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Fibrinogen and fibrin are novel substrates for *Fasciola hepatica* cathepsin L peptidases

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ABSTRACT

Cathepsin peptidases form a major component of the secreted proteins of the blood-feeding trematodes *Fasciola hepatica* and *Schistosoma mansoni*. These peptidases fulfill many functions, from facilitating infection to feeding and immune evasion. In this study, we examined the *Fasciola* cathepsin L peptidases FhCL1, FhCL2, and FhCL3 and the schistosomal cathepsin peptidases SmCB1 and SmCL3 for their anticoagulant properties. Although no direct anticoagulant effect of these peptidases was observed, we discovered that cathepsin peptidases from *Fasciola*, but not from *Schistosoma*, were able to degrade purified fibrinogen, with FhCL1 having the highest fibrinogenolytic activity. Additionally, FhCL1 and FhCL2 both efficiently degraded fibrin. The lack of a direct anticoagulant or fibrinolytic effect of these peptidases is explained by their inhibition by plasma components. However, within the parasite gut, high concentrations of these peptidases could induce an anticoagulant environment, facilitating blood-feeding for extended periods.

Secreted peptidases play a crucial role in the life cycle of trematodes, both in the immature and mature stages. Of special interest are cathepsin peptidases, which are papain-like cysteine peptidases, that make up a large proportion of the transcriptome of trematodes (10–27%) [1] and their secretome [2]. These secreted cathepsin peptidases fulfill a broad range of functions from facilitating infection, acquisition of nutrients, tissue migration, and immune suppression [1]. In addition, secretomes of blood-feeding parasites, such as leeches or hookworm species, often contain a repertoire of peptides and peptidases with anticoagulant properties, to allow blood-feeding for extensive periods [3,4].

Fasciola and *Schistosoma* species are well studied examples of trematodes that feed on blood components. Cathepsins are the major components of the secretome of both *Fasciola hepatica* and *Schistosoma mansoni* [5–7]. In this study, we examined the anticoagulant capacities of several cathepsin peptidases from these blood-feeding trematodes: *F. hepatica* Cathepsin L 1 (FhCL1), FhCL2, FhCL3, *S. mansoni* Cathepsin B 1 (SmCB1), and *S. mansoni* Cathepsin L 3 (SmCL3). Cathepsin L peptidases are the most abundantly secreted peptidases from *F. hepatica*. FhCL3 is known to cleave collagen and is mostly secreted by newly excysted juveniles (NEJs) upon penetration through the gut wall, accounting for 37% of the NEJs secretome [7]. FhCL1 and FhCL2 are major components of bile duct-residing, adult parasite secretions, and represent 69% and 22% of the adult secretome, respectively [7]. They are known to be involved in the cleavage of hemoglobin, collagen, IgG, fibronectin, and laminin [8–11]. SmCB1 is the most abundant cysteine peptidase in adult *S. mansoni* residing in the mesenteric veins and is, like FhCL1 and FhCL2, involved in the cleavage of hemoglobin, IgG, serum albumin, but also α-2-macroglobulin [5,12]. SmCL3 is a gut-associated, but not secreted, peptidase of schistosomula and adult *S. mansoni* and is involved in the digestion of host proteins such as serum albumin and hemoglobin [13]. All five peptidases were recombinantly expressed in *Pichia pastoris* and activated as previously described [6,13–15]. Anticoagulant properties of these peptidases were first examined with clot lysis time assays [16], with thrombin generation assays [17], and with fibrin formation assays in which fibrin was formed in 3x diluted trisodiumcitrate anticoagulated plasma by addition of 4 μM phospholipids (40% phosphatidylcholines, 40% phosphatidylethanolamines, and 20% phosphatidylserines), 10 mM $CaCl_2$, in the absence or presence of tissue factor (1 pM or 5 pM). However, no anticoagulant effect of the peptidases was found with these assays (data not shown).

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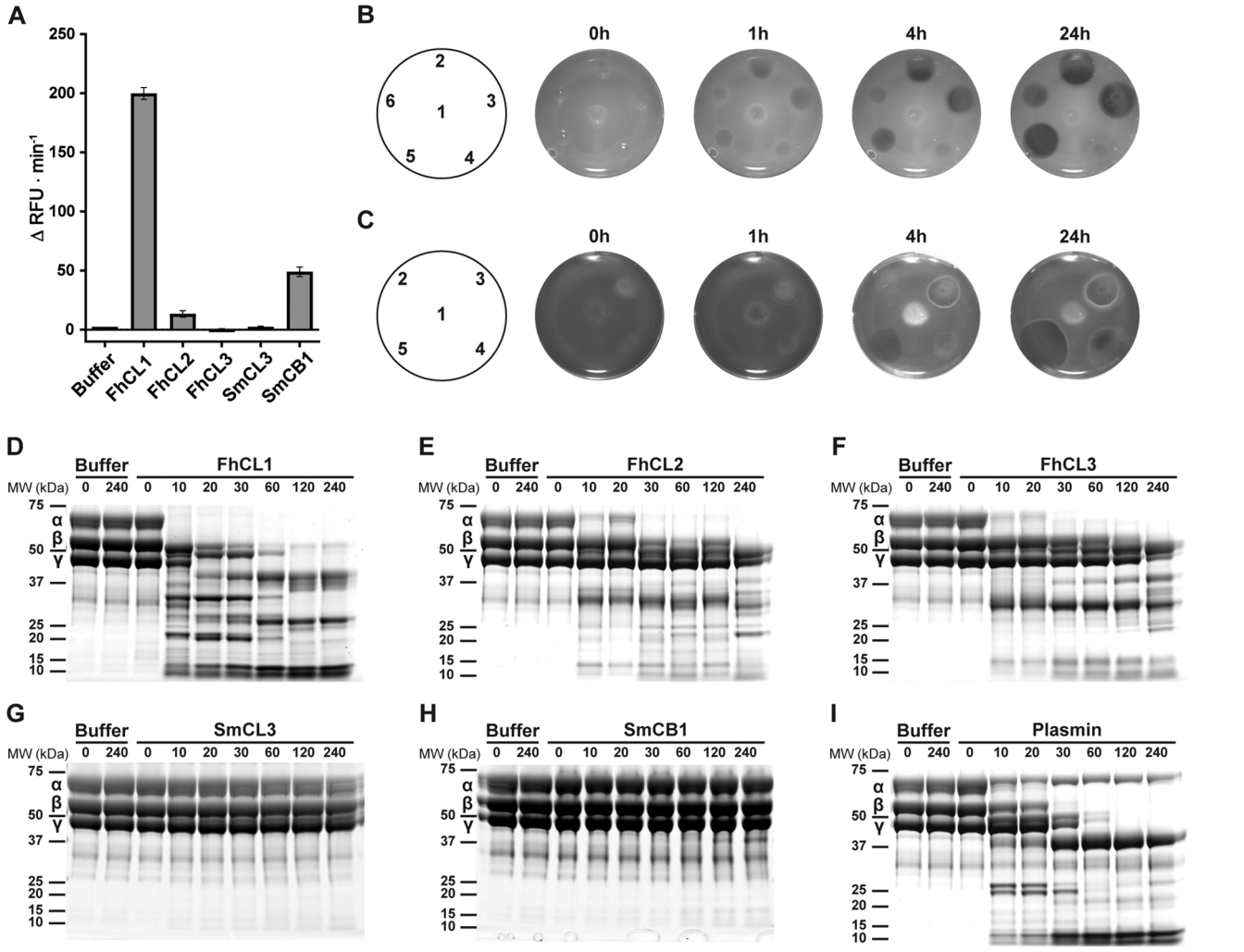

Fig. 1. Fibrinogenolytic and fibrinolytic activities of secreted peptidases of *F. hepatica* and *S. mansoni*.
A) Cleavage of plasmin substrate (Boc-Val-Leu-Lys-AMC, Bachem, Bubendorf, Switzerland) in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) by 20 nM of peptidases of *F. hepatica* (FhCL1, FhCL2, and FhCL3) and *S. mansoni* (SmCL3 and SmCB1). Recombinant peptidases were produced in *Pichia pastoris* and purified as previously described [6,13,14] and activated for 10 min at 37 °C in reaction buffer (2.5 mM EDTA, 0.1 M Sodium Citrate, 2 mM DTT, pH 5.0). SmCB1 was activated with activation buffer (0.1 M sodium acetate, 0.1% PEG6000, 1 mM EDTA, 2.5 mM DTT, 10 mg/ml Dextran sulphate (Mw 500 kDa), pH 4.5) for 2 h at 37 °C [15]. Fluorescence was monitored for 120 min at 37 °C on a Spectromax M^2 fluorescence microplate reader (MTX Lab Systems, Bradenton, FL, USA) equipped with a 390 nm excitation filter and 460 nm emission filter. Initial rates of fluorescence are represented as mean ± SD. All values represent three replicates of duplo measurements. Fibrinolytic activity of *F. hepatica* (B) and schistosomal (C) peptidases was assessed on a fibrin gel (representative image of three experiments). Fibrin gels were prepared by coagulating 4.9 mg/ml purified human fibrinogen (Enzyme research laboratories, South Bend, IN, USA) in HBS with 1 U/ml human alpha-thrombin (Haematologic Technologies, Essex Junction, VT, USA) followed by addition of peptidase samples and subsequent incubation at 37 °C for up to 24 h. B) Fibrinolytic activity of 10 µl aliquots of reaction buffer (2.5 mM EDTA, 0.1 M Sodium Citrate, 2 mM DTT, pH 5.0) (1), FhCL1 (10 µM in reaction buffer) (2), FhCL2 (10 µM in reaction buffer) (3), FhCL3 (10 µM in reaction buffer) (4), plasmin (streptokinase-activated plasminogen, 100 µg/ml in reaction buffer) (5), and plasmin (streptokinase-activated plasminogen, 10 µg/ml in reaction buffer) (6), spotted on a fibrin gel. C) Fibrinolytic activity of 10 µl aliquots of reaction buffer (1), SmCL3 (3 µM in reaction buffer) (2), SmCB1 (250 nM in reaction buffer) (3), plasmin (streptokinase-activated plasminogen, 100 µg/ml in reaction buffer) (4), and plasmin (streptokinase-activated plasminogen, 10 µg/ml in reaction buffer) (5) spotted on a fibrin gel. D–I) Fibrinogenolytic activity of *F. hepatica* and schistosomal secreted peptidases and plasmin was determined on Coomassie brilliant blue stained 10% SDS-PAGE gels with a modified fibrinogenolytic assay [18]. Purified fibrinogen (2 µg/ml in HBS) was incubated at 37 °C with 100 nM FhCL1 (D), 100 nM FhCL2 (E), 100 nM FhCL3 (F), 100 nM SmCL3 (G), 25 nM SmCB1 (H), or 100 nM Plasmin (streptokinase-activated plasminogen) (I), respectively. Reactions were terminated by adding denaturing solution (SDS-Page sample buffer containing 6.7 M urea, 2% SDS, and 25 mM dithiothreitol) and heating at 100 °C for 10 min (representative image of three experiments). The numbers at the top of each lane represent the time (in minutes) when samples were taken during the digestion.

In order to assess whether the expressed peptidases may have a fibrinolytic effect, cleavage of the plasmin substrate Boc-val-leu-lys-AMC was examined (Fig. 1A). FhCL1, FhCL2 and SmCB1 were able to cleave the plasmin substrate, generating a fluorescent signal. This indicates a potential fibrinolytic activity of these peptidases.

To examine this potential fibrinolytic activity, lysis of fibrin was assessed for the *F. hepatica* and schistosomal peptidases on a fibrin gel using plasmin as a positive control (Fig. 1B, C). FhCL1 and FhCL2 demonstrated lysis of the fibrin gel after the incubations as visible by the formation of transparent circles (Fig. 1B). FhCL3 did not lyse the fibrin gel, consistent with the absence of cleavage of the plasmin substrate (Fig. 1B). We also found that none of the schistosomal peptidases was able to lyse the fibrin gel within 24 h (Fig. 1C), despite the ability of SmCB1 to cleave plasmin substrate (Fig. 1A).

Next, fibrinogenolytic activity of the studied cysteine peptidases was assessed through analysis of the hydrolysis of purified fibrinogen with SDS-PAGE, as described previously [18] (Fig. 1D–H), and compared to cleavage of fibrinogen by plasmin (Fig. 1i). Interestingly, all three tested *F. hepatica* peptidases were able to cleave fibrinogen, as demonstrated by degradation of the fibrinogen α-chain (66 kDa), β-chain (52 kDa) and γ-chain (46.5 kDa) (Fig. 1D–F). FhCL1 has a larger fibrinogenolytic activity than FhCL2 and FhCL3 and is capable of degradation of the fibrinogen α-chain, β-chain, and γ-chain. FhCL2 and FhCL3 demonstrate only minor cleavage of the γ-chain and slower

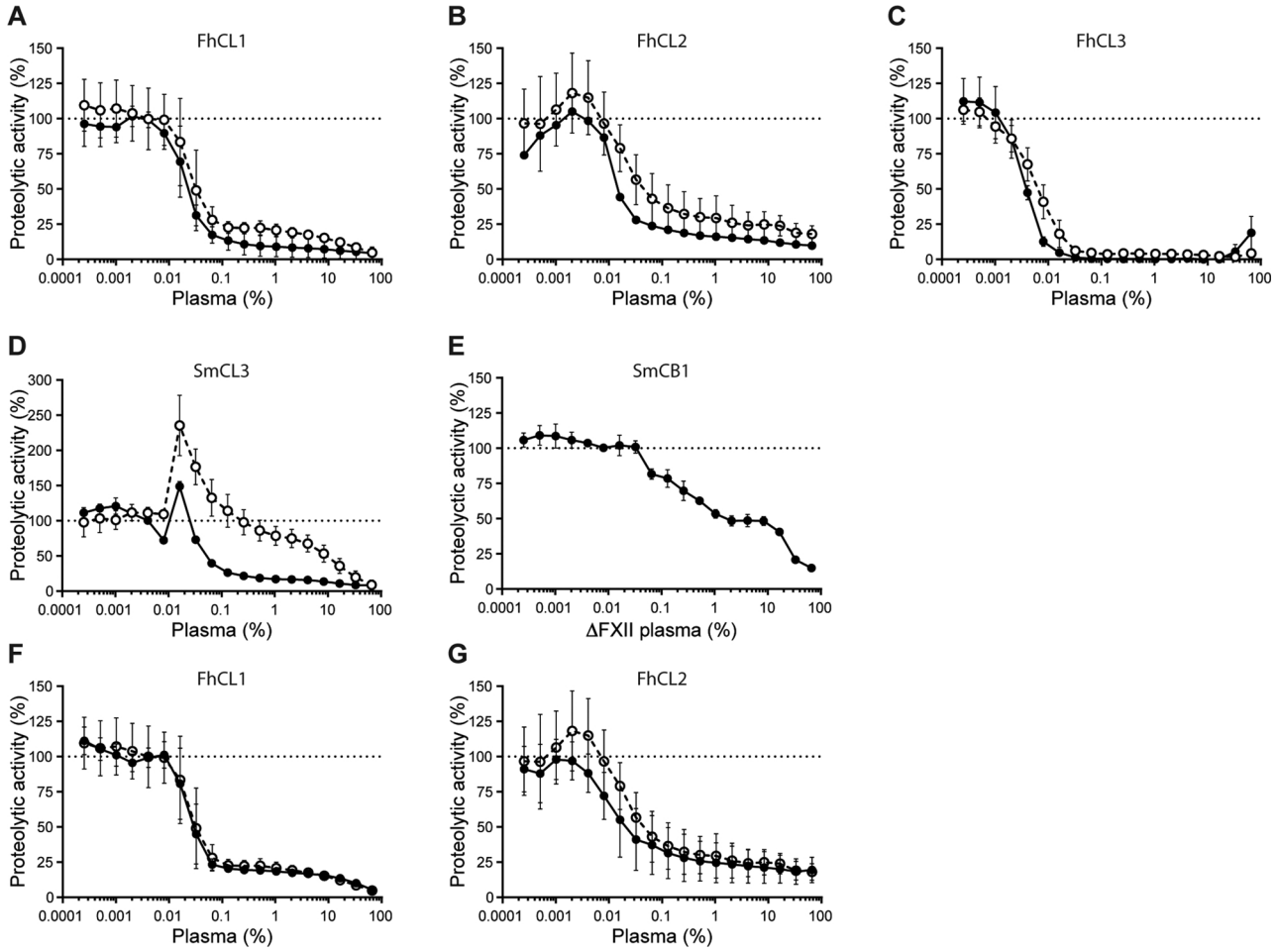

Fig. 2. Proteolytic activity of *F. hepatica* and schistosomal secreted peptidases is inhibited by human plasma.
Proteolytic activity of FhCL1 (20 nM) (A) or FhCL2 (20 nM) (B) on Boc-Val-Leu-Lys-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of citrated (open circles) or heparinized human platelet poor plasma from healthy donors (closed circles). C) Proteolytic activity of FhCL3 (20 nM) on Z-Pro-Arg-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of citrated (open circles) or heparinized human platelet poor plasma from healthy donors (closed circles). D) Proteolytic activity of SmCL3 (20 nM) on Z-Phe-Arg-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of citrated (open circles) or heparinized human platelet poor plasma from healthy donors (closed circles). Proteolytic activity of dextran sulfate-activated SmCB1 (20 nM) on Z-Phe-Arg-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of FXII depleted human plasma. Proteolytic activity of FhCL1 (20 nM) (F) or FhCL2 (20 nM) (G) on Boc-Val-Leu-Lys-AMC substrate (Bachem, Bubendorf, Switzerland) in HBS in the presence of citrated human platelet poor plasma from healthy donors without (open circles) or with addition of glutathione (3.3 mM of which 90% was the reduced form) (closed circles). Fluorescence was monitored for 120 min at 37 °C on a Spectromax M^2 fluorescence microplate reader (MTX Lab Systems, Bradenton, FL, USA) equipped with a 390 nm excitation filter and 460 nm emission filter. Proteolytic activity is displayed as a percentage (mean ± SD) of the activity without addition of plasma (100%, dotted line). All values represent three replicates of duplo measurements.

cleavage of the α-chain and β-chain compared to FhCL1. For all *F. hepatica* peptidases cleavage of the α-chain proceeded faster than cleavage of the β-chain and γ-chain. The cleavage pattern of the *F. hepatica* peptidases showed major differences with that of plasmin, indicating distinct cleavage sites of these peptidases. Plasmin seemed to degrade first the β-chain and γ-chain, with the α-chain still present after 4 h of incubation. Lastly, consistent with the lack of fibrinolytic activity, both schistosomal peptidases did not demonstrate any fibrinogenolytic activity (Fig. 1G,H).

These results show that FhCL1 compared to FhCL2, is much more active in degrading the plasmin-substrate (Fig. 1A) and fibrinogen (Fig. 1D,E), but their activity towards fibrin is similar (Fig. 1B). Therefore, the activity of these peptidases is strongly dependent on the actual substrate. Differences in substrate specificity have been described for the *F. hepatica* peptidases [6], where FhCL1 most efficiently cleaves substrates with hydrophobic residues at the P2 position (including Z-FR-NHMec and Boc-VLK-NHMec) while FhCL2 and FhCL3 have a preference for Pro-containing substrates and they cleave substrates with Phe and Leu in the P2 position much less effectively. Likewise, different K_m values of SmCB1 and SmCL3 for the Z-FR-AMC substrate have been described [12,13], indicating differences in substrate specificity for substrates with a hydrophobic residue at the P2 position.

From these experiments it is clear that the studied *F. hepatica* peptidases, but not the schistosomal peptidases, contain both fibrinogenolytic (FhCL1, FhCL2 and FhCL3) and fibrinolytic (FhCL1 and FhCL2) activity on purified fibrinogen and fibrin. This, however, contrasts with our first findings that these peptidases do not affect fibrin formation and clot lysis times in plasma. Therefore, we next sought an explanation for this discrepancy by examining the effect of plasma on the proteolytic activity of these peptidases (Fig. 2). Proteolytic activity of FhCL1, FhCL2, FhCL3, and SmCL3 on fluorogenic peptide substrates was examined in the presence of either citrated or heparinized plasma (Fig. 2A–D). Since SmCB1 was activated by dextran sulphate [15] and dextran sulphate is a non-physiological trigger for contact system activation *in vitro*, factor XII depleted citrated plasma was used to examine proteolytic activity of SmCB1 in plasma (Fig. 2E). Examination of proteolytic activity showed that all five examined peptidases were inhibited by plasma components at low concentrations (< 0.1% plasma). This inhibition of the peptidases by plasma components explains the absence of anticoagulant effects observed in the performed clot lysis and thrombin generation assays.

Reduced glutathione (GSH) has been described as an activating agent for FhCL1 through reduction of the active site cysteine [19]. Therefore, we also examined whether addition of GSH to plasma could counteract the inhibitory effect of plasma components for FhCL1 and FhCL2 (Fig. 2F,G). However, addition of GSH did not reverse the effect of plasma components. Most likely the GSH did not have an additional effect to the DTT that was already present in the buffer used to activate the peptidases.

In the present study, we report the discovery of fibrinogen and fibrin as novel substrates for the *F. hepatica* cathepsin peptidases FhCL1 and FhCL2 and the fibrinogenolytic activity of FhCL3. We also examined two schistosomal cathepsin peptidases, SmCL3 and SmCB1, for their fibrinogenolytic and fibrinolytic activity and found that fibrinogen and fibrin are not substrates for these peptidases. As all tested peptidases are inhibited by plasma components, we suggest that they most likely do not affect blood coagulation in their host. However, secretion of *F. hepatica* cathepsin L peptidases at high concentrations in the parasite gut implies that this may locally overcome the inhibitory effect of plasma components and induce an anticoagulant environment in the gut through cleavage of fibrinogen and fibrin, allowing blood-feeding by the parasite for extended periods. Our finding that schistosomal peptidases are not able to cleave fibrinogen or fibrin is in line with the presence of other anticoagulant and fibrinolytic strategies adopted by schistosomes (reviewed in [20]). This allows schistosomes to counteract fibrin formation at their surface, allowing blood-feeding and survival of the parasite within its host.

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References

[1] M.W. Robinson, J.P. Dalton, S. Donnelly, Helminth pathogen cathepsin proteases: it's a family affair, Trends Biochem. Sci. 33 (2008) 601–608, http://dx.doi.org/10.1016/j.tibs.2008.09.001.

[2] C. Stack, J.P. Dalton, M.W. Robinson, The phylogeny, structure and function of trematode cysteine proteases, with particular emphasis on the *Fasciola hepatica* cathepsin L family, Adv. Exp. Med. Biol. 712 (2011) 116–135, http://dx.doi.org/10.1007/978-1-4419-8414-2_8.

[3] M. Salzet, Anticoagulants and inhibitors of platelet aggregation derived from leeches, FEBS Lett. 492 (2001) 187–192.

[4] M. Ledizet, L.M. Harrison, R.A. Koskia, M. Cappello, Discovery and pre-clinical development of antithrombotics from hematophagous invertebrates, Curr. Med. Chem. Cardiovasc. Hematol. Agents 3 (2005) 1–10.

[5] C.R. Caffrey, J.H. McKerrow, J.P. Salter, M. Sajid, Blood n guts: an update on schistosome digestive peptidases, Trends Parasitol. 20 (2004) 241–248, http://dx.doi.org/10.1016/j.pt.2004.03.004.

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

[7] M.W. Robinson, R. Menon, S.M. Donnelly, J.P. Dalton, S. Ranganathan, An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen Fasciola hepatica: proteins associated with invasion and infection of the mammalian host, Mol. Cell. Proteomics 8 (2009) 1891–1907, http://dx.doi.org/10.1074/mcp.M900045-MCP200.

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

[9] P. Berasaín, F. Goñi, S. McGonigle, A. Dowd, J.P. Dalton, B. Frangione, C. Carmona, Proteinases secreted by *Fasciola hepatica* degrade extracellular matrix and basement membrane components, J. Parasitol. 83 (1997) 1–5.

[10] P. Berasain, C. Carmona, B. Frangione, J.P. Dalton, F. Goñi, *Fasciola hepatica*: parasite-secreted proteinases degrade all human IgG subclasses: determination of the specific cleavage sites and identification of the immunoglobulin fragments produced, Exp. Parasitol. 94 (2000) 99–110, http://dx.doi.org/10.1006/expr.1999.4479.

[11] C.M. Stack, C.R. Caffrey, S.M. Donnelly, A. Seshaadri, J. Lowther, J.F. Tort, P.R. Collins, M.W. Robinson, W. Xu, J.H. McKerrow, C.S. Craik, S.R. Geiger, R. Marion, L.S. Brinen, J.P. Dalton, Structural and functional relationships in the virulence-associated cathepsin L proteases of the parasitic liver fluke, *Fasciola hepatica*, J. Biol. Chem. 283 (2008) 9896–9908, http://dx.doi.org/10.1074/jbc.M708521200.

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

[13] J. Dvorák, S.T. Mashiyama, M. Sajid, S. Braschi, M. Delcroix, E.L. Schneider, W.H. McKerrow, M. Bahgat, E. Hansell, P.C. Babbitt, C.S. Craik, J.H. McKerrow, C.R. Caffrey, SmCL3, a gastrodermal cysteine protease of the human blood fluke *Schistosoma mansoni*, PLoS Negl. Trop. Dis. 3 (2009) e449, http://dx.doi.org/10.1371/journal.pntd.0000449.

[14] C.M. Stack, J.P. Dalton, M. Cunneen, S. Donnelly, De-glycosylation of Pichia pastoris-produced *Schistosoma mansoni* cathepsin B eliminates non-specific reactivity with IgG in normal human serum, J. Immunol. Methods 304 (2005) 151–157, http://dx.doi.org/10.1016/j.jim.2005.07.019.

[15] A. Jílková, M. Horn, P. Rezáčová, L. Marešová, P. Fajtová, J. Brynda, J. Vondrášek, J.H. McKerrow, C.R. Caffrey, M. Mareš, Activation route of the *Schistosoma mansoni* cathepsin B1 drug target: structural map with a glycosaminoglycan switch, Structure 22 (2014) 1786–1798, http://dx.doi.org/10.1016/j.str.2014.09.015.

[16] T. Lisman, P.G. de Groot, J.C.M. Meijers, F.R. Rosendaal, Reduced plasma fibrinolytic potential is a risk factor for venous thrombosis, Blood 105 (2005) 1102–1105, http://dx.doi.org/10.1182/blood-2004-08-3253.

[17] H.C. Hemker, P. Giesen, R. Al Dieri, V. Regnault, E. de Smedt, R. Wagenvoord, T. Lecompte, S. Béguin, Calibrated automated thrombin generation measurement in clotting plasma, Pathophysiol. Haemost. Thromb. 33 (2003) 4–15, http://dx.doi.org/10.1159/000071636.

[18] P. Geng, L. Lin, Y. Li, Q. Fan, N. Wang, L. Song, W. Li, A novel fibrin(ogen)olytic trypsin-like protease from Chinese oak silkworm (*Antheraea pernyi*): purification and characterization, Biochem. Biophys. Res. Commun. 445 (2014) 64–70, http://dx.doi.org/10.1016/j.bbrc.2014.01.155.

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

[20] M.M. Mebius, P.J.J. van Genderen, R.T. Urbanus, A.G.M. Tielens, P.G. de Groot, J.J. van Hellemond, Interference with the host haemostatic system by schistosomes, PLoS Pathog. 9 (2013) e1003781, http://dx.doi.org/10.1371/journal.ppat.1003781.