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Type I cystatin (stefin) is a major component of *Fasciola gigantica* excretion/secretion product☆

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ABSTRACT

In the present study we describe type 1 cystatin, a cysteine protease inhibitor, as a major released antigen of the tropical liver fluke *Fasciola gigantica* (FgStefin-1). Immunohistochemical analysis showed that FgStefin-1 is abundant in (a) tissue of tegumental type, including oral and ventral sucker, pharynx, genital atrium, metraterm, cirrus and (b) the intestinal epithelium. Faint staining was observed in the epithelia of ovary and proximal uterus. Immunoblots showed the presence of FgStefin-1 in the parasite's excretion/secretion (ES) product and immunodepletion demonstrated that FgStefin-1 herein is partially complexed with cathepsin L. Furthermore, quantitation of FgStefin-1 in comparison to cathepsin L in ES product and crude worm extract of adults supports a major external function of FgStefin-1 with an estimated 50% being released in at least equimolar amounts to cathepsin L. Sera of an experimentally infected rabbit reacted with recombinant FgStefin-1 starting 8 weeks postinfection. Activity analyses of recombinant FgStefin-1 showed nanomolar inhibition constants for mammalian cathepsin B, L, and S cysteine proteases and released cysteine proteases of the parasite. The protein is active over a wide pH range and is heat stable. Our results suggest protective functions of FgStefin-1, regulating intracellular cysteine protease activity, and possibly protection against extracellular proteolytic damage to the parasite's intestinal and tegumental surface proteins. Considering inhibition kinetics and previously demonstrated immunomodulatory properties of cystatin in parasitic nematodes a comparable function of FgStefin-1 is suggested and is at present under investigation.

1. Introduction

Cathepsin B and L cysteine proteases are established as important and abundantly produced antigens in the trematode genus *Fasciola* and have been implicated in parasite nutrition, protection, and host invasion (for a recent review see [1]). Expression pattern and biological function of their counterparts, cysteine protease inhibitors of the cystatin family (MEROPS [2] inhibitor family I25, clan IH) are at present less well researched in *Fasciola* and in trematodes in general. Cystatins were early on classified into a superfamily containing three distinct families, type 1: stefins, single-domain cytoplasmic cystatins, type 2: cystatins, single-domain secreted cystatins containing a C-terminal disulphide bond, and type 3: kininogens, multi-domain cystatins [3]. The first human stefin was purified from polymorphonuclear granulocytes and characterized as an inhibitor of cysteine proteases [4]. In trematodes sequence and inhibitory activity of a homologous protein from *Schistosoma mansoni* was described by Morales et al., [5] and Khaznadji et al., [6] characterized sequence, temporal expression, and inhibitory activity of a multi-domain cystatin from *F. hepatica*. At present, neither immunohistochemical data demonstrating the distribution of cystatin in parasite tissues nor data regarding possible effects of trematode cystatins on the host immune system exist. In contrast, substantial immunomodulatory effects of type 2 cystatins from parasitic nematodes have been demonstrated in the last years [7–14]. They were found to decrease T cell responses by inhibition of proteases participating in MHC class II antigen processing and presentation and to increase interleukin 10 and nitric oxide production (reviewed in [15]). While nematode genomes contain multicopy cathepsin B and L gene families which are differentially expressed during the development a possible function of cystatin in inhibition of these cysteine proteases has not been studied yet [16–19]. In addition to parasitic helminths cystatins have also been investigated in arthropod pests. Human cystatin A was found to interfere with allergenic cysteine proteases of the house dust mite [20] and cystatins released by blood feeding ticks were shown to have a

☆ *Note*: Nucleotide sequence data reported in this paper is available in the EMBL, GenBank and DDJB databases under the accession number FJ827152.

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function in feeding as well as antiinflammatory and immunosuppressive activity [21,22].

In the present study we have molecularly characterized a *F. gigantica* cystatin homologous to the schistosomal protein described by Morales et al., [5]. Analyses were conducted to provide the first data on this protein in the genus *Fasciola* and to add to the information available from *Schistosoma*. This includes the inhibition properties against mammalian and endogenous cysteine proteases and stability of the protein. Tissue-specific distribution and release as an abundant ES antigen are demonstrated and quantitatively analyzed in comparison to cathepsin L, the major released cysteine protease in the adult parasite. This work will lead to a better understanding of the biological function of type 1 cystatin in *Fasciola* and trematodes in general and in the future we will perform additional analyses to investigate whether FgStefin-1 has immunomodulatory properties as has been demonstrated for nematode cystatins.

2. Materials and methods

2.1. Parasites

Adult *F. gigantica* were freshly collected from bile ducts and gall bladders of naturally infected cattle killed at a local slaughterhouse. Metacercariae were obtained from naturally infected snails (*Radix* spp.). Juvenile parasites were obtained from ICR mice orally infected with metacercariae. They were collected from the liver parenchyma 2, 4, and 6 weeks postinfection. Serum samples of the infected mice were collected preinfection and weekly postinfection. Parasites not used immediately were washed in 0.85% normal saline solution and kept frozen in liquid nitrogen until processed in further experiments.

2.2. Nucleic acids

Total RNA from 2-, 4-, and 6-week-old juvenile and adult *F. gigantica* as well as genomic DNA from adult *F. gigantica* were prepared by standard methods as described previously [23] and stored at −20 °C until use.

2.3. Molecular cloning and sequence analysis

A partial 333 bp FgStefin-1 cDNA was amplified by PCR from an adult stage *F. gigantica* cDNA library [24] using primers (forward 5′-AATCACTAAAAATGATGTGC-3′, reverse 5′-ATAGGAGTACCGGTCATG-3′) based on an EST sequence from *F. hepatica* (http://www.sanger.ac.uk/Projects/Helminths/). The cDNA fragment was used as a DIG-labelled probe (PCR DIG Labeling Mix, Roche) to isolate full length cDNAs from *F. gigantica* adult and metacercarial stage cDNA libraries by standard screening methods. Their nucleotide sequences where determined using the services of Pacific Science Ltd., Thailand and NSTDA Bioservice Unit, Thailand. Sequence analyses were performed using EMBOSS 6.0.1 [25], SignalP 3.0 [26], and the NCBI-BLAST server (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search for and obtain homologous type 1 cystatin sequences. ClustalW 2.0.1 [27] was used to calculate a multiple alignment starting with 94 type 1 cystatin sequences. Sequence parts extending at the N- and C-termini beyond the FgStefin-1 sequence were deleted and the aligned sequences filtered to remove sequences that shared more than 90% identity. The remaining 53 sequences were realigned and used to create a sequence logo [28] representative for type 1 cystatin in the animal kingdom. The alignment itself is available as supplemental data (Fig. S1) and contains accession numbers and species information.

2.4. Southern, Northern and reverse transcriptase-PCR analyses

For Southern analysis, 10 μg each of genomic DNA was digested with restriction endonucleases (Fermentas Life Sciences) PstI, SalI, or PstI/SalI and size-separated by electrophoresis in a 0.7% agarose gel in TBE buffer. Lambda DNA digested with EcoRI and HindIII was used as a size standard. In Northern analysis, 30 μg of total RNA from adults was size-separated in a 1.2% agarose gel containing 6.5% formaldehyde in 1× MOPS buffer. A high range RNA molecular weight marker (Fermentas Life Sciences) was used to determine sizes of hybridizing RNAs. DNA and RNA were blotted to Nylon membranes (Hybond-N Plus, Amersham Biosciences) and immobilized by baking at 80 °C for 1 h. Hybridization of filter-bound nucleic acids was done at 50 °C (DNA) or 55 °C (RNA) in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, 0.1 mg/ml herring sperm DNA for 15 h with DIG-labeled FgStefin-1 DNA probes generated by PCR (PCR DIG Labeling Mix, Roche). Enzymatic detection by alkaline phosphatase was performed according to the instruction manual (DIG Detection Kit, Roche). To perform RT-PCR total RNA from 2-, 4-, 6-week-old juveniles and adult *F. gigantica* was extracted in TRIzol reagent (Invitrogen). The extracted RNA (1 μg) was treated with DNase (Promega) at 37 °C for 30 min and the DNase was then inactivated by heating at 65 °C for 10 min in the presence of 20 mM EGTA. The DNase treated total RNA was reverse transcribed by RevertAid™ M-MuLV reverse transcriptase (Fermentas Life Sciences) using an oligo$(dT)_{18}$ primer for 1 h at 42 °C. The RT-product was used to amplify a 333 bp DNA fragment of FgStefin-1 by a standard PCR setup (30 cycles at 94 °C, 45 °C, 72 °C, 1 min each step) with the primers as detailed in Section 2.3. The RT-PCR products were size-separated in a 1% agarose gel.

2.5. Expression of recombinant FgStefin-1 in E. coli and production of polyclonal anti-FgStefin-1 antisera

The protein encoding fragment of the FgStefin-1 cDNA was inserted into the pQE30 vector (QIAGEN) using BamHI and PstI restriction sites. These recognition sites were introduced by PCR using forward primer 5′-GGA TCC atc gag ggt cgc ATG ATG TGC GGC GGC TGC-3′ and reverse primer 5′-CTG CAG GAG TACCGA TCA TGA TC-3′. The forward primer introduced in addition a factor Xa cleavage site (lower case characters). *E. coli* M15 was transformed with pQE30-FgStefin-1 and the expression of recombinant protein was induced by addition of IPTG (1 mM final concentration). Soluble recombinant protein was purified by Ni-NTA affinity-chromatography under native conditions following protocols 9 and 12 as outlined in the QIAExpressionist manual (QIAGEN). Following purification, the N-terminal His-tag was removed by cleavage with factor Xa (MoBiTec) according to the supplied instructions and subsequent binding to Ni-NTA sepharose. Factor Xa was removed by affinity capture on Xarrest™ Agarose (Novagen) and rFgStefin-1 was finally dialyzed against PBS, pH 7.2 and stored at −20 °C for use in further studies. Anti-sera against rFgStefin-1 were prepared by intraperitoneal immunization of ICR mice three times in 3-week intervals with 10 μg of purified rFgStefin-1 emulsified in complete Freund's adjuvant for the first immunization and in incomplete Freund's adjuvant for the following immunizations. Preimmune and immunized sera were collected for further immunological studies.

2.6. Parasite antigen preparation

Excretory/secretory (ES) product was prepared from freshly collected adult parasites. The parasites were washed, preincubated in PBS, pH 7.2 for 1 h at 37 °C, and transferred into fresh PBS for 1 h at 37 °C. Insoluble material and eggs in the buffer were removed by centrifugation at 5000 × *g* and 4 °C for 20 min. The ES product in the supernatant was concentrated using Amicon Ultra centrifugal fil-