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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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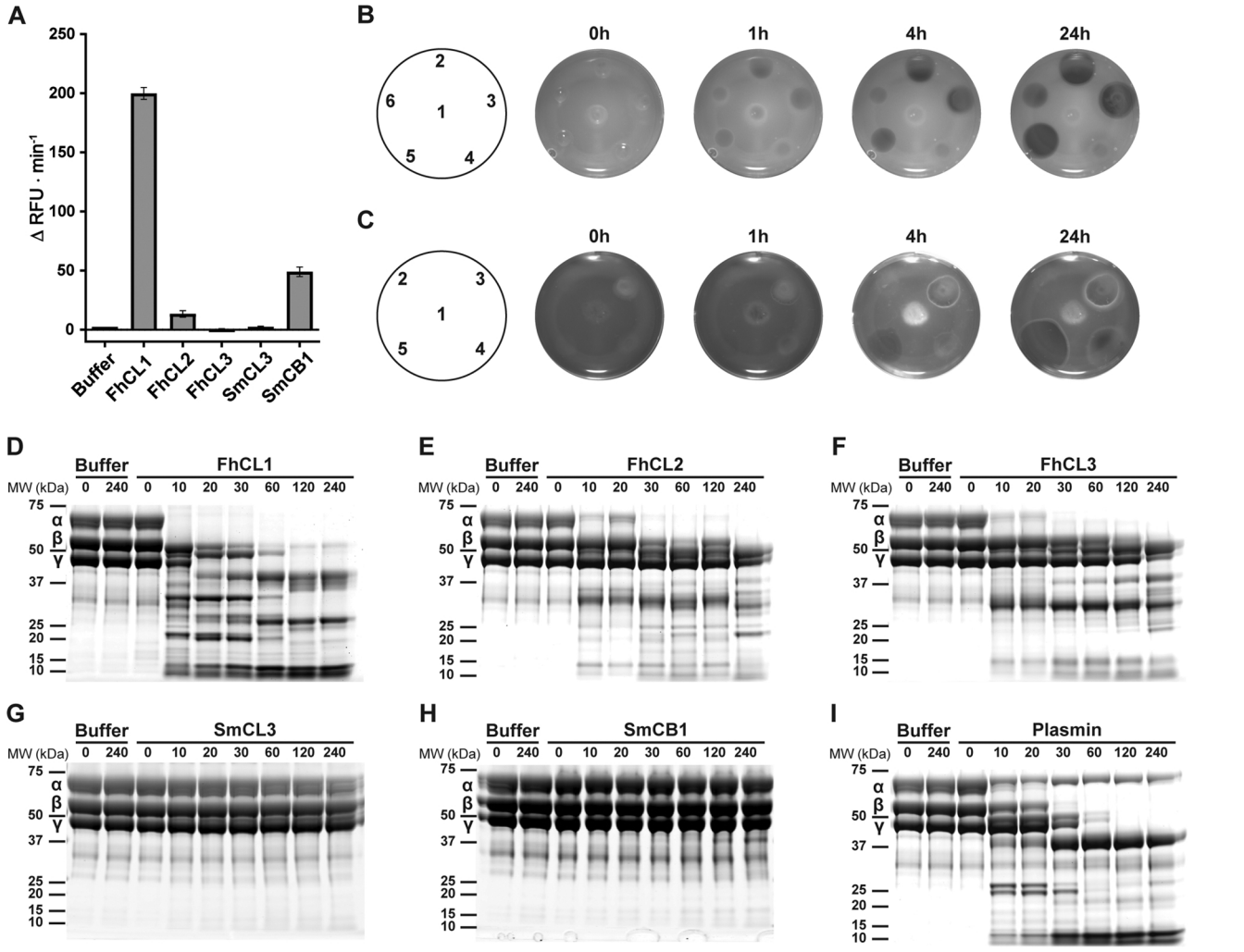

Fig. 1. Fibrinogenolytic and fibrinolytic activities of secreted peptidases of *F. hepatica* and *S. mansoni*.
A) Cleavage of plasmin substrate (Boc-Val-Leu-Lys-AMC, Bachem, Bubendorf, Switzerland) in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) by 20 nM of peptidases of *F. hepatica* (FhCL1, FhCL2, and FhCL3) 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