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The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen☆

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ABSTRACT

Secreted cysteine proteases are major players in host–parasite interactions; in *Fasciola hepatica*, a distinct group of cathepsins L was found to be predominantly expressed in the juvenile stages, but their enzymatic properties were unknown. Cathepsin L3 (FhCL3) is a main component of the juvenile secretory products and may participate in invasion. To characterize the biochemical properties, the proenzyme was expressed in the methylotrophic yeast *Hansenula polymorpha* and the mature enzyme was obtained from the culture medium. FhCL3 exhibited optimal activity and stability at neutral pH and a noticeable restricted substrate specificity with 70-fold preference for Tos-Gly-Pro-Arg-AMC over typical cathepsin substrates with hydrophobic or aliphatic residues in the S2 position. Accordingly, FhCL3 efficiently cleaved type I collagen over different pH and temperature conditions, but it did not cleave immunoglobulin. While most cathepsin cysteine proteinases are unable to digest collagen, mammalian cathepsin K, adult *F. hepatica* FhCL2 and the plant zingipain can also cleave collagen and substrates with Pro in P2 position, but only FhCL3 and zingipain hydrolyze these substrates with the highest efficiency. Molecular modeling and structural comparisons of the collagen cleaving cathepsins indicated that the strong substrate selectivity observed might be due to steric restrictions imposed by bulky aromatic residues at the S2–S3 subsites. The remarkable similarities of the active site clefts highlight the evolutive constrains acting on enzyme function. The presence of a collagen cleaving enzyme in *F. hepatica* juvenile stages is suggestive of a role in tissue invasion, an essential feature for the establishment of the parasites in their host.

1. Introduction

The identification of the major virulence factors is a primary goal in parasite research since they can eventually lead to their use in the development of specific control measures, either through drugs or immunogens. In parasitic trematodes secreted cysteine proteases have been recognized as major players in host–parasite interaction with proposed roles in host colonization, traversing tissue barriers, evading immune responses, and digesting nutrients [1,2]. Therefore these enzymes might be partly responsible for the pathogenic effects of the infection, which translate in the case of zoonotic species in health and sanitary issues and in relevant economic losses. The infection with *Fasciola hepatica* or *F. gigantica* is responsible for fasciolosis, a debilitating liver infection affecting more than 700 million livestock animals worldwide, and resulting in severe economic losses conservatively estimated at US$ 3.2 billion annually, not to mention the increase of the incidence of human fasciolosis in several regions that lead to its recognition as an emerging zoonotic disease by the World Health Organization [3].

It has long been established that *F. hepatica* has several cathepsin like cysteine proteases that prevail in secretions of different life stages of the fluke's cycle, while phylogenetic analysis demonstrated the presence of a complex multigenic family [2,4]. However, up to recently there was no clear evidence of differential expression of these enzymes. Proteomic analysis of adult secretions identified a restricted set of cathepsin L like enzymes that might start their expression during the stage of liver migration [5]. Using combined molecular, biochemical and proteomic approaches a subset of cysteine proteases including at least three cathepsin B like and two cathepsin L like enzymes was identified as predominantly expressed by the newly excysted juveniles (NEJ) [6]. The differential expression patterns observed might correlate with functional diversification within the cathepsin family. Due to their restrictive expression the NEJ enzymes might be the ones involved in the

Abbreviations: AMC, 7-amino-4-methyl coumarin; FhCL, *Fasciola hepatica* cathepsin L; Tos, Tosyl; Z, benzyloxycarbonyl.

☆ *Note:* Nucleotide sequence data reported in this paper is available in the GenBank™ database under the accession number EU287914.

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initial steps of mammalian host infection, the excystment of metacercariae and invasion through the intestinal wall as suggested by previous reports [7–9].

The NEJ cathepsins L are encoded by an early divergent clade of genes bearing substitutions in relevant positions of their active site, and may have consequently different enzymatic properties than the predominant counterparts in the adult flukes [5,6]. Secreted protein profiles and proteinase activities showed subtle changes *in vitro* during the first 48 h after excystment, with prevalence of cathepsin L-like activity. The predominant cathepsin found in the early NEJ excretion/secretion products (E/S) by proteomic analysis is proFhCL3, progressively changing to the mature enzyme during the first 48 h of NEJ development. Noticeably, at this stage the hydrolysis of an oligopeptide substrate with Pro in the P2 position is more efficient than the more typical cathepsin substrates bearing Phe or Leu in that position [6].

The property of hydrolyzing substrates with Pro in P2 is shared with mammalian cathepsin K, an enzyme involved in bone resorption by osteoclasts [10]. The vertebrate enzyme has the unusual ability of cleaving collagen at the Gly-Pro-Xaa repeat sequence (where Xaa is frequently hydroxyproline). A Tyr residue at position 67 within the S2 subsite of cathepsin K has been indicated as the principal responsible for this activity through stabilizing interactions with the Pro residue in the substrate [11]. Besides cathepsin K, only few other papain-like proteases have collagenolytic activity. Three ginger rizhome cysteine proteases (CP-II or zingipain, GP2 and GP3) have a marked preference for substrates with Pro in the P2 position, a fact that has been related to size exclusion due to the occurrence of a bulky tryptophan in the same position occupied by Tyr in cathepsin K [12,13]. The other C1A protease capable of collagen cleavage is FhCL2, secreted by the adult stages of *F. hepatica*. This enzyme has the same residues as mammalian cathepsin K at relevant positions in the S2 subsite, being a clear example on evolutive constrains [14]. While a collagenolytic activity in adult liver flukes might be relevant for diverse functions, the presence of a similar activity in early invasive stages is essential for the establishment of the parasite in the host.

As the initial stages of invasion are the main objectives of the protective immune responses, and the fluke pathology is mainly due to liver erosion during juvenile fluke migration, FhCL3 may be a relevant target for vaccine and chemotherapy design [15]. In order to further characterize this enzyme, we functionally cloned a cDNA encoding FhCL3, and characterized the recombinant enzyme. Tested with synthetic substrates the enzyme showed a marked preference for Pro residues in the selectivity S2 pocket of the active site. Accordingly, we demonstrated efficient collagenase activity, consistent with a role of FhCL3 in the tissue invasion process. Molecular modeling of the active site structure of cathepsins with collagenase activity compared to more typical C1A enzymes allowed the discussion of possible evolutive restrictions acting on enzyme function.

2. Materials and methods

2.1. Expression and purification of FhCL3 in the yeast Hansenula polymorpha

The FhCL3 cDNA fragment comprising most of the proenzyme coding sequence and the 3′ UTR was amplified by PCR from NEJ cDNA using the forward primer 5′-GCGGA**TCCAATGATGTGTCA-TGGCACGAG**TGGAAGCG-3′ (*Bam* HI site underlined; first 8 residues added to the propeptide in bold) and a *Sal* I adapter oligodT reverse primer (5′-GGCCACGCGTCGACAGTACTTTTTTTTTTTTTTTV-3′, *Sal* I site underlined). The resulting fragment was cloned into pCR TOPO4 vector (Invitrogen). After total digestion with *Bam* HI and *Sal* I the fragment of the expected size was inserted in frame with the yeast α-factor into the X4-Mfα-ScPas3 vector for expression and secretion in *H. polymorpha* [16]. FhCL1 was sub-cloned by amplification from a pre-existing adult cDNA clone using primers 5′-TAC GGA TCC AAT GAY GAT TTG TGG CAT-3′ forward and 5′-CGA TTT CCG TGA TAA GCT TCT GTC GAC TCA-3′ reverse (*Bam* HI and *Sal* I cloning sites underlined), and expressed in the same system. Vector and yeast strains were kindly provided by Dr. R.J.S. Baerends and Dr. J.A.K.W. Kiel, Molecular Cell Biology Lab, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands. Plasmid were linearized with *Stu* I to transform the yeast strain NCYC 495 (*leu* 1.1) by electroporation and the plasmid was integrated into the yeast genome as previously described [17]. Yeast transformants were cultured in 50 ml of yeast rich medium (YEPD) to an OD_{600} of 5–6. Cells were harvested by centrifugation at 2000 × *g* for 10 min, and resuspended in buffered minimal media (0.67% yeast nitrogen base; 0.1 M phosphate buffer pH 6.0; 1% methanol) for induction at an initial OD_{600} of 1. Cultures were maintained at 37 °C with orbital agitation until an OD_{600} of 6–10 was reached (approximately 48 h incubation). After cell harvesting the supernatant was concentrated 20–30-fold in an Amicon concentrator with an ultrafiltration membrane of 10 kDa cut-off. Cathepsin enriched fractions were obtained by anion exchange chromatography (HiTrap QXL 5 ml GE Healthcare) from concentrated supernatants dialyzed against buffer Tris–HCl 0.1 M pH 7. Briefly, the column was equilibrated with Tris–HCl 0.1 M pH 7, and bound enzyme was eluted in a 0 to 0.5 M NaCl gradient. Elution fractions were assayed for FhCL3 activity using the fluorogenic peptide Tos-GPR-AMC and analyzed by SDS-PAGE electrophoresis. The FhCL3 and FhCL1 gel bands, migrating with an apparent molecular weight of 26–27 kDa were analyzed by tryptic digestion and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) in a Voyager-DE-PRO mass spectrometer (Perspective Biosystems, Framingham, MA, Biochemical and Analytical Proteomic Unit, Institute Pasteur Montevideo) and verified to correspond to the mature FhCL1 and FhCL3, respectively. Purified enzyme was quantified by the BCA method [18].

2.2. Enzymatic assays

Enzyme concentration was determined by active-site titration with E-64c. Kinetic parameters were determined in reaction buffer containing 50 mM sodium phosphate, pH 6.5, 1 mM dithiothreitol, 1 mM EDTA at 24 °C, typically final enzyme concentrations were in the 10^{-9} M range; and the substrate was added after 10 min of incubation of enzyme in reaction buffer. Proteolytic activity was monitored by the hydrolysis of 7-amino-4-methyl coumarin (AMC) from the synthetic peptide substrates Z-FR-AMC, Z-VLK-AMC, and Tos-GPR-AMC. Reaction rates with different substrate concentrations were measured as the slope of the progress curves obtained by continuous recording in a FluoStar spectrofluorimeter at 345 excitation and 440 emission wavelengths, using an AMC standard curve for product concentration calculation. Kinetic constants, k_{cat} and K_M, were estimated by non-linear regression analysis of the Michaelis–Menten plot. For pH activity profile, buffers pH 3.0–5.5 were prepared by mixing 0.5 M citric acid and 0.5 M sodium citrate; those at pH 6.0–9.0 were prepared by mixing 0.5 M Na_2HPO_4 and 0.5 M NaH_2PO_4. The stability assay was performed by incubation of 0.1 μM FhCL3 in buffer Tris–HCl 0.1 M pH 7 with 0.2 M NaCl in a water bath at 37 °C for different lengths of time. Residual activities were calculated as percentages of the activity of 0.1 μM FhCL3 without incubation on duplicated experiments.

2.3. Digestion of type I collagen and immunoglobulin G1

Protein digestion was analyzed by incubating 10 μg type 1 rat tail collagen (kindly provided by Dr. S. Chifflet, Dpto Bioquímica,