choe et al., 2006). Proto jsme provedli srovnání sekvencí získaných katepsinů L a zaměřili se na kompozici S2 kapsy aktivního místa, která byla u některých více

odlišná. Katepsiny L *E. nipponicum* se na základě rozdílné polarity a náboje S2 kapsy rozdělily do 3 skupin. Jako příklad zde uvádím EnCL6, kde negativně nabitý Asp v pozici 205 (číslování podle papainu) v S2 kapse může katepsinu L dát specifitu podobnou katepsinům B. Ortology EnCL6 mají amfipatický Gly133 v S2 kapse, který je typický pro katepsin S nebo pro „plerocerkoid growth factor" (PGF) u larválního stádia tasemnice Spirometra. U EnCL2 naznačila kompozice S2 kapsy, že se může jednat o potenciální kolagenázu/elastázu. Přítomnost aromatického zbytku Tyr/Trp67 v aktivním místě je typická pro katepsin L2 a L3 u F. hepatica a lidský katepsin K, které ochotně štěpí kolagen a elastin (Lecaille *et al.,* 2002; Corvo *et al.,* 2009). Navíc exprese EnCL2 u dospělého stádia je velmi nízká. Široké zastoupení katepsinů L s různou mírou exprese u dospělého stádia *E. nipponicum* pravděpodobně naznačuje důležitost těchto enzymů v různých dějích životního cyklu parazita včetně rozdílných životních stadií jako je např. onkomiracidium, diporpa nebo dospělec. U endoparazitů typu *F. hepatica* se katepsiny L podílejí, kromě trávení, na tkáňové migraci, úniku před imunitní odpovědí, excystaci metacerkarií nebo na produkci vajíček (Tort *et al.,* 1999; Dalton *et al.,* 2006). I u *Schistosoma mansoni* bylo detekováno více katepsinů L, kde SmCL1/F, SmCL2 a SmCL3 se podílejí na trávení hemoglobinu (Caffrey *et al.,* 2004, Dvořák *et al.,* 2009). SmCL2, kromě podílu na trávení, byl v jedné studii detekován i v pohlavní soustavě samic (Michel *et al.,* 1995), což však další studie vyvrací (Bogitsh *et al.,* 2001). U *E. nipponicum* byly biochemicky charakterizovány pouze dva katepsiny L z deseti identifikovaných v transkriptomu, a ty se pravděpodobně účastní trávení. Zbylé enzymy mohou mít rozdílnou lokalizaci, ale i rozdílnou funkci v organizmu. Sekrece proteolytických enzymů do vnějšího prostředí, jako toho využívá např. *F. hepatica* při tkáňové migraci, se u ektoparazita žijícího na žábrách kapra zdá být nepravděpodobná. Nicméně oba katepsiny L byly detekovány v exkrečně sekrečních produktech dospělce. Mohou tedy hrát teoreticky roli i v interakcích s hostitelem. Zajímavé je, že EnCL1 narozdíl od EnCL3 neobsahuje N-terminální signální sekvenci typickou pro sekretované proteiny. Nepřítomnost signální sekvence u katepsinů L byla popsána i u několika dalších organizmů jako je např. ploštice *Rhodnius prolixus* (Lopez-Ordoñez *et al.,* 2001). U lidského katepsinu L byla prokázána přímá sekreční dráha díky aminokyselinové sekvenci –SXPXV lokalizované v C-terminální sekvenci enzymu (Chauhan *et al.,* 1998). V transkriptomu dospělce *E. nipponicum* byl nalezen i další katepsin L (EnCL5), který neobsahuje signální sekvenci. Doposud není jasné, jaké intramolekulární signály by

mohly být zahrnuty v sekreci těchto enzymů a zda jsou skutečně sekretovány. K odhalení mechanizmu sekrece by mohla napomoci lokalizace EnCL5 spolu s ověřením, zda je přítomný i v exkrečně sekrečních produktech, s následným porovnáním jejich primární struktury s EnCL1. Nicméně z výsledků lokalizace a detekci EnCL1v sekretomu je patrné, že k sekreci dochází. Oba charakterizované katepsiny L byly imunohistochemicky lokalizovány ve střevě dospělce, nicméně u protilátek nebyla ověřována zkřížená reaktivita. Pro potvrzení výsledků byly navrženy specifické RNA próby a byla provedena RNA in situ hybridizace, která lokalizovala oba katepsiny L uvnitř trávicích buněk střeva. Nicméně sense RNA próby, které byly použity jako kontrola, reagovaly stejně jako antisense RNA próby. Z literatury je známo, že více než 30 % transkribovaných genů u člověka má antisense transkripci (Ozsolak *et al.,* 2010) a podobně je tomu i u jiných organizmů (Osato *et al.,* 2003; Steigele *et* Nieselt, 2005; Henke *et al.,* 2015). Vláknově specifickou RT-PCR byla ověřena přítomnost obou transkriptů ve vzorcích. Přítomnost přirozeně se vyskytujících antisense transkriptů by mohla sloužit k regulaci genové transkripce (Pelechano *et* Steinmetz, 2013). Zároveň nebyla provedena kolokalizace obou enzymů najednou, takže nejsme schopni říct, jestli se EnCL1 nachází ve stejných kompartmentech/váčcích jako EnCL3, což by bylo dobré do budoucna ověřit.

Vzhledem k tomu, že příjem a trávení krve se neobejde bez reakcí s koagulačním systémem, testovali jsme extrakty dospělců *E. nipponicum* na přítomnost těchto látek. Ve vzorku solubilního proteinového extraktu dospělce se podařilo detekovat inhibiční aktivitu vůči peptidázám zahrnutým v koagulační kaskádě, jako je faktor Xa a trombin, a zároveň i proti plazminu, který je součástí fibrinolýzy. Z výsledků je patrné, že E. nipponicum může mít více než jeden inhibitor serinových peptidáz a zdá se, že extrakt, který je komplexním vzorkem, obsahuje spektrum inhibitorů s rozdílnými funkcemi a vlastnostmi (což se prokázalo i v další studii, kde jsme se zabývali charakterizací jednoho ze serpinů *E. nipponicum* a jejímž jsem spoluautorem – (Roudnický *et al.,* 2018)). V transkriptomu dospělce byly nalezeny serpiny, annexiny, proteiny s Kunitz a Kazal doménami. My jsme se v rámci práce zaměřili na inhibitory Kunitzova typu, jichž bylo v transkriptomu objeveno nejméně šest rozdílných (nepublikováno). Několik Kunitz proteinů mělo různé substituce v P1 pozici a buď vykazovaly vysokou podobnost anebo se lišily v místech mimo aktivní doménu. Data byla ověřena sekvenací (Mikeš a Havelková, ústní sdělení). Jeden z nich (EnKT1) vykazoval vysokou podobnost s textilininem, antihemorhagickým faktorem z jedu pakobry východní *Pseudonaja*

textilis. Proto byl EnKT1 vyprodukován v rekombinantní formě a biochemicky a funkčně charakterizován. Inhibiční efekt EnKT1 vůči faktoru Xa byl prokázán, ale ještě větší inhibice byly zaznamenána proti plazminu a plazmovému kallikreinu in vitro. Antikoagulační aktivita EnKT1 byla potvrzena tromboelastografií, zatímco antifibrinolytickou aktivitu se prokázat nepodařilo. Z těchto výsledků je patrné, že se může jednat o potenciální antikoagulant cílený proti faktoru Xa. Pro ověření naší hypotézy byla použita krev pacienta s deficitem faktoru Xa, která jasně ukázala, že EnKT1 neměl žádný efekt na prodloužení času koagulace oproti testovaným krvím u zdravých osob, u kterých probíhala koagulace krve podstatně rychleji (Manikkan, 2012). Faktor Xa je klíčový prvek v koagulační kaskádě všech obratlovců včetně ryb a hraje hlavní roli při přeměně protrombinu na trombin (Krishnaswamy, 2013). Byť s přesvědčivým výsledkem, experiment byl proveden pouze s krví jednoho pacienta s deficitem faktoru Xa a proto by bylo dobré pokus ještě zopakovat. Testy byly prováděny na lidské krvi v nemocnici, kde na přístrojích nelze používat rybí krev – proto by bylo dobré dále ověřit i antikoagulační efekt EnKT1 v systému s rybí krví. Nicméně mechanizmy a molekulární nástroje hemokoagulace jsou u obratlovců značně konzervované (Doolittle *et* Surgenor 1962 v Jiang *et* Doolittle, 2003; Tavares-Dias *et* Oliviera, 2009).

Abychom dokázali lépe odhadnout funkci EnKT1 u dospělce *E. nipponicum*, byl EnKT1 lokalizován – a to uvnitř trávicích buněk střeva. Nejdříve byla použita metoda RNA in situ hybridizace, kde docházalo k barvení kontrolní sense próby, stejně jako v případě katepsinů L. Proto jsme pro potvrzení použili metodu imunolokalizace, kde bylo zapotřebí zesílit detekční signál a EnKT1 byl detekován opět uvnitř trávicích buněk, nikoliv však v lumen střeva dospělce. Nicméně EnKT1 obsahuje signální sekvenci a jeho přítomnost byla potvrzena i v exkrečně sekrečních produktech. Je možné, že se v lumen střeva nachází pouze v malém množství a jeho signál byl pod detekčním limitem, anebo může být v komplexu s jiným proteinem. Dále se podařilo prokázat, že EnKT1 je schopen inhibovat rybí komplement in vitro a jedná se tak o první parazitární Kunitz protein, u kterého byla schopnost blokovat komplement prokázána. Rybí komplement, na rozdíl od savčího, vykazuje širší diverzitu komplementových složek (více ortologů či kopií genů některých složek) a je schopen aktivace v širokém rozmezí teplot. Tím se stává velmi silným vrozeným obranným mechanizmem imunity u ryb (Boshra *et al.,* 2006). Proto inhibice komplementu bude evidentně pro rybí parazity velmi důležitá. Funkční testy byly prováděny pouze na rybí krvi. Proto by bylo dobré ověřit, zda EnKT1 má schopnost

inhibovat i lidský komplement, a zdá má tedy další biomedicínský potenciál, což se v rámci práce z časových důvodů testovat nepodařilo. EnKT1 nejlépe blokoval lidský plazmový kallikrein v testech in vitro. U plazmového kallikreinu se dlouhou dobu předpokládalo, že hraje významnou roli v koagulaci krve, jako část kontaktního systému, který může aktivovat vnitřní cestu koagulace (Dennis *et al.,* 1995, Wu, 2015). Kallikrein-kininový systém reprezentuje kaskádu, která vede k produkci bradykininu štěpením vysokomolekulárního kininogenu. Může mít tedy vliv na tvorbu zánětu, ovlivnit tlak krve anebo bolest (Bryant *et* Shariat-Madar, 2009). Na druhou stranu rybí krev se liší od lidské tím, že neobsahuje plazmový kallikrein (Wong *et* Takei, 2013). Proto schopnost EnKT1 inhibovat plazmový kallikrein u ryb z pohledu parazita nemá žádný význam. Nicméně schopnost inhibovat plazmový kallikrein v lidské krvi by mohla být využita v medicíně podobně jako je tomu u aprotininu nebo textilininu-1 (Flight *et al.,* 2005). Dále jsme prokázali schopnost EnKT1 inhibovat plasmin in vitro, ale neschopnost inhibovat fibrinolýzu lidské krve in vitro. Zajímavé výsledky nás přivedly na propojení kaskád koagulace, fibrinolýzy a komplementu, které spolu úzce spolupracují (Rittirsch *et al.,* 2008). Je známo, že plazmin, kromě fibrinolýzy, může mít i další funkce, jako např. spolu s faktorem Xa mohou hrát roli jako C3 a C5 konvertázy při aktivaci komplementu (Amara *et al.,* 2010). Proto schopnost EnKT1 inhibovat plazmin i faktor Xa může být klíčovým prvkem pro zabránění aktivace komplementu a může sloužit jako ochrana buněk a tkání parazita. Nicméně je třeba ověřit, zda za inhibicí funkce komplementu stojí tento efekt, nebo interakce s jinými peptidázami, např. MASP-1, MASP-2, C1a. Ve funkčních testech byla urychlená přeměna plasminogenu na plasmin vyvolána přidáním tPA (tkáňový aktivátor plasminogenu). Je tedy možné, že před aktivací plasminu byl již EnKT1 sekvestrován faktorem Xa. Proto ve srovnání s aprotininem, který své antifibrinolytické vlastnosti prokázal, ale s faktorem Xa nereaguje (Flight *et al.,* 2009), by EnKT1 při stejných koncentracích fibrinolýzu nemusel zablokovat. Bylo by tedy vhodné hypotézu ověřit a měření zopakovat i s vyššími koncentracemi EnKT1. Zajímavé výsledky se objevily i při měření hodnot maximální pevnosti sraženiny (MCF, maximum clot firmness) v přítomnosti EnKT1. Nízké hodnoty MCF poukazují na snížený počet nebo funkci destiček, nízkou hladinu fibrinogenu anebo poruchy polymerace fibrinu v důsledku snížené aktivity faktoru XIII. Výsledky by tedy mohly naznačit, že EnKT1 může mít vliv i na funkci krevních destiček nebo aktivitu faktoru XIII. Nicméně hypotézu je třeba ověřit.

Závěrem lze shrnout, že ze získaných dat nám vyplývá, že EnKT1 by mohl být bifunkční protein, který v průběhu sání parazita blokuje koagulaci krve a zároveň brání buňky střeva před aktivitou komplementu. Současně jsou nejspíše molekulární složky těchto efektorových drah v hostitelské krvi štěpeny účinnými proteázami parazita, zejména katepsiny L, vyskytujícími se i v lumen střeva.

Je tedy patrné, že monogenea mají celou řadu zajímavých molekulárních nástrojů, kterými dokážou efektivně získávat a zpracovávat nasátou krev a případně interagovat se svými hostiteli. Proto detailní popis struktury a funkcí těchto molekul může vést k objasnění interakcí mezi parazitem a hostitelem, a to hlavně z pohledu patogeneze a rybí imunity. Na druhou stranu může vést i k nalezení molekul s potenciálním uplatněním v medicíně nebo při vývoji vakcín cílených proti parazitům.

Prohlášení spoluautorů

Mgr. Lucie Jedličková se podílela na všech publikacích uvedených ve své dizertační práci:

Publikace č. 1: **Major acid endopeptidases of the blood-feeding monogenean *Eudiplozoon nipponicum* (Heteronchoinea: Diplozoidae).**

- Sdílené spoluautorství

- Měření peptidázových aktivit v solPE a ESP *E. nipponicum*. Získání kompletních sekvencí katepsinů L pomocí degenerovaných primerů, PCR a RACE PCR a jejich bioinformatická analýza. Podíl na interpretaci dat a sepisování publikace.

Publikace č. 2: **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.**

- Produkce rekombinantních katepsinů L, jejich purifikace a aktivace. Funkční charakterizace katepsinů L a jejich lokalizace ve tkáni dospělce *E. nipponicum* pomocí RNA *in situ* hybridizace a imunohistochemie. Podíl na bioinformatické anotaci struktur enzymů, interpretaci dat a sepisování publikace.

Publikace č. 3: **A novel Kunitz protein with proposed dual function from *Eudiplozoon nipponicum* (Monogenea) impairs haemostasis and action of complement *in vitro*.**

- Produkce rekombinantního Kunitz inhibitoru, purifikace a jeho funkční charakterizace. Lokalizace pomocí metody RNA *in situ* hybridizace ve tkáni dospělého červa. Podíl na interpretaci dat a sepisování publikace.

…..……..

RNDr. Libor Mikeš, Ph.D.

Školitel a spoluautor publikací

Seznam zkratek

ESP - exkrečně sekreční produkty

solPE - solubilní proteinový extrakt

iKT - inhibitor Kunitzova typu

CP - cysteinové peptidázy

iCL - inhibitor cathepsinu L

AK - aminokyseliny

Cat L/B/C/D - kathepsiny L/B/C/D

SP - signální peptid

LAP - leucin amino peptidáza

MBL - lektin vázající manózu

SCP - serinová karboxypeptidáza

MAC - membránový útočný komplex (membrane attack complex)

tPA - tkáňový aktivátor plasminogenu

MCF - maximální pevnost sraženiny (maximum clot firmness)

Použitá literatura

Acosta, D., Goñi, F. and Carmona, C. (1998). Characterization and partial purification of a leucine aminopeptidase from *Fasciola hepatica*. *J. parasitol.* **84**, 1-7.

Acosta, D., Cancela, M., Piacenza, L., Roche, L., Carmona, C. and Tort, J. F. (2008). *Fasciola hepatica* leucine aminopeptidase, a promising candidate for vaccination against ruminant fasciolosis. *Mol. Biochem. Parasitol*. **158**, 52-64. doi: 10.1016/j.molbiopara.2007.11.011.

Alberts, B., Johnson, A., Lewis, J. Raff, M., Roberts, K. and Walter, P. (2002). Innate immunity. In *Molecular Biology of the Cell* (4th edition), pp.1298 - 1307. Garland Science, New York.

Amara, U., Flierl, M. A., Rittirsch, D., Klos, A., Chen, H., Acker, B., Brückner, U. B., Nilsson, B., Gebhard, F., Lambris, J.D. and Hubert-Lang, M. (2010). Molecular intercommunication between the complement and coagulation systems. *J. Immunol* **185**, 5628-36.

Atkinson, H. J., Babbitt, P. C. and Sajid, M. (2009). The global cysteine peptidase landscape in parasites. *Trends Parasitol*. **25**, 573-81. doi: 10.1016/j.pt.2009.09.006.

Azar, D. and Nel, A. (2012). Evolution of hematophagy in "non-biting midges" (Diptera: Chironomidae). *Terr. Arthropod Rev*. **5**, 15-34. doi: 10.1163/187498312X620577.

Barrett, A. and Rawlings, N. (1996). Families and clans of cysteine peptidases. *Perspect. Drug Dis. Design*. **6**, 1-11. doi: 10.1006/abbi.1995.1227.

Barrett, A. J., Rawlings, N. D. and Woessner, J. F. (1998). *Handbook of Proteolytic Enzymes*. pp. 1666. Academic Press. doi: 10.1016/B978-0-12-382219-2.00442-7.

Barrett, A. J., Rawlings, N. D. and Woessner, J. F. (2004). *Handbook of Proteolytic Enzymes: Second Edition*. pp. 984. Academic Press. doi: 10.1016/C2009-0-03628-9.

Beutler, B. (2004). Innate immunity: an overview. *Mol. Immunol.* **40**, 845-859.

Boeger, W. A. and Kritsky, D. C. (2001). Phylogenetic relationships of the Monogenoidea. In *Systematics association special volume,* **60**, 92-98.

Bogitsh, B. J. and Carter, S. (1977): *Schistosoma manson*i: ultrastructural studies on the esophageal secretory granules. *J. Parasitol.* **63**, 681–686.*****

Bogitsh, B. J., Dalton, J. P., Ciaran, P. B. and Brindley, P. J. (2001): Gut–associated immunolocalization of the *Schistosoma manosni* cysteine proteases, SmCL1 and SmCL2. *J. Parasitol.* **87**, 237–241.

Boon, G. D. (1993). An overview of hemostasis. *Toxicol. Pathol.* **21**, 170-9 doi: 10.1177/019262339302100209.

Boshra, H., Li, J. and Sunyer, J.O. (2006). Recent advances on the complement system of teleost fish. *Fish Shellfish Immunol*. **20**, 239-262.

Boshra, H., Gelman, A.E. and Sunyer, J. O. (2004). Structural and functional characterization of complement C4 and C1s-like molecules in teleost fish: insights into the evolution of classical and alternative pathways. *J. Immunol.* **173**: 349-359.

Brindley, P. J, Kalinna, B. H., Dalton, J. P., Day, S. R., Wong, J. Y. M., Smythe, M. L. and McManus, D. P. (1997). Proteolytic degradation of host hemoglobin by schistosomes. *Mol. Biochem. Parasitol.* **89**, 1–9.

Bryant, J.W. and Shariat-Madar, Z. (2009). Human plasma kallikrein-kinin system: Physiological and biochemical parameters. *Cardiovasc. Hematol. Agents Med. Chem.* **7**(3): 234–250.

Bozas, S.E., Panaccio, M., Creaney, J., Dosen, M., Parsons, J. C., Vlasuk, G. V., Walker, I. D. and Spithill, T. W. (1995). Characterization of a novel Kunitz-type molecule from the trematode *Fasciola hepatica*. *Mol. Biochem. Parasitol.* **74**, 19-29.

Broze, G. J. and Majerus, P. W. (1980). Purification and properties of human coagulation factor VII. *J. Biol. Chem*. **255**, 1242-7.

Brömme, D., Bonneau, P. R., Lachance, P. and Storer, A. C. (1994). Engineering the S2 subsite specificity of human cathepsin S to a cathepsin L- and cathepsin B-like specificity". *J. Biol. Chem.* **269**, 30238-42.

Buchmann, K. and Bresciani, J. (2006). Monogenea Phylum Platyhelminthes. In *Fish Diseases and Disorders Volume 1. 2nd edition.*, pp. 297–344. CABI, Wallingford.

Caffrey, C. R., McKerrow, J. H., Salter, J. P. and Sajid, M. (2004). Blood "n" guts: an update on schistosome digestive peptidases. *Trends Parasitol.* **20**, 241–8. doi: 10.1016/j.pt.2004.03.004.

Caffrey, C. R., Goupill, L., Rebello, K. M., Dalton, J. P. and Smith, D. (2018). Cysteine proteases as digestive enzymes in parasitic helminths. *PLOS Negl. Trop. Dis.* 12, e0005840. doi.org/10.1371/journal.pntd.0005840

Cantacessi, C., Mulvenna, J., Young, N.D., Kasny, M., Horak, P., Aziz, A., Hofmann, A., Loukas, A., Gasser, R.B. (2012). A deep exploration of the transcriptome and "excretory/secretory" proteome of adult Fascioloides magna. *Mol. Cell Proteomics* **11**, 1340-53.

Carmona, E., Dufour, É., Plouffe, C., Takebe, S., Mason, P., Mort, J. S. and Ménard, R. (1996). Potency and selectivity of the cathepsin L propeptide as an inhibitor of cysteine proteases. *Biochemistry*. **35**, 8149-57. doi: 10.1021/bi952736s.

Corvo, I., Cancela, M., Cappetta, M., Pi-Denis, N., Tort, J.F. and Roche, L. (2009). The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen. *Mol. Biochem. Parasitol.* **167**:41–7.

Coulombe, R., Grochulski, P., Sivaraman, J., Ménard, R., Mort, J. S. and Cygler, M. (1996). Structure of human procathepsin L reveals the molecular basis of inhibition by the prosegment. *EMBO J.* **15**, 5492-503. doi: 066/14.

Dalton, J. P. ed. (1999). *Fasciolosis*. pp. 544. CABI, Wallingford.

Dalton, J. P., McGonigle, S., Rolph, T. P. and Andrews, S. J. (1996). Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. *Infect. Immun.* **64**, 5066–5074.

Dalton, J. P., Neill, S. O., Stack, C., Collins, P., Walshe, A., Sekiya, M., Doyle, S., Mulcahy, G., Hoyle, D., Khaznadji, E., Moiré, N., Brennan, G., Mousley, A., Kreshchenko, N., Maule, A. G. and Donnelly, S. M. (2003). *Fasciola hepatica* cathepsin L-like proteases: biology, function, and potential in the development of first generation liver fluke vaccines. In *Int. J. Parasitol.* **33**, 1173-81. doi: 10.1016/S0020-7519(03)00171-1.

Dalton, J. P., Skelly, P. and Halton, D. W. (2004): Role of the tegument and gut in nutrient uptake by parasitic platyhelminths. *Can. J. Zoo.* **82**, 211–232.

Dalton, J. P., Caffrey, C. R., Sajid, M., Stack, C. M., Donnelly, S., Loukas, A., Don, T., McKerrow, J., Halton, D. W. and Brindley, P. J. (2006). Proteases in trematode biology. In *Parasitic Flatworms: Molecular Biology, Biochemistry, Immunology and Physiology*. pp. 348-368. CABI. Cambridge, UK.

Delcroix, M., Sajid, M., Caffrey, C. R., Lim, K.-C. C., Dvořák, J., Hsieh, I., Bahgat, M., Dissous, C. and Mckerrow, J. H. (2006). A multienzyme network functions in intestinal protein digestion by a platyhelminth parasite. *J. Biol. Chem.* **281**, 39316–29. doi: 10.1074/jbc.M607128200.

Dennis, M.S., Herzka, A. and Lazarus, R.A. (1995). Potent and selective kunitz domain inhibitors of plasma kallikrein designed by phage display. *J. Biol. Chem.* **207**, 25411–25417.

Dvořák, J., Mashiyama, S. T., Sajid, M., Braschi, S., Delcroix, M., Schneider, E. L., McKerrow, W. H., Bahgat, M., Hansell, E., Babbitt, P. C., Craik, C. S. C., McKerrow, J. H., Caffrey, C. R. (2009): SmCL3, a gastrodermal cysteine protease of the human blood fluke *Schistosoma mansoni*. *PLoS Negl. Trop. Dis.* **3**, e449.

Dy, C. Y., Buczek, P., Imperial, J. S., Bulaj, G. and Horvath, M. P. (2006). Structure of conkuntzin-S1, neurotoxin and Kunitz-fold disulfide variant from cone snail. *Acta Crystallogr. D. Biol. Crystallogr.* **62**, 980-90. doi:10.1107/S0907444906021123

Falcón, C. R., Masih, D., Gatti, G., Sanchez, M. C., Motrán, C. C. and Cervi, L. (2014). *Fasciola hepatica* Kunitz type molecule decreases dendritic cell activation and their ability to induce inflammatory responses. *PLoS One* **9**, e114505. doi: 10.1371/journal.pone.0114505.

Fisheries and Aquaculture Department (2017). http://www.fao.org/fishery/about/en.

Flight, S. M., Johnson, L. A., Trabi, M., Gaffney, P., Lavin, M., de Jersey, J., Masci, P. (2005). Comparison of texilinin-1 with aprotinin as serine protease inhibitor and as antifibrinolytic agents. *Patophysiol. Haemost. Thromb.* **34**, 188-93.

Flight, S. M., Johnson, L. A., Du, Q. S., Warner, R. L., Trabi, M., Gaffney, P. J., Lavin, M. F., de Jersey, J. and Masci, P. P. (2009). Textilinin-1, an alternative anti-bleeding agent to aprotinin: Importance of plasmin inhibition in controlling blood loss. *Br. J. Haematol*. **145**, 207–11. doi: 10.1111/j.1365-2141.2009.07605.x.

Fló, M., Margenat, M., Pellizza, L., Graña, M., Durá N, R., Bá Ez, A., Salceda, E., Soto, E., Alvarez, B. and Ferná Ndez, C. (2017). Functional diversity of secreted cestode Kunitz proteins: Inhibition of serine peptidases and blockade of cation channels. *PLoS Pathog.* **13**, 1–33. doi: 10.1371/journal.ppat.1006169.

Francischetti, I. M. B., Valenzuela, J. G., Andersen, J. F., Mather, T. N. and Ribeiro, J. M. C. (2002). Ixolaris, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of the tick, *Ixodes scapularis*: identification of factor X and factor Xa as scaffolds for the inhibition of factor VIIa/tissue factor complex. *Blood* **99**, 3602–12. doi: 10.1182/blood-2001-12-0237.

Franta, Z., Sojka, D., Frantova, H., Dvorak, J., Horn, M., Srba, J., Talacko, P., Mares, M., Schneider, E., Craik, Ch. S., McKerrow, J. H., Caffrey, C. R., Kopacek, P. (2011): *Ir*CL1 – The haemoglobinolytic cathepsin L of the hard tick, *Ixodes ricinus*. *Int. J. Parasitol.* **41**, 1253–1262.

Gasque, P. (2004). Complement: a unique innate immune sensor for danger signals. *Mol. Immunol.* **41**, 1089-1098.

Genderen, H. O. van, Kenis, H., Hofstra, L., Narula, J. and Reutelingsperger, C. P. M. (2008). Extracellular annexin A5: functions of phosphatidylserine-binding and two-dimensional crystallization. *Biochim. Biophys. Acta* **1783**, 953–63. doi: 10.1016/j.bbamcr.2008.01.030.

Gillespie, R. D., Mbow, M. L. and Titus, R. G. (2000). The immunomodulators of bloodfeeding artropod saliva. *Parasite Immunol.* **22**, 319-31.

Gillmor, S. A., Craik, C. S. and Fletterick, R. J. (1997). Structural determinants of specificity in the cysteine protease cruzain. Protein Science **6**, 1603-1611.

Gonzalez, S., Fló, M., Margenet, M., Durán, R., Gozalez-Sapienza, G., Grana, M., Parkinson, J., Maizels, R. M., Salinas, G., Alvarez, G. and Fernández, C. (2009). A family of diverse Kunitz inhibitors from *Echinococcus granulosus* potentially involved in host-parasite cross-talk. *Plos One* 4, e7009. doi: 10.1371/journal.pone.0007009.

Grzesiak, A.,Helland, R., Smalås, A. O., Krowarsch, D., Dadlez, M. and Otlewski, J. (2000). Substitutions at the P(1) position in BPTI strongly affect the association energy with serine proteinases. *J. Mol. Biol.* **301**, 205–17. doi: 10.1006/jmbi.2000.3935.

Gunčar, G., Pungerčič, G., Klemenčič, I., Turk, V. and Turk, D. (1999). Crystal structure of MHC class II-associated p41 Ii fragment bound to cathepsin L reveals the structural basis for differentiation between cathepsins L and S. *EMBO J*. **18**, 793-803. doi: 10.1093/emboj/18.4.793.

Halton, D. W. (1997). Nutritional adaptations to parasitism within the platyhelminthes. *Int. J. Parasitol.* **27**, 693–704. doi: 10.1016/S0020-7519(97)00011-8.

Hatta, T., Kazama, K., Miyoshi, T., Umemiya, R., Liao, M., Inoue, N., Xuan, X., Tsuji, N., Fujisaki, K. (2006): Identification and characterisation of a leucine aminopeptidase from the hard tick *Haemaphysalis longicornis*. *Int. J. Parasitol.* **36**, 1123–1132.

Hawdon, J. M., Datu, B. and Crowell, M. (2003). Molecular cloning of a novel multidomain Kunitz-type proteinase inhibitor from the hookworm *Ancylostoma caninum*. *J. Parasitol.* **89**, 402–407. doi: 10.1645/0022-3395(2003)089[0402:MCOANM]2.0.CO;2.

Henke, C., Strissel, P.L., Schubert, M.T., Mitchell, M., Stolt, C.C., Faschingbauer, F., Beckmann, M.W. and Strick, R. (2015). Selective expression of sense and antisense transcripts of the sushi-ichi-related retrotransposon-derived family during mouse placentogenesis. *Retrovirology* **12**:9. doi: 10.1186/s12977-015-0138-8.

Hirazawa, N., Umeda, N., Hatanka, A. and Kuroda, A. (2006). Characterization of serine proteases in the monogenean Neobenedenia girellae. *Aquaculture* **255**, 188–195.

Hodová, I., Matějusová, I., Gelnar, M., Matejusova, I. and Gelnar, M. (2010). The surface topography of *Eudiplozoon nipponicum* (Monogenea) developmental stages parasitizing carp (*Cyprinus carpio* L.). *Cent. Eur. J. Biol.* **5**, 702-709. doi: 10.2478/s11535-010-0040-2.

Holland, M. C. H. and Lambris, J. D. (2002). The complement system in teleosts. *Fish Shellfish Immunol*. **12**, 399-420.

Horn, M., Nussbaumerová, M., Šanda, M., Kovářová, Z., Srba, J. J., Franta, Z. Z., Sojka, D., Bogyo, M., Caffrey, C. R., Kopáček, P., Mareš, M. (2009). Hemoglobin digestion in blood-feeding ticks: mapping a multipeptidase pathway by functional proteomics. *Chem. Biol.* **16**, 1053–63. doi: 10.1016/j.chembiol.2009.09.009.

Hořejší, V., Bartůňková, J., Brdička, T. and Spíšek, R. (2005). Základy imunologie. (3. vydání), pp. 279. Triton. Praha.

Hovius, J. W. R., Levi, M. and Fikrig, E. (2008). Salivating for knowledge: potential pharmacological agents in tick saliva. *PLoS Med.* **5**, e43. doi: 10.1371/journal.pmed.0050043.

Huber-Lang, M., Sarma, J.V., Zetoune, F. S., Rittirsch, D., Neff., T. A., McGuire, S. R., Lambris, J. D., Warner, R. L., Flierl, M. A., Hoesel, L. M., Gebhard, F., Younger, J. G., Drouin, S. M., Wetsel, R. A. and Ward, P. A. (2006). Generation of C5a in the absence of C3: new complement activtion pathway. *Nat. Med*. **12**, 682-7.

Huntington, J. A. (2013). Thrombin inhibition by the serpins. *J. Thromb. Haemost.* **11**, 254–264. doi: 10.1111/jth.12252.

Champagne, D. E., Smartt, C. T., Ribeiro, J. M. and James, A.A. (1995). The salivary gland-specific apyrase of the mosquito *Aedes aegypti* is a member of the 5'-nucleotidase family. *Proc. Natl. Acad. Sci. U S A* **92**, 694-698. doi: 10.1073/pnas.92.3.694.

Champagne, D. E. (2005). Antihemostatic molecules from saliva of blood-feeding arthropods. *Pathophysiol. Haemost. Thromb.* **34**, 221-7. doi: 10.1159/000092428

Chand, H. S., Schmidt, A. E., Bajaj, S. P. and Kisiel, W. (2004). Structure-function analysis of the reactive site in the first Kunitz-type domain of human tissue factor pathway inhibitor-2. *J. Biol. Chem.* **279**, 17500–17507. doi: 10.1074/jbc.M400802200.

Chauhan, S.S., Ray, D., Kane, S.E., Willingham, M.C. and Gottesman, M.M. (1998). Involvement of carboxy-terminal amino acids in secretion of human lysosomal protease cathepsin L. *Biochemistry.* **37**(23):8584-94.

Choe, Y., Leonetti, F., Greenbaum, D.C., Lecaille, F., Bogyo, M., Brömme, D., Ellman, J.A. and Craik, C.S. (2006). Substrate profiling of cysteine proteases using a combinatorial peptide library identifies functionally unique specificities. *J. Biol. Chem.* **281**, 12824-32.

Illy, C., Quraishi, O., Wang, J., Purisima, E., Vernet, T. and Mort, J. S. (1997). Role of the occluding loop in cathepsin B activity. *J. Biol. Chem.* **272**, 1197-202. doi: 10.1074/jbc.272.2.1197.

Janeway, C. A. and Medzihov, R. (2002). Innate Immune Recognition. *Annu. Rev. Immunol.* **20,** 197-216.

Jiang, Y. and Doolittle, R. F. (2003). The evolution of vertebrate blood coagulation as viewed from a comparison of puffer fish and sea squirt genomes. *Proc. Natl. Acad. Sci.* U.S.A. **100**, 7527-7532.

Kamphuis, I. G., Kalk, K. H., Swarte, M. B. A. and Drenth, J. (1984). Structure of papain refined at 1.65 Å resolution. *J. Mol. Biol*. **179**, 233-56. doi: 10.1016/0022-2836(84)90467-4.

Karrer, K. M., Peiffer, S. L. and DiTomas, M. E. (1993). Two distinct gene subfamilies within the family of cysteine protease genes. *Proc. Natl. Acad. Sci. U S A* **90**, 3063-7. doi: 10.1073/pnas.90.7.3063.

Kašný, M., Mikeš, L., Hampl, V., Dvořák, J., Caffrey, C. R., Dalton, J. P. and Horák, P. (2009). Chapter 4. Peptidases of Trematodes. *Adv. Parasitol*. **69**, 205-97. doi: 10.1016/S0065-308X(09)69004-7.

Kawatsu, H. (1978). Studies on the anemia of fish-IX, Hypochromic microcytic anemia of crucian carp caused by infestation with a trematode, *Diplozoon nipponicum*. *Bull. Japan. Soc. Sci. fish* **44**, 1315–1319. doi: 10.2331/suisan.44.1315.

Kazimírová, M. and Štibrániová, I. (2013). Tick salivary compounds: their role in modulation of host defences and pathogen transmission. *Front. Cell. and Infect. Microbiol*. **3**, 43. doi: 10.3389/fcimb.2013.00043.

Keizer, M. P., Pouw, R. B., Kamp, A.M., Patiwael, S., Marsman, G., Hart, M. H., Zeerleder, S., Kuipers, T. W. and Wouters, D. (2015). TFPI inhibits lectin pathway of complement activation by direct interaction with MASP-2. *Eur. J. Immunol.* **45**, 544-50. doi: 10.1002/eji.201445070.

Konstanzová, V., Koubková, B., Kašný, M., Ilgová, J., Dzika, E. and Gelnar, M. (2015). Ultrastructure of the digestive tract of *Paradiplozoon homoion* (Monogenea). *Parasitol. Res.* **114**, 1485–94. doi: 10.1007/s00436-015-4331-4.

Krarup, A., Wallis, R., Preisanis, J. S., Gál, P. and Sim, R. B. (2007). Simultaneous activation of complement and coagulation by MBL-associated serine protease, *PLoS One* **18**, e623. doi: 10.1371/journal.pone.0000623

Krishnaswamy, S. (2013). The transition of prothrombin to thrombin. *J. Thromb. Haemost*. **11**, 265–276.

Krowarsch, D., Dadlez, M., Buczek, O., Krokoszynska, I., Smalas, A. O. and Otlewski, J. (1999). Interscaffolding additivity: binding of P1 variants of bovine pancreatic trypsin inhibitor to four serine proteases. *J. Mol. Biol.* **289**, 175–86. doi: 10.1006/jmbi.1999.2757.

Krowarsch, D., Cierpicki, T., Jelen, F. and Otlewski, J. (2003). Canonical protein inhibitors of serine proteases. *Cell. Mol. Life. Sci.* **60**, 2427-44.

Laskowski, M. and Kato, I. (1980). Protein Inhibitors of Proteinases. *Annu. Rev. Biochem.* **49**, 593–626. doi: 10.1146/annurev.bi.49.070180.003113.

Lara, F. A., Lins, U., Paiva-Silva, G., Almeida, I. C., Braga, C. M., Miguens, F. C., Oliveira, P. L. and Dansa-Petretski, M. (2003): A new intracellular pathway of haem detoxification in the midgut of the cattle tick *Boophilus microplus*: aggregation inside a specialized organelle, the hemosome. *J Exp. Bio.* **206**, 1707–1715.

Lecaille, F., Choe, Y., Brandt, W., Li, Z., Craik, C.S. and Brömme, D. (2002). Selective inhibition of the collagenolytic activity of human cathepsin K by altering its S2 subsite specificity. *Biochemistry* **41**(26):8447-54.

Lee, J. H., Kim, C. H., Shin, Y. P., Park, H. J., Park, S., Lee, H. M., Kim, B. S. and fboeLee, I. H. (2010). Characterization of Kunitz-type protease inhibitor purified from hemolymph of *Galleria mellonella* larvae. *Insect Biochem. Mol. Biol.* **40**, 873–882. doi: 10.1016/j.ibmb.2010.08.007.

Leung, L. L. and Morser, J. (2016). Plasmin as a complement C5 convertase. *EBioMedicine* **11**, 20-1. doi: 10.1016/j.ebiom.2016.03.015.

Littlewood, D. T. and Bray, R. A. eds. (2001). *Interrelationships of the Platyhelminthes*. Taylor and Francis, London, pp 356.

Lopez-Ordoñez, T., Rodriguez, M.H. and Hernández-Hernández, F. D. (2001). Characterization of a cDNAencoding a cathepsin L-like protein of Rhodnius prolixus. *Insect Mol. Biol.* **10**, 505–511.

López-Otín, C. and Bond, J. S. (2008). Proteases: Multifunctional enzymes in life and disease. *J. Biol. Chem*. **283**, 30433-7. doi: 10.1074/jbc.R800035200.

Lowther, J., Robinson, M. W., Donnelly, S. M., Xu, W., Stack, C. M., Matthews, J. M. and Dalton, J. P. (2009). The importance of pH in regulating the function of the F*asciola hepatica* cathepsin L1 cysteine protease. *PLoS Negl. Trop. Dis.* **3**, e369. doi: 10.1371/journal.pntd.0000369.

Lubenow, N. and Greinacher, A. (2001). Drugs for the prevention and treatment of thrombosis in patients with heparin-induced thrombocytopenia. *Am. J. Cardiovasc. Drugs* **1**, 429-43. doi: 10.2165/00129784-200101060-00003

Macedo-Ribeiro, S., Almeida, C., Calisto, B. M., Friedrich, T., Mentele, R., Stürzebecher, J., Fuentes- Prior, P. and Pereira, P. J. (2008). Isolation, cloning and structural characterisation of boophilin, a multifunctional Kunitz-type proteinase inhibitor from the cattle tick. *PLOS One* **3**, e1624.

Macfarlane, R. G. (1964). An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature* **202**, 498-9. doi: 10.1038/202498a0.

Mans, B. J. (2011). Evolution of vertebrate hemostatic and inflammatory control mechanisms in blood-feeding arthropods. *J. Innate Immun*. **3**, 41-51. doi: 10.1159/000321599.

Marcilla, A., De La Rubia, J. E., Sotillo, J., Bernal, D., Carmona, C., Villavicencio, Z., Acosta, D., Tort, J., Bornay, F. J., Esteban, J. G. and Toledo, R. (2008). Leucine aminopeptidase is an immunodominant antigen of *Fasciola hepatica* excretory and secretory products in human infections. *Clin. Vaccine Immunol.***15**, 95-100. doi: 10.1128/CVI.00338-07.

Markwardt, F., Kaiser, B. and Richter, M. (1992). Haemostyptic effects of batroxobin with regard to hirudin treatment. *Thromb. Res*. **68**, 475-82. doi: 10.1016/0049-3848(92)90060-N.

Manikkan, A.T. (2012). Factor X deficiency: an uncommon presentation of AL amyloidosis. *Ups. J. Med. Sci*. **117**, 457–459.

Matějusová, I., Koubková, B., D'Amelio, S. and Cunningham, C. O. (2001). Genetic characterization of six species of diplozoids (Monogenea; Diplozoidae). *Parasitology* **123**, 465–74. doi: 10.1017/S0031182001008617.

McCarthy, E., Stack, C., Donnalley, S.M., Doyle, S., Mann, V.H., Brindley, P.J., Stewart, M., Day, T.A., Maule, A.G. and Dalton, J.P. (2004): Leucine aminopeptidase of the human blood flukes, *Schistosoma mansoni* and *Schistosoma japonicum*. *Int. J. Parasitol.* **34**, 703–14.

McDougall, H. C. (2012). Identifying "hidden" antigens in the liver fluke, *Fasciola hepatica*. *PhD thesis*. pp. 265. University of Glasgow, UK.

McIntyre, G. F., Godbold, G. D. and Erickson, A. H. (1994): The pH – dependent membrane association of procathepsin L is mediated by a 9–Residue Sequence within the propeptide. *J. Biol. Chem.* **269**, 567–572.

McManus, D. P. and Dalton, J. P. (2006). Vaccines against the zoonotic trematodes *Schistosoma japonicum*, *Fasciola hepatica* and *Fasciola gigantica*. *Parasitology* **133**, S43-61. doi: 10.1017/S0031182006001806.

McVeigh, P., Maule, A. G., Dalton, J. P. and Robinson, M. W. (2012). *Fasciola hepatica* virulence-associated cysteine peptidases: A systems biology perspective. *Microbes Infect.* **14**, 301–310. doi: 10.1016/j.micinf.2011.11.012.

Mendes-Sousa, A. F., Vale, V. F., Queiroz, D. C., Pereira-Filho, A. A., da Silva, N. C. S., Koerich, L. B., Moreira, L. A., Pereira, M. H., Sant'Anna, M. R., Araújo, R. N., Andersen, J., Valenzuela, J. G. and Gontijo, N. F. (2018). Inhibition of the complement system by saliva of Anopheles (Nyssorhynchus) aquasalis. *Insect Biochem. Mol. Biol*. **92**, 12-20. doi: 10.1016/j.ibmb.2017.11.004.

Michel, A., Ghoneim, H., Resto, M., Klinkert, M. Q. and Kunz, W. (1995): Sequence, characterization of a cysteine proteinase cathepsin L in *Schistosoma mansoni*. *Mol. Biochem. Parasitol.* **73**, 7–18.

Millers, E.-K. I., Trabi, M., Masci, P. P., Lavin, M. F., de Jersey, J. and Guddat, L. W. (2009). Crystal structure of textilinin-1, a Kunitz-type serine protease inhibitor from the venom of the Australian common brown snake (*Pseudonaja textilis*). *FEBS J.* **276**, 3163–75. doi: 10.1111/j.1742-4658.2009.07034.x.

Milstone, A. M., Harrison, L. M., Bungiro, R. D., Kuzmic, P. and Cappello, M. (2000). A broad spectrum Kunitz type serine protease inhibitor secreted by the hookworm *Ancylostoma ceylanicum*. *J. Biol. Chem.* **275**, 29391–9. doi: 10.1074/jbc.M002715200.

Mottram, J. C., Helms, M. J., Coombs, G. H. and Sajid, M. (2003). Clan CD cysteine peptidases of parasitic protozoa. *Trends Parasitol.* **19**, 182-7.

Mulenga, A., Misao, O. and Sugimoto, C. (2003): Three serine proteinases from midguts of the hard tick *Rhipicephalus appendiculatus*; cDNA cloning and preliminary characterization. *Exp. Appl. Acarol.* **29**, 151–164.

Musil, D., Zucic, D., Turk, D., Engh, R. A., Mayr, I., Huber, R., Popovic, T., Turk, V., Towatari, T., Katunuma, N. and al., et (1991). The refined 2.15 A X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity. *EMBO J.* **10**, 2321-30.

Nakao, M., Matsuro, J., Obo, R., Fujiki, K., Nonaka, M., Yano, T. (2000). Molecular cloning and protein anaylsis of divergent forms of the complement component C3 from a bony fish, the common carp (*Cysprinus carpio*): presence of variants lacking the catylitic histidine. *Eur. J. Immunol.* **30**, 858-66. doi: 10.1002/1521-4141(200003)30:3<858::AID-IMMU858>3.0.CO;2-M

Oliva, M. L. V, Silva, M. C. C., Sallai, R. C., Brito, M. V and Sampaio, M. U. (2010). A novel subclassification for Kunitz proteinase inhibitors from leguminous seeds. *Biochimie* **92**, 1667–73. doi: 10.1016/j.biochi.2010.03.021.

Osato, N., Yamada, H., Satoh, K., Ooka, H., Yamamoto, M., Suzuki, K., Kawai, J., Carninci, P., Ohtomo, Y., Murakami, K., Matsubara, K., Kikuchi, S. and Hayashizaki, Y. (2003) Antisense transcripts with rice full-length cDNAs. *Genome Biol.* **5**:R5.

Ozsolak, F., Kapranov, P., Foissac, S., Kim, S.W., Fishilevich, E., Monaghan, A.P., John, B. and Milos, P.M. (2010). Comprehensive polyadenylation site maps in yeast and human reveal pervasive alternative polyadenylation. *Cell* **143**(6):1018-29. doi: 10.1016/j.cell.2010.11.020.

Palta, S., Saroa, R. and Palta, A. (2014). Overview of the coagulation system. *Indian J. Anaesth.* **58**, 515-23. doi: 10.4103/0019-5049.144643.

Pecka, M. (2004). *Laboratorní hematologie v přehledu, Fyziologie a patologie hemostázy*, pp. 237. Finder, Český Těšín.

Pečínková, M., Matějusová, I., Koubková, B. and Gelnar, M. (2007). Investigation of *Paradiplozoon homoion* (Monogenea, Diplozoidae) life cycle under experimental conditions. *Parasitol. Int*. **56**, 179-83. doi: 10.1016/j.parint.2007.01.010.

Peigneur, S., Billen, B., Derua, R., Waelkens, E., Debaveye, S., Béress, L. and Tytgat, J. (2011). A bifunctional sea anemone peptide with Kunitz type protease and potassium channel inhibiting properties. *Biochem. Pharmacol.* **82**, 81–90. doi: 10.1016/j.bcp.2011.03.023.

Pelechano, V. and Steinmetz, L.M. (2013). Gene regulation by antisense transcription. *Nat. Rev. Genet.* **14**(12):880-93. doi: 10.1038/nrg3594.

Petersen, S. V., Thiel, S., Jensen, L., Vorup- Jensen, T., Koch, C. and Jensenius, J. C. (2000). Control of the classical and the MBL pathway of complement activation. *Mol. Immunol.* **37**, 803-11.

Prevot, P. P., Adam, B., Boudjeltia, K. Z., Brossard, M., Lins, L., Cauchie, P., Brasseur, R., Vanhaeverbeek, M., Vanhamme, L. and Godfroid, E. (2006). Anti-hemostatic effects of a serpin from the saliva of the tick *Ixodes ricinus*. *J. Biol. Chem*. **281**, 26361-9. doi: 10.1074/jbc.M604197200.

Ranasinghe, S. and McManus, D. P. (2013). Structure and function of invertebrate Kunitz serine protease inhibitors. *Dev. Comp. Immunol.* **39**, 219–227. doi: 10.1016/j.dci.2012.10.005.

Ranasinghe, S. L., Fischer, K., Gobert, G. N. and Mcmanus, D. P. (2015a). Functional expression of a novel Kunitz type protease inhibitor from the human blood fluke *Schistosoma mansoni*. *Parasit. Vectors* **8**, 1–10. doi: 10.1186/s13071-015-1022-z.

Ranasinghe, S. L., Fischer, K., Zhang, W., Gobert, G. N. and McManus, D. P. (2015b). Cloning and Characterization of Two Potent Kunitz Type Protease Inhibitors from *Echinococcus granulosus*. *PLoS Negl. Trop. Dis.* **9**, e0004268. doi: 10.1371/journal.pntd.0004268.

Ranasinghe, S. L., Fischer, K., Gobert, G. N. and McManus, D. P. (2015c). A novel coagulation inhibitor from *Schistosoma japonicum*. *Parasitology* **142**, 1663–1672. doi: 10.1017/S0031182015001328.

Ranasinghe, S. L., Duke, M., Harvie, M., McManus, D. P. (2017). Kunitz-type protease inhibitor as a vaccine candidate against schistosomiasis mansoni. *Int. J. Infecet. Dis.* **66**, 26-32. doi: 10.1016/j.ijid.2017.10.024.

Rao, Y. and Yang, T. (2007).): cDNA cloning, mRNA expression and recombinant expression of a cathepsin L–like cysteine protease from *Neobenedenia melleni* (Monogenea: Capsalidae). *Aguaculture* **207**, 41–53.

Rasche, H. (2001). Haemostasis and thrombosis: an overview. *Eur. Heart J. Suppl.* **3**, Q3–Q7.

Rawlings, N. D. and Barrett, A. J. (1999). MEROPS: the peptidase database. *Nucleic Acids Res.* **27**, 205–218.

Rawlings, N., Morton, F., Kok, C., Kong, J. and Barrett, A. (2008). MEROPS: the peptidase database. *Nucleic Acids Res.* **36**, D320-5.

Rawlings, N. D., Barrett, A. J. and Bateman, A. (2010). MEROPS: the peptidase database. *Nucleic Acids Res.* **38**, D227–D233.

Rawlings, N. D., Barrett, A. J., Thomas, P. D., Huang, X., Bateman, A., Finn, R. D. (2018). The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and comparison with peptidases in the Panther database. *Nucleic Acid Res.* **46**, D624-D632.

Reed, P., Francis-Floyd, R., Klinger, R. and Petty, D. (2009). Monogenean Parasites of Fish. *University of Florida IFAS extension* 1–4.

Ribeiro, J. M. C. and Francischetti, I. M. B. (2003). Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives. *Annu. Rev. Eentomol*. **48**, 73-88. doi: 10.1146/annurev.ento.48.060402.102812.

Rittirsch, D., Flierl, M.A. and Ward, P. A. (2008). Harmful molecular mechanisms in sepsis. *Nat. Rev. Immunol.* **8**, 776-87.

Roberts, L. S. and Janovy, J. (2005). Foundation of parasitology, 7th edition. pp 702, McGraw-Hill, New York.

Robinson, M. W., Dalton, J. P. and Donnelly, S. (2008). Helminth pathogen cathepsin proteases: it's a family affair. *Trends Biochem. Sci*. **33**, 601-8. doi: 10.1016/j.tibs.2008.09.001.

Robinson, M. W., Corvo, I., Jones, P. M., George, A. M., Padula, M. P., To, J., Cancela, M., Rinaldi, G., Tort, J. F., Roche, L. and Dalton, J. P. (2011). Collagenolytic activities of the major secreted cathepsin L peptidases involved in the virulence of the helminth pathogen, *Fasciola hepatica*. *PLoS Negl. Trop. Dis.* **5**, e1012. doi: 10.1371/journal.pntd.0001012.

Roudnický, P., Vorel, J., Ilgová, J., Benovics, M., Norek, A., Jedličková, L., Mikeš, L., Potěšil, D., Zdráhal, Z., Dvořák, J., Gelnar, M., Kašný, M. (2018). Identification and partial characterization of a novel serpin from *Eudiplozoon nipponicum* (Monogenea, Polyopisthocotylea). *Parasite* **25**, 61.

Sajid, M. and McKerrow, J. H. (2002). Cysteine proteases of parasitic organisms. *Mol. Biochem. Parasitol*. **120**, 1-21. doi: 10.1016/S0166-6851(01)00438-8.

Sajid, M., McKerrow, J. H, Hanssell, E., Mathieu, M. A., Lucas, K. D., Hsich, I., Greenbaum, D., Bogyo, M., Salter, J. P., Lim, K. C., Franklin, Ch., Kim, J. H.,Caffrey, C. R. (2003): Functional expression and characterization of *Schistosoma mansoni* cathepsin B and its *trans*-activation by endogenous asparaginyl endopeptidase. *Mol. Biochem. Parasitol.* **131**, 65–75.

Sakanari, J. A., Nadler, S. A., Chan, V. J., Engel, J. C., Leptak, C. and Bouvier, J. (1997). *Leishmania major*: comparison of the cathepsin L- and B-like cysteine proteae genes with those of other trypanosomatids. *Exp Parasitol* **85**,63-76.

Shigetomi, H., Onogi, A., Kajiwara, H., Yoshida, S., Furukawa, N., Haruta, S., Tanase, Y., Kanayama, S., Noguchi, T., Yamada, Y., Oi, H. and Kobayashi, H. (2010). Anti-inflammatory actions of serine protease inhibitors containing the Kunitz domain. *Inflamm Res* **59**, 679–687. doi: 10.1007/s00011-010-0205-5.

Shinnar, A. E., Butler, K. L. and Park, H. J. (2003). Cathelicidin family of antimicrobial peptides: Proteolytic processing and protease resistance. *Bioorg. Chem*. **31**, 425-36. doi: 10.1016/S0045-2068(03)00080-4.

Schmaier, A. H. (2016). The contact activation and kallikrein/kinin systems: Pathophysiologic and physiologic activities. *J. Thromb. Haemost*. **14**, 28-39. doi: 10.1111/jth.13194.

Schroeder, H., Skelly, P. J., Zipfel, P. F., Losson, B. and Vanderplasschen, A. (2009). Subversion of complement by hematophagous parasites. *Dev. Comp. Immunol.* **33**, 5-13.

Smith, D., Tikhonova, I. G., Jewhurst, H. L., Drysdale, O. C., Dvořák, J., Robinson, M. W., Cwiklinski, K. and Dalton, J. P. (2016). Unexpected activity of a novel Kunitz-type inhibitor: inhibition of cysteine proteases but not serine proteases. *J. Biol. Chem.* **291**, 19220–34. doi: 10.1074/jbc.M116.724344.

Smith, A. M., Dowd. A. J., McGonigle, S., Keegan, P. S., Gerard, B., Trudgett, A. and Dalton, J. P. (1993). Purification of a cathepsin L-like proteinase secreted by adult *Fasciola hepatica*. *Mol. Biochem. Parasitol.* **62**, 1-8.

Smyth, J. D. and Halton, D. W. (1983a). The Monogenean - functional morphology. In *The physiology of trematodes*, pp. 250–288. Cambridge University Press. Cambridge, UK.

Smyth, J. D. and Halton, D. W. (1983b). The Monogenean - physiology. In *The Physiology of Trematodes*, pp. 289–320. Cambridge University Press, Cambridge, UK.

Sojka, D., Franta Z., Horn, M., Hajdušek, O., Caffrey, C. R., Mareš, M. and Kopáček, P. (2008): Profiling of proteolytic enzymes in the gut of the tick *Ixodes ricinus* reveals an evolutionary conserved network of aspartic and cysteine peptidases. *Parasit. Vectors* **7**, 1–13.

Sojka, D., Franta, Z., Horn, M., Caffrey, C. R., Mareš, M. and Kopáček, P. (2013). New insights into the machinery of blood digestion by ticks. *Trends Parasitol.* **29**, 276-85. doi: 10.1016/j.pt.2013.04.002.

Sonenshine, D. E. (1991): Biology of ticks. pp. 472. *Oxford University Press* Vol. 1, UK.

Steigele, S. and Nieselt, K. (2005). Open reading frames provide a rich pool of potential natural antisense transcripts in fungal genomes. *Nucleic Acids Res*.**33**:5034-44.

Sunyer, J.O., Zarkadis, I. K., Sahu, A. and Lambris, J. D. (1996). Multiple forms of complement C3 in trout that differ in binding to complement activators. *Proc. Natl. Acad. Sci. USA.* **93**, 8546-51.

Sunyer, J. O., Tort, L. and Lambris, J. D. (1997). Diversity of the third form of complement, C3, in fish: functional characterization of five forms of C3 in the diploid fish *Sparus aurata*. *Biochem. J.* **326**, 877-81.

Sunyer, J. O. and Lambris, J. D. (1998). Evolution and diversity of the complement system of poikilothermic vertebrates. *Immunol. Rev.* **166**, 39-57.

Šlechtová, J. (2007). Hemostáza - jak ji možná neznáte. *Klin. Biochem. Metab.* **15**, 97–101.

Tavares-Dias, M., and Oliveira, S. R. (2009). A review of the blood coagulation system of fish. *Braz. J. Bioscien.* **7**, 205-224.

Tort, J., Brindley, P. J., Knox, D., Wolfe, K. H. and Dalton, J. P. (1999). Proteinases and associated genes of parasitic helminths. *Adv. Parasitol.* **43**, 161–266.

Tosi, M. F. (2005). Innate immune responses to infection. *J. Allergy Clin. Immunol.* **116**, 241-9.

Trojan, S. (2003). Fyziologie krve. In *Lékařská fyziologie (4. vydání)*, pp. 140–151. GRADA, Praha.

Tsujimoto, H., Kotsyfakis, M., Francischetti, I. M. B., Eum, J. H., Strand, M. R. and Champagne, D. E. (2012). Simukunin from the salivary glands of the black fly *Simulium vittatum* inhibits enzymes that regulate clotting and inflammatory responses. *PloS One* **7**, e29964. doi: 10.1371/journal.pone.0029964.

Turk, B., Turk, D. and Turk, V. (2000). Lysosomal cysteine proteases: more than scavengers. *Biochim. Biophys. Acta* **1477**, 98–111.

Turk, V., Stoka, V., Vasiljeva, O., Renko, M., Sun, T., Turk, B. and Turk, D. (2012). Cysteine cathepsins: From structure, function and regulation to new frontiers. *Biochim. Biophys. Acta* **1824**, 68-88. doi: 10.1016/j.bbapap.2011.10.002.

Valigurová, A., Hodová, I., Sonnek, R., Koubková, B. and Gelnar, M. (2011). *Eudiplozoon nipponicum* in focus: monogenean exhibiting a highly specialized adaptation for ectoparasitic lifestyle. *Parasitol. Res.* **108**, 383–94. doi: 10.1007/s00436-010-2077-6.

van de Locht, A., Stubbs, M.T., Bode, W., Friedrich, T., Bollschweiler, C., Höffken, W. and Huber, R. (1996). The ornithodorin-thrombin crystal structure, a key to the TAP enigma? *EMBO J*. **15**, 6011-7.

Vasiljeva, O., Reinheckel, T., Peters, C., Turk, D., Turk, V. and Turk, B. (2007). Emerging Roles of Cysteine Cathepsins in Disease and their Potential as Drug Targets. *Curr. Pharm. Des.* **13**, 387-403. doi: 10.2174/138161207780162962.

Vernet, T., Berti, P. J., de Montigny, C., Musil, R., Tessier, D. C., Ménard, R., Magny, M. C., Storer, A. C. and Thomas, A.C. (1995). Processing of the papain precursor. The ionization state of a conserved amino acid motif within the Pro region participates in the regulation of intramolecular processing. *J. Biol. Chem.* **270**, 10838-10846.

Wex, T., Levy, B., Wex, H. and Brömme, D. (1999). Human cathepsins F and W: A new subgroup of cathepsins. *Biochem. Biophys. Res. Commun.* **259**, 401-7. doi: 10.1006/bbrc.1999.0700.

Whittington, I. D. and Kearn, G. C. (2011). Hatching Strategies in Monogenean (Platyhelminth) Parasites that Facilitate Host Infection. *Integr. Comp. Biol.* **51**, 91–9.

Wiedmer, T., Esmon, C. T. and Sims, P. J. (1986). Complement proteins C5b-9 stimulate procoagulant activity through platelet prothrombinase. *Blood* **68**, 875-80.

Williamson, A. L., Brindley, P. J., Knox, D. P., Hotez, P. J. and Loukas, A. (2003). Digestive proteases of blood-feeding nematodes. *Trends Parasitol.* **19**, 417–23. doi: 10.1016/S1471-4922(03)00189-2.

Williamson, A. L., Lecchi, P., Turk, B. E., Choe, Y., Hotez, P. J., McKerrow, J. H., Cantley, L. C., Sajid, M., Craik, C. S. and Loukas, A. (2004). A multi-enzyme cascade of hemoglobin proteolysis in the intestine of blood-feeding hookworms. *J. Biol. Chem.* **279**, 35950–35957. doi: 10.1074/jbc.M405842200.

Wong, M. K. and Takei, Y. (2013). Lack of plasma kallikrein-kinin system cascade in teleosts. *PLOS One* **8**, e811057. doi: 10.1371/journal.pone.0081057.

Wu, Y. (2015). Contact pathway of coagulation and inflammation. *Thromb. J.* 13,1-9.

Yuan, C.-H., He, Q.-Y., Peng, K., Diao, J.-B., Jiang, L.-P., Tang, X. and Liang, S.-P. (2008). Discovery of a distinct superfamily of Kunitz-type toxin (KTT) from tarantulas. *PloS One* **3**, e3414. doi: 10.1371/journal.pone.0003414.

Zhang, S. and Cui, P. (2014). Complement system in zebrafish. *Dev.Comp.Immunol*. **46**, 3-10. doi: 10.1016/j.dci.2014.01.010

Zhao, R., Dai, H., Qiu, S., Li, T., He, Y., Ma, Y., Chen, Z., Wu, Y., Li, W. and Cao, Z. (2011). SdPI, the first functionally characterized Kunitz-type trypsin inhibitor from scorpion venom. *PloS One* **6**, e27548. doi: 10.1371/journal.pone.0027548.

Zhou, J., Zhang, Y.-Y., Li, Q.-Y. and Cai, Z.-H. (2015). Evolutionary History of Cathepsin L (L-like) Family Genes in Vertebrates. *Int.J. Biol. Sci.* **11**, 1016-1025. doi: 10.7150/ijbs.11751

Zipfel, P. F. and Skerka, C. (2009). Complement regulators and inhibitory proteins. *Nat. Rev. Immunol.* **9**, 729-40. doi: 10.1038/nri2620.

Zurawski, T. H., Mousley, A., Mair, G. R., Brennan, G. P., Maule, A. G., Gelnar, M. and Halton, D. W. (2001). Immunomicroscopical observations on the nervous system of adult *Eudiplozoon nipponicum* (Monogenea: Diplozoidae). *Int. J. Parasitol*. 31, 783-92. doi: 10.1016/S0020-7519(01)00192-8.

Zurawski, T. H., Mousley, A., Maule, A. G., Gelnar, M. and Halton, D. W. (2003). Cytochemical studies of the neuromuscular systems of the diporpa and juvenile stages of *Eudiplozoon nipponicum* (Monogenea: Diplozoidae). *Parasitology* **126**, 349-57. doi: 10.1017/S0031182002002871.

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