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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 confirmed 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 (*En*KT1) 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 *En*KT1 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 *En*KT1 based on the inhibition of Factor Xa were confirmed by thromboelastography, but no effect on fibrinolysis was observed. Moreover, we discovered that *En*KT1 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 anti-haemostatic 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 *En*KT1, 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 prepared 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 μg 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 Bouiń s solution (Sigma-Aldrich, USA) at room temperature for 1 h and transferred to a fresh solution overnight (4 °C). 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 μg 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 μl in 96-well black flat bottom plates (Nunc, Thermo Scientific). The reaction was started by adding 100 μl of 40 μM substrate in the same buffer. Controls did not contain solPE. The production of free fluorophore was measured in 2 min cycles at 28 °C for 30 min using an Infinite M200 fluorimeter (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 (*En*KT1) 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 *En*KT1 (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 Scientific, 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 *Nde*I-*Xho*I enzymes (commissioned work performed by the company GenScript, USA). A 6x His-tag was incorporated on the C-terminus of *En*KT1. The construct was transformed into BL21 Star™(DE3) *E. coli* (Invitrogen, USA). The expression of recombinant *En*KT1 (r*En*KT1) 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 sarcosine, 10 mM imidazole, pH 8.0. The solution was sonicated on ice (10 W, 3 × 30 s), centrifuged at 10,000*g* for 10 min at 4 °C, and filtered through a 0.22 μm filter. Recombinant *En*KT1 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 r*En*KT1 were produced in an ICR/CD1 mouse (ENVIGO, Italy) that was injected s.c. three times at intervals of 14 days with 30 μg of purified r*En*KT1 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 r*En*KT1was detected on blots using iBody4, a biotinylated copolymer containing nitrilotriacetic acid-bound nickel cations (Šácha et al., 2016). Purified r*En*KT1 (2 μg) was run in a 4–12% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (PVDF, Bio-Rad). Monospecific polyclonal antibodies against r*En*KT1 and control sera (both diluted 1:100) were verified on immunoblots. The r*En*KT1 (1 μg/well) and worm soluble protein extract (20 μg/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 μg, Sigma-Aldrich 93614) was incubated in the presence of r*En*KT1 (1 μg) 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: r*En*KT1 ≈ 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 r*En*KT1 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 r*En*KT1 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*) – *En*CL1 (GenBank accession number KP793605), *En*CL3 (GenBank accession number KP793606), and *En*CB (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 pre-incubated with different concentrations of r*En*KT1 (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* (r*En*KT1).

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 International GmbH, Germany) ROTEM® system reagent for re-calcifying 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 (final 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 r*En*KT1 and aprotinin (positive control, Sigma-Aldrich) used in the test were 200 nM, 2 μM and 4 μM. 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 r*En*KT1 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, Denmark). Each well contained 100 μl of 20% fish serum in PBS premixed with various amounts of purified r*En*KT1 (0.08–4 μM concentration). After 10 min incubation at room temperature, 100 μl of erythrocyte suspension were added. Controls did not contain either r*En*KT1 or fish serum, or both. Additional controls were performed with heat-inactivated serum (56 °C, 30 min) and with bovine serum albumin (BSA) used instead of r*En*KT1 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 (*Sac*I/*Nco*I 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 deparaffinated in xylene, rehydrated and treated by proteinase K (final concentration 0.75 μg/ml, 17 min, 37 °C). Hybridisation was performed with RNA probes for 19 h at 42 °C employing a modified protocol as described previously (Quack et al., 2009). After that, sections were stringently washed 4 × 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 μm 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 (phosphate buffered saline/0.25% Triton X-100), incubated for 1 h at 37 °C with horseradish peroxidase (HRP)-labeled rabbit-anti-mouse IgG secondary antibody (ThermoFisher) diluted 1:2000, and washed again 3 × 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

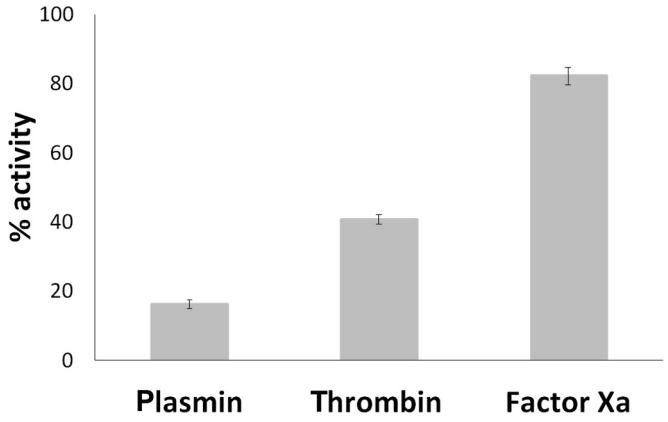

Fig. 1. Inhibitory effects of *Eudiplozoon nipponicum* soluble protein extract (solPE) on peptidases involved in haemostasis/fibrinolysis. Peptidases were preincubated with 5 μg of solPE. Activity was measured with appropriate substrates VPR-AMC (thrombin), IEGR-AMC (FXa) and ALK-AMC (plasmin). Bars 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. *En*KT1 possesses arginine residue in P1 (R_{81}) and alanine in P1′ (A_{82}) positions. BlastP revealed that *En*KT1 (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 *En*KT1 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 *En*KT1, 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 *En*KT1 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 *En*KT1 expressed in *E. coli* was purified 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 μg) was detected on PVDF membrane by iBODY4 binding to His-tag (Fig. 3). A reaction of mouse anti-r*En*KT1 antibodies was confirmed on a western blot of r*En*KT1 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 *En*KT1 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-r*En*KT1 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

r*En*KT1 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 r*En*KT1 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 r*En*KT1 on fibrin clot formation and lysis in whole human blood. Purified r*En*KT1 slowed the coagulation process in a concentration-dependent manner – the clotting time was prolonged by ≈60% in the presence of 4 μM 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 r*En*KT1 (0.2–4 μM) 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 μM (Table 3). On the other hand, we observed some inhibitory effect of r*En*KT1 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 μM r*En*KT1 prolonged the time needed for complete lysis by 33% and 2 μM r*En*KT1 by 145%, relevant to the control without r*En*KT1 (Fig. 7). r*En*KT1 alone had no effect on erythrocyte integrity even at a 20 μM 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 histological 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

MGSKLILSMALLAMAVATLWIAEVSGGVPKFHSGGQMSGGAHKFLLGGQMSGEVPKFLLDGQTTASPL
STCQLPQMVGMCRASFPRFYFDGKKCTEFIYGGCGGNANNFQTKAECESTCPVV*

Fig. 2. Complete amino acid sequence of Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (*En*KT1). Signal sequence on *En*KT1 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′ positions of the reactive site are indicated by white letters. Solid underlining indicates peptides identified by mass spectrometry in excretory/secretory products of adult *E. nipponicum*.

Table 2
Primary structures of the P6–P5′ region and P18′ residue of the active loop of Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (*En*KT1) textilinin-1, and aprotinin.

	P6	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	P5′	P18′
*En*KT1	**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 *En*KT1 (GenBank accession number MF346930), textilinin-1 (GenBank accession number Q90WA1.1), and aprotinin (GenBank accession number P00974.2)].

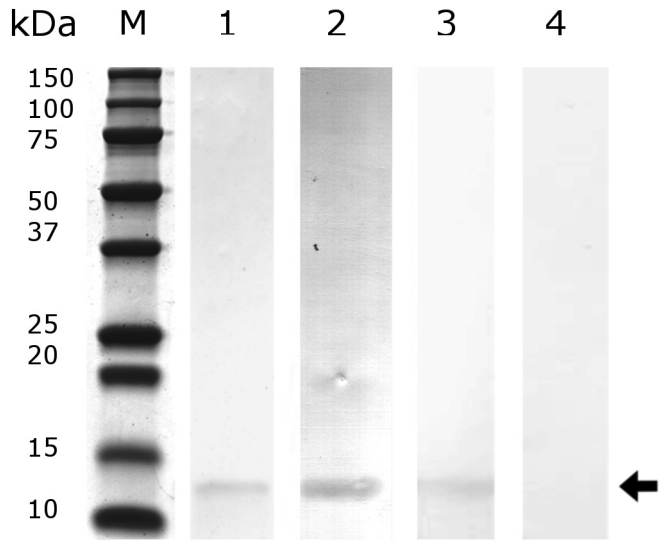

Fig. 3. Detection of the purified recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (r*En*KT1) by SDS-PAGE and blotting. Lane 1, r*En*KT1 in 4–12% gel stained with Coomassie Brilliant Blue R-250. Lane 2, r*En*KT1 band labelled by an iBody4 affinity probe on a blot. Lane 3, r*En*KT1 band was detected by mouse anti-r*En*KT1 antibodies on a blot. Lane 4, control blot with pre-immune serum.

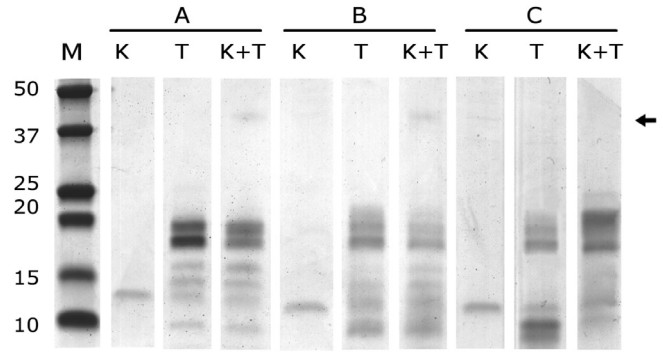

Fig. 4. Complex formation of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (r*En*KT1) with trypsin. (A) Blots were run under non-reducing conditions. (B) Blots were run under reducing conditions. (C) Blots were run under reducing conditions with a boiled sample. The r*En*KT1-trypsin complex is indicated by an arrow. Low MW bands in lanes with trypsin correspond to intermediate products of trypsin autohydrolysis. K, *En*KT1 alone. T, trypsin alone. K + T, *En*KT1 + trypsin.

a sense probe; however, strand-specific RT-PCR also revealed antisense 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 *En*KT1 with specific antibodies on histological sections of the adult worms showed the presence of *En*KT1 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, predominantly 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 platyhelminths 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 peptidolytic activities of three serine peptidases involved in haemostasis/fibrinolysis in vertebrates – 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 containing a spectrum of inhibitors with different specificities/functions. 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 *En*KT1, 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 *En*KT1 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 *En*KT1 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 r*En*KT1 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-Rodríguez et al., 2009). To test the inhibitory specificity of r*En*KT1, 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

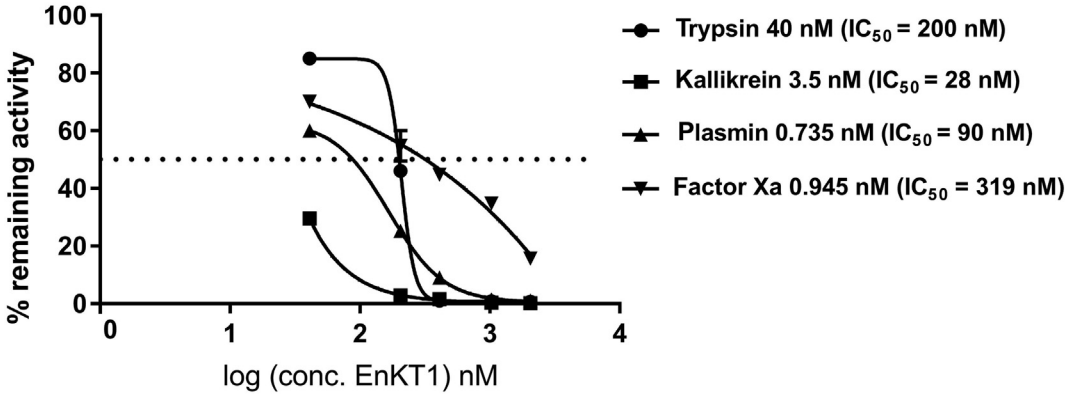

Fig. 5. Relative inhibition of trypsin, plasma kallikrein, plasmin and Factor Xa by recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (r*En*KT1). The dotted line indicates 50% inhibition of peptidase activity (IC_{50}). Relative inhibition values are related to the enzymes' activities at 0 nM concentration of r*En*KT1 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 r*En*KT1 in the calculations. Bars represent S.D.

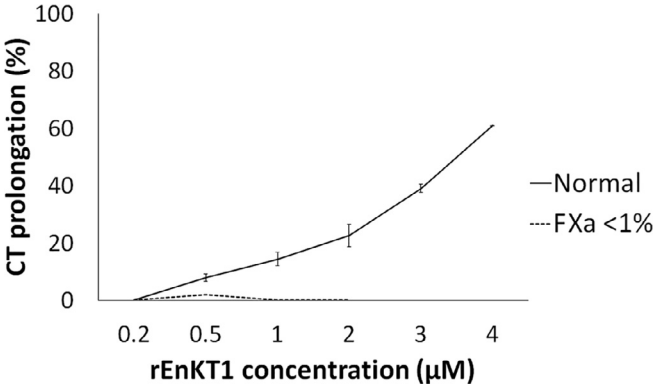

Fig. 6. The effect of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (r*En*KT1) on formation of a fibrin clot in whole human blood. The black line shows relative prolongation of coagulation time in the presence of different concentrations of r*En*KT1 in relation to the control. The dashed line shows no effect of r*En*KT1 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).

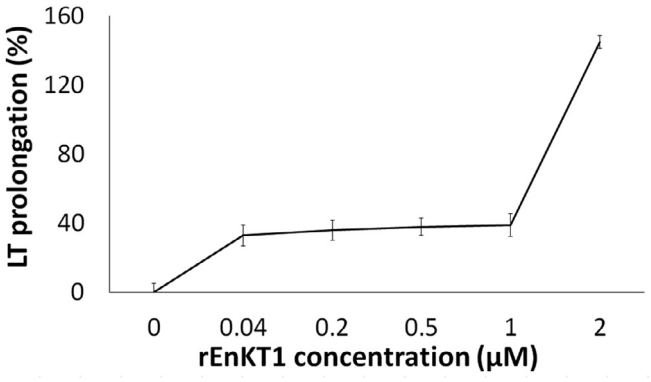

Fig. 7. Concentration-dependent effect of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (r*En*KT1) on lysis of rabbit erythrocytes by goldfish complement. LT, lysis time (time needed for lysis of rabbit erythrocytes in 1% suspension, in the presence of 10% goldfish serum). Bars represent S.D.

Table 3
The effect of recombinant Kunitz-type inhibitor 1 from *Eudiplozoon nipponicum* (r*En*KT1) 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 µM	18.7 ± 6.1	56 ± 1.4
Aprotinin, 4 µM	14.3 ± 4.0	54.5 ± 2.1
r*En*KT1, 200 nM	100 ± 0.0	30 ± 7.0
r*En*KT1, 2 µM	99.6 ± 0.5	42.5 ± 2.1
r*En*KT1, 4 µM	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 r*En*KT1 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 r*En*KT1 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 r*En*KT1 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 *En*KT1 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 *En*KT1 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′ sites (Manithody et al., 2012). However, it is difficult to elucidate the role of individual amino acid residues within the active loop of *En*KT1 in binding

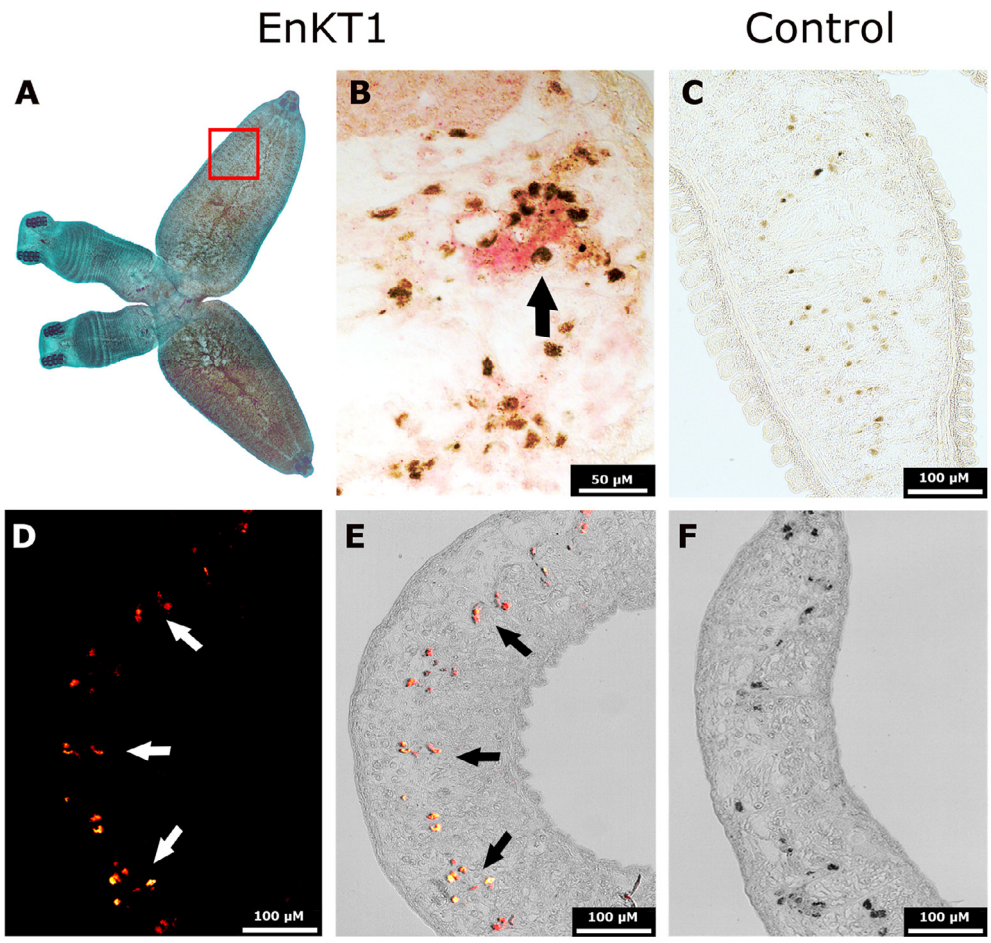

Fig. 8. Localisation of transcripts of Kunitz-type inhibitor 1 gene from *Eudiplozoon nipponicum* (*Enkt1*) and *En*KT1 protein on histological sections of *E. nipponicum* adult worms. (A) Image of whole worms (pair) with an area marked by a square which indicates the approximate position within a worm's 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-r*En*KT1 antibodies within haematin cells (fluorescence); (E) the same as D with merged fluorescence + bright field; (F) control with pre-immune serum. Arrows indicate localisation of *En*KT1 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′ (polar versus polar positive), and P5′ (polar positive versus polar) (see Table 2). So, it seems that some of these residues may support the ability of *En*KT1 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 *En*KT1.

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 r*En*KT1 in vitro with an IC_{50} in the nanomolar range. Surprisingly, no effect was observed on fibrinolysis in 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 r*En*KT1 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 r*En*KT1 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 r*En*KT1 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 r*En*KT1 could hypothetically indicate that *En*KT1 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 goldfish serum, another function of *En*KT1 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 parasite's gut, and help in this way to avoid damage to the cells of gastrodermis. There is also a possibility that *En*KT1 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 *En*KT1, 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 immunohistochemistry using anti-r*En*KT1 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 *En*KT1 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, *En*KT1 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, *En*KT1 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, *En*KT1 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. *En*KT1, 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 *En*KT1, 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, 5628–5636.

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, W597–W603.

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

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. 297–344.

Corral-Rodríguez, M.Á., Macedo-Ribeiro, S., Barbosa Pereira, P.J., Fuentes-Prior, P., 2009. Tick-derived Kunitz-type inhibitors as antihemostatic factors. Insect Biochem. Mol. Biol. 39, 579–595.

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, 25411–25417.

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, 188–193.

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., Graña, M., Durá, 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, 254–264.
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 deficiency: an uncommon presentation of AL amyloidosis. Ups. J. Med. Sci. 117, 457–459.
Manithody, C., Yang, L., Rezaie, A.R., 2012. Identification 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, 1667–1673.
Peigneur, S., Billen, B., Derua, R., Waelkens, E., Debaveye, S., Béress, 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://fisheries.tamu.edu/files/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. Inflamm. Res. 59, 679–687.
Schmaier, A.H., 2016. The contact activation and kallikrein/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, 1–9.
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 first functionally characterized Kunitz-type trypsin inhibitor from scorpion venom. PloS One 6, e27548.