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Brief Report

Evaluation of Gelatinolytic and Collagenolytic Activity of *Fasciola hepatica* Recombinant Cathepsin-L1

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Background: Cysteine proteases of the liver fluke, *Fasciola hepatica*, participate in catabolism of proteins, migration of the fluke through host tissues and combat host immune system.
Objectives: In this study, we evaluated proteolytic activity of *F. hepatica* recombinant cathepsin L1 (rCL1) against gelatin and collagen as common substrates.
Material and Methods: The coding sequences of *F. hepatica* CL1 were cloned and expressed in *Escherichia coli*, in our previous study. The rCL1 was purified by nickel affinity chromatography with a HisTrap Column. The protein concentrations of the purified fractions were determined by Bradford assay. Rat collagen type-1 was treated with distinct amounts of rCL1 at 37 °C, overnight, and the byproduct was analyzed by SDS-PAGE. Furthermore, we used bovine skin gelatin as zymography substrate to evaluate the gelatinolytic activity of the purified rCL1.
Results: Recombinant CL1 was capable to digest intact type-1 collagen within 24 h and the gelatinlytic activity of rCL1 was visible at approximately 37 kDa region, with optimal activity at acidified conditions (pH 4).
Conclusion: Findings provide a possible mechanism by which a major secretory molecule of *F. hepatica* could be involved in parasite survival as well as its pathogenesis.
Key words: Cathepsin L1; Collagen; *Fasciola hepatica*; Gelatin; Recombinant Enzyme

1. Background

Fasciolosis is a zoonotic disease commonly transmitted by metacercariae-infected herbs or vegetables. *Fasciola (F) hepatica* and *F. gigantica* may infect both livestock and humans and has recently emerged as a serious pathogen (1). Recently proteomic analyses of *F. hepatica* life stages have provided valuable information on the expression of key proteases which are potentially involved in host-parasite interactions (2,3). The findings showed that cysteine proteases play a major role in these interactions and cathepsin L (CL) family which is composed of five distinct clades (FhCL1, FhCL2, FhCL3, FhCL4 , and FhCL5) is the most important one. These virulence-associated cysteine proteases show a firm temporal regulation in their expression levels during the parasite's development in the mammalian host (2). For example, FhCL3 is highly expressed in newly excysted juvenile Fasciola that initiate the infection, but once the juvenile Fasciola travel across the intestinal wall, the expression of FhCL3 is down-regulated (2,3). As the parasite migrates through the liver parenchyma and becomes adult within the bile ducts, the expression of FhCL1, FhCL2, and FhCL5 is up-regulated (2,3).

Cathepsin L1 (CL1) is a major excretory cysteine protease which is commonly produced by the parasite and participates in the catabolism of proteins, migration of the fluke through the host intestine and penetration of the parasite to the Glisson's capsule of the liver through the cleaving of interstitial matrix proteins such as fibronectin, laminin, and native collagens of the host tissues. It also provides mechanisms for combating with host immune system, thus playing an essential role in the pathogenicity of the parasite. Additionally, it has recently been revealed that CL1 may suppress Th1 immune responses in laboratory animals and dispose them to simultaneous bacterial co-infections (4, 5). Therefore, this protease could be a target of intervention strategies.

The crucial role of *F. hepatica* cysteine proteases, specially cathepsins in the pathogenesis of this parasite, including the invasion of tissues, acquisition of nutrients, and immunomodulation has made them key targets for the development of novel vaccines and drugs (6).

2. Objectives

In this study, we evaluated the proteolytic activity of a previously produced *F. hepatica* recombinant CL1 against gelatin and collagen as common protein substrates, to confirm the functionality of the produced enzyme.

3. Materials and Methods

3.1. Cathepsin L1 Source

The coding sequence of *F. hepatica* CL1 (Gen Bank accession No. U62288.2) was previously cloned in pET-21 and the recombinant protein was expressed in *Escherichia coli* BL21 (DE3) cells; the product was then purified using HisTrap nickel affinity chromatography column (7). In this study, following a re-expression and purification steps, the quantity and quality of the purified fractions were determined by SDS-PAGE and Bradford assay, respectively.

Moreover, livers from *F. hepatica*-infected sheep were collected from the Kahrizak slaughterhouse (Tehran, Iran) and the adult flukes were collected and washed with sterile phosphate-buffered saline (PBS). Then, the live flukes were incubated in RPMI medium (1 fluke per 2 mL of medium; Gibco-BRL, Gaithersburg, MD, USA), containing 100 $IU.mL^{-1}$ of penicillin, 100 $\mu g.mL^{-1}$ of streptomycin and sufficient amounts of anti-protease cocktail at 37 °C, overnight in a humidified CO_2 incubator as previously described (7). Then, the excretory-secretory (E/S) product of the parasites was collected and centrifuged at 10,000 x g for 30 min at 4 °C.

3.2. Purification of rCL1 by FPLC Method

The rCL1 was also purified by anion exchange chromatography using AKTA PRIM plus Fast Protein Liquid Chromatography (FPLC) system (GE healthcare Biosciences, Uppsala, Sweden). Briefly, DEAE-Sepharose 6B resin was equilibrated with 20 mM Tris-HCl pH 8.0 as the starting buffer. The bacterial lysate (rCL1 expressing *E. coli* BL21) was dialyzed against the starting buffer and loaded on the column. The contents were fractionated using the starting buffer supplied by increasing gradient amounts of sodium chloride as elution buffer. Similar peaks from several runs were pooled and concentrated by lyophilization. The contents of the fractions were analyzed by subjecting to 12% SDS-PAGE slabs in parallel to molecular weight standards.

3.3. Collagenase Activity

The collagenolytic activity of the rCL1 was initially checked by directly mixing the purified rCL1 with a commercial collagen. In brief, rat type-1 collagen (Calbiochem, UK) was dissolved to a final concentration of 1 $\mu g.mL^{-1}$, then 15 µg of the collagen was mixed with 0.5 µg of the purified rCL1 or acetate buffer (as control), and the contents were incubated for 24 h at 37 °C. Finally, digestion of the collagen was analyzed on 12% SDS-PAGE (8).

3.4. Gelatin Zymography

Gelatin zymography or gelatin substrate polyacrylamide gel electrophoresis was used for the evaluation of the gelatinolytic activity of the rCL1 and the E/S components of *F. hepatica* as its initial native source. Resolving gel (12%) containing 0.3% gelatin was prepared as previously described (9, 10). Recombinant CL1 and E/S were mixed with non-reducing sample buffer and appropriate volumes of the prepared sample were loaded onto the gel without heating. Following electrophoresis, the slab was washed for a total of an hour in 2.5% Triton X-100, prepared in 0.1 M sodium phosphate pH 6.0, to remove SDS. The slab was then incubated overnight at 37 °C in the activation buffer (0.1 M sodium phosphate pH 6.0) and stained with Coomassie Brilliant blue R-250. After de-staining, the gels were scanned and the proteolysis areas were analyzed by densitometry.

4. Results

4.1. Purification of rCL1 by Anion Exchange Chromatography Using FPLC

One typical protein peak was observed following the purification of the recombinant protein by anion exchange chromatography of *F. hepatica* rCL1 (**Fig. 1A**) or affinity chromatography method. The fractions corresponding to identical peaks were collected. Silver nitrate staining of the SDS-PAGE showed a single band with an apparent 37 kDa size (**Fig. 1B**).

4.2. Collagen Activity

Evaluation of collagenase activity showed that the purified recombinant enzyme was capable to completely digest the intact rat type 1 collagen alpha chains within 24 h incubation at 37 °C, while the addition of PBS in the negative control did not result in any alteration in the contents of the substrate (**Fig. 2A**).

4.3. Gelatin Zymography: For Gelatinolytic Analysis We Used Gelatin Substrate.

Recombinant and native products were run on the zymography gel and following an overnight activation step, the enzyme gelatinolytic activity was visualized on de-stained gels. Under non-reducing conditions, rCLI was capable to degrade gelatin and produce a distinct unstained region at 37 kDa (**Fig. 2B**), while, E/S could digest gelatin in different regions, resulting a smear (**Fig. 2C**).

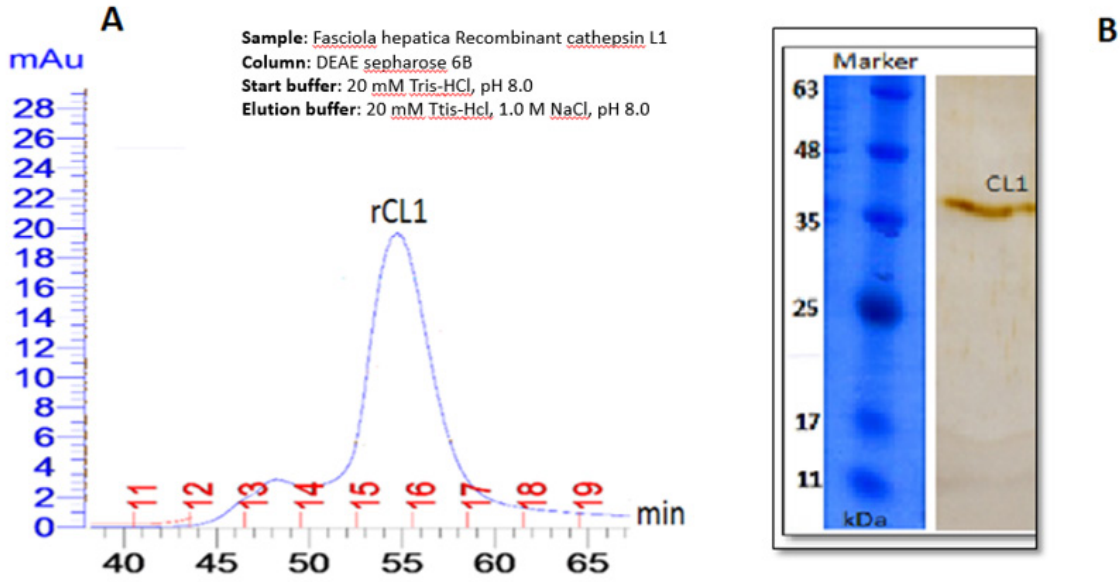

Fig 1. Anion exchange chromatography of *F. hepatica* rCL1. A: FPLC chromatogram of F. hepatica rCL1 obtained by anion exchange chromatography on a DEAE Sepharose CL-6B column. A typical peak was obtained when the NaCl in the elution buffer exceeded 40-50%. **B:** Fraction was electrophoresed on 12% polyacrylamide gels and visualized by silver nitrate staining.

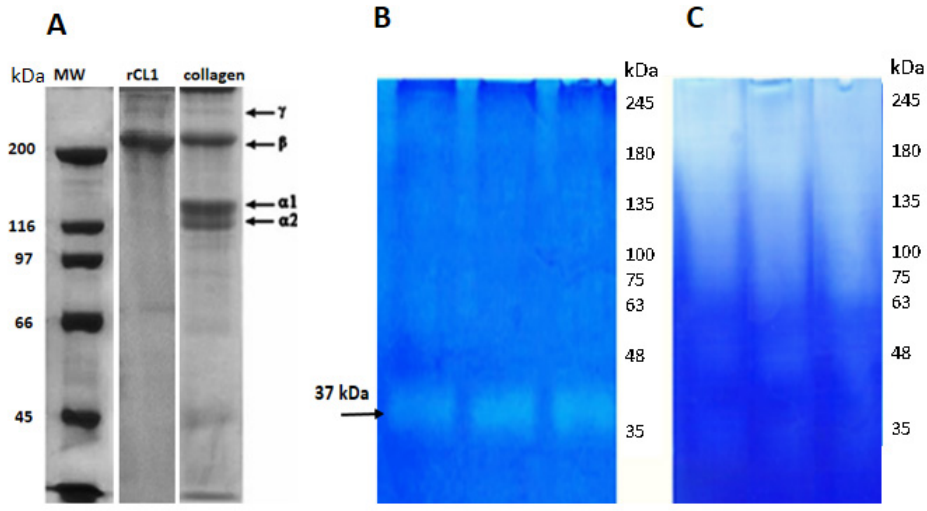

Fig 2. Proteolytic activity of Fasciola hepatica recombinant cathepsin L1 (rCL1) and E/S. A: Collagenase activity of rCL1 Similar amounts of rat type 1 collagen was mixed with PBS (Lane 1), or rCL1 (lane 2) and incubated for 24 h at 37°C. The products were run on SDS-PAGE and stained with Coomassie blue G250. **B: Gelatine zymography of rCL1** SDS-PAGE gel was incorporated with gelatin, then F. hepatica rCL1 or excretory/secretory products were run without heating (native electrophoresis). The rCL1 digested gelatin substrate at 37 kDa region. **C: Gelatine zymography of E/S** Excretory/secretory product digested the gelatin in different regions and resulted various bands.

4. Discussion

Cathepsin L1 is a secretory product of *F. hepatica* and is a major protease found in excretory-secretory metabolites of this fluke, which is potentially harmful for humans or sheep (11). It facilitates penetration and migration of fasciola in host tissues such as liver and intestine, and plays a crucial role in the survival of living flukes in physiologic conditions. In this regard, we analyzed the proteolytic impact of this enzyme on two substrates, namely collagen and gelatin (12). In accordance with the findings of Corvo (13), Howell (14) and some other researchers (15, 16), our study showed that rCl 1 could degrade collagen after 24 h incubation and was able to digest α_1 and α_2 chains of collagen.

Collagenase activity of *F. hepatica* CL1 goes back to its catalytic activity on P2 position of collagen which is a proline and leucine rich sequence. This function is similar to the collagenase activity of human cathepsin K

and cathepsin LS on type II collagen (derived from bovine nasal septum) and type VI collagen (derived from basement membranes). Therefore, *F.hepatica* can damage host tissues and facilitate migration of the parasite through liver, bile duct, and other organs.

Cathepsin F was discovered in other trematodes like *Paragonimus westermani* and *Clonorchis sinensis,* too (15), and serve similar functions such as simplification of migration through the host tissue and achievement of nutrients by the hydrolysis of hemoglobin and collagens (17, 18). In *Schistosoma* spp., both cathepsin L and cathepsin F have been identified (19, 20) but it is believed that the cathepsin L-like cysteine proteases of the *Fasciola* spp. have significant effects on this parasite pathogenicity, distinct from those of the other trematode parasites.

Fasciola CLI may have greater participation in pathogenicity and host immunomodulation, e.g., by the cleavage of immunoglobulins and suppression of Th1 responses (21, 22). This function is specific only to the *Fasciola* cathepsin L family and has not been detected in other trematodes.

Gelatinase activity of CL1 was also well observed in acidic conditions (pH 6). E/S caused a protein smear in zymography gel, while rCL1 produced a single reactive band at 37 kDa region in similar conditions, which were in line with the findings of Dowd et al (23). The main gelatinase activity of CL1 occurs in acidified conditions with pH ranging from 4 to 7. In this study, zymography was performed at pH 6 and the functionality of the target protein was nearly identical to that found in Dowd et al study (23).

Gelatin is a non-specific substrate which could be decomposed by many proteolytic enzymes including serine, cysteine, aspartate and cysteine proteases as well as metalloproteases which may be found in E/S of *F. hepatica*. Therefore, in gelatin zymography, the presence of any type of proteolysis enzyme may result in the digestion of gelatin (15). Notably, we applied purified form of a recombinant form of CL1 which showed enzyme activity in parallel to its previously approved immunogenicity (7).

5. Conclusion

Findings provide a mechanism by which *F. hepatica* synthesizes and secretes fully-activated proteases which is essential for *Fasciola* survival as a parasite. The data obtained in this study showed a mechanism which may facilitate migration of *F. hepatica* in host tissues and provides evasion mechanism from host immune system. Based on the mentioned pathogenicity and considering their well-known immunogenicity, it seems that *Fasciola* cathepsins may be used as vaccine candidates in experimental and ruminant hosts to determine their protection against liver flukes.

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Conflict of Interest

None declared.

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