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Identification and functional characterization of CsStefin-1, a cysteine protease inhibitor of *Clonorchis sinensis*☆

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ABSTRACT

Cathepsin Fs of *Clonorchis sinensis* (CsCFs) are major secreted proteins that are expressed in the intestine of the parasite and play pivotal roles in parasite nutrition and host–parasite interactions. However, strict regulation of their activities is also essential to minimize inadequate superfluous damage to the parasite and host. In this study, we identified and characterized a novel cysteine protease inhibitor of *C. sinensis*, CsStefin-1, as a modulator of CsCFs. CsStefin-1 was shown to be a typical cysteine protease inhibitor of family 1 cystatins that lacks the N-terminal signal peptide and C-terminal cysteine residues required for disulfide bond formation. Phylogenetic and structural analyses also showed that CsStefin-1 is a family 1 intracellular cystatin. Bacterially expressed CsStefin-1 effectively inhibited various cysteine proteases, including human cathepsin B, human cathepsin L, papain, and CsCFs. CsStefin-1 was active over a wide pH range and was highly stable under physiological conditions. CsStefin-1 also inhibited the processing of CsCFs. CsStefin-1 was expressed throughout various developmental stages of the parasite from metacercaria to adult worm and the protein was detected in worm extract, but not in the excretory and secretory products of adult worm. Immunolocalization analysis showed that CsStefin-1 was mainly localized to the intestinal epithelium, where CsCFs are actively synthesized. Our results collectively suggest the regulatory functions of CsStefin-1, modulation of CsCFs activity and processing, to protect the parasite from superfluous damage by the endogenous cysteine proteases.

1. Introduction

Clonorchis sinensis is a liver fluke that causes clonorchiasis in humans. It is prevalent in Far East Asian countries including China, Korea, Japan, and Taiwan and is estimated to have infected 35 million people worldwide [1]. Humans are usually infected with the parasite by ingesting raw or undercooked fresh water fish that contain metacercariae. Metacercariae are excysted in the duodenum, whereupon they migrate into the bile duct and mature. Adult worms cause mechanical irritation and pathological changes in the epithelium of bile ducts. Heavy, chronic infections might be complicated by several hepatobiliary diseases such as cholangitis, cholelithiasis, and cholangiectasis. A high correlation between *C. sinensis* infection and incidence as well as mortality of cholangiocarcinoma suggests a strong association between the parasite and cholangiocarcinoma [2–5].

Cathepsin Fs of *C. sienesis* (CsCFs) are multigene family enzymes that share similar structural and biochemical properties. They are expressed throughout the various developmental stages of the parasite and their expression levels increased gradually in accordance with maturation [6,7]. CsCFs are apparently synthesized in the epithelial cells lining the intestine of the parasite, after which they are actively secreted into the intestinal lumen [6,7]. Their primary biological function is likely associated with nutrient acquisition of the parasite by hydrolyzing various host proteins, but the fact that CsCFs are eventually secreted by the parasite as major components of the excretory and secretory products (ESP) also suggests extracorporeal roles when outside of the parasite [6,7]. Although CsCFs play pivotal roles in parasite nutrition and host–parasite interactions, strict regulation of their activities is also essential to minimize inadequate superfluous damage to the parasite and host. Such regulation is generally achieved in part by a diverse array of endogenous protease inhibitors [8,9]. In this regard, characterization of a counterpart regulator of CsCFs is important for in-depth understanding of the biology and pathogenesis of CsCFs.

Cystatins are protein inhibitors of cysteine protease that ubiquitously present in a wide range of organisms. They participate in a diverse amount of important physiological roles, including protection of cells/organisms from inappropriate endogenous or external

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proteolysis and regulation of intracellular or extracellular protein breakdown. Cystatins are classified into three major families based on primary structure: stefins (family 1), cystatins (family 2), and kininogens (family 3) [10–13]. Stefins are generally intracellular, non-glycosylated, single-domain proteins that lack disulfide bonds, whereas cystatins are predominantly extracellular proteins that possess two disulfide bonds at the C-terminus and may be glycosylated and/or phosphorylated. Both family 1 and family 2 inhibitors are low molecular weight proteins of 10–15 kDa. Kininogens are larger intravascular proteins consisting of multi-domain cystatins.

In this study, we identified a novel cysteine protease inhibitor of *C. sinensis*, named CsStefin-1, and characterized its biochemical and functional properties. CsStefin-1 was a typical intracellular cystatin of family 1 that effectively inhibited various cysteine proteases, including CsCFs. CsStefin-1 was expressed throughout the various developmental stages of *C. sinensis* and was mainly localized to the intestinal epithelial cells of the parasite. Our results collectively suggest that the regulatory functions of CsStefin-1, modulating activity and processing of CsCFs, to protect the parasite from inadequate superfluous damages due to the endogenous cysteine proteases.

2. Materials and methods

2.1. Parasite

C. sinensis metacercariae were collected from naturally infected *Pseudorasbora parva* obtained from Korea. Rats were experimentally infected by oral administration of 100 metacercariae and were sacrificed 6 weeks post-infection. Adult worms were collected from bile ducts and then washed five times with cold physiological saline to remove any contamination of hosts. The collected worms were used immediately for RNA preparation or stored at −70 °C until use.

2.2. Identification and cloning of the gene encoding a cysteine protease inhibitor of C. sinensis (CsStefin-1)

The nucleotide sequence of CsStefin-1 was identified during expressed sequence tags (EST) analysis of the cDNA library of *C. sinensis* adult worms. The nucleotide sequences of clones randomly selected from the *C. sinensis* cDNA library were determined using the universal T7 promoter primer [7]. The homology patterns of the ESTs were analyzed against the non-redundant database using the BLASTX program at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). Five clones, all of which contained a characteristic cystatin motif and showed high levels (up to 50%) of sequence identity with presently known cystatins of helminth parasites, including *Schistosoma mansoni* (EU014295) and *Fasciola gigantica* (AY815084), were identified and selected for further sequencing analysis using the T3 promoter primer. Finally, a full-length gene sequence encoding CsStefin-1 was identified. To clone the full-length gene of CsStefin-1, the mRNA of *C. sinensis* was isolated from adult worms using an Oligotex mRNA purification kit (Qiagen, Valencia, CA, USA). Single-stranded cDNA was synthesized using a BD SMART™ RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. The full-length gene of CsStefin-1 was then amplified from the cDNA by polymerase chain reaction (PCR) using oligonucleotide primers flanking the open reading frame (ORF) of the gene. The forward primer was 5′-ATGCCAATATGCGGTGGCATTAGTGCT-3′ and the reverse primer was 5′-TCAAAAATAATCCAACGGATCTGTGTC-3′. The amplification reaction was performed using the following thermal cycling profile: 94 °C for 4 min, 30 cycles at 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min, followed by a 72 °C extension for 10 min. The PCR product was analyzed on a 1.5% agarose gel, gel-purified and ligated into the T&A Cloning vector (Real Biotech Corporation, Banqiaa City, Taiwan). The ligation mixture was transformed into *Escherichia coli* DH5α competent cells, and positive clones were screened by PCR to verify the presence of plasmids containing the appropriate insert. The nucleotide sequence of the insert was determined by automated sequencing. Analysis of primary structures of the deduced amino acid sequence was conducted with DNASTAR (DNASTAR, Madison, WI, USA), PSORT (http://www.psort.org/) and Signal P (http://www.cbs.dtu.dk/services/SignalP/). In order to clone the genomic sequence of CsStefin-1, genomic DNA was extracted from *C. sinensis* adult worms using a Genomic DNA Purification Kit (Real Biotech Corporation). The DNA was then PCR amplified using a specific primer set for CsStefin-1 (5′-ATGCCAATATGCGGTGGCATTAGTGCT-3′ and 5′-TCAAAAATAATCCAACGGATCTGTGTC-3′). The PCR product was gel-purified, cloned into the T&A Cloning vector (Real Biotech Corporation), and transformed into *E. coli* DH5α. The nucleotide sequence was determined from both strands as described above.

2.3. Phylogenetic and homology modeling analyses

The phylogenetic tree was constructed using the neighbor-joining method with MEGA 4 (http://www.megasoftware.net). Bootstrap proportions were used to assess the robustness of the tree with 1000 bootstrap replications. Three-dimensional homology modeling of CsStefin-1 was performed using the Modeller (http://salilab.org/modeller/) based on the crystal structure of human stefin B (pdb: **1STF**). Subsequent analysis, visualization, and preparation of the model were performed using UCSF Chimera (http://www.cgl.ucsf.edu/chimera/).

2.4. Expression and purification of recombinant CsStefin-1

The full-length gene encoding CsStefin-1 was amplified by PCR using the following primers: 5′-GGATCCATGCCAATATGCGGTGGCATT-3′ containing a 5′ *Bam* HI site and 5′-CTGCAGTCAAAAATAATCCAACGGATC-3′ containing a 5′ *Pst* I site. The purified PCR product was ligated into the T&A Cloning vector (Real Biotech Corporation) followed by transformation into *E. coli* DH5α. The resulting plasmid DNA was then digested with the appropriate restriction enzymes, ligated into the pQE-30 expression vector (Qiagen), and then transformed into *E. coli* M15 [pREP4] cells (Qiagen). Bacteria were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37 °C for 3 h. The cells were collected, suspended in native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), sonicated on ice, and then centrifuged at 4 °C for 20 min at 12,000 × *g*. The recombinant protein was purified by nickel–nitrilotriacetic acid (Ni–NTA) chromatography (Qiagen) by following the manufacturer's instructions. The purification and purity of the recombinant protein was determined by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.5. Production of antisera for CsStefin-1 (anti-CsStefin-1)

For production of anti-CsStefin-1, balb/c mice were immunized with purified CsStefin-1 (50 μg) three times every 2 weeks. The antigen was emulsified with equal volumes of Freund's complete adjuvant (Sigma) for the first immunization or Freund's incomplete adjuvant (Sigma) for the following two boostings. Two weeks after the final boosting, the mice were sacrificed and the sera were collected. The specificity of anti-CsStefin-1 was confirmed by immunoblot analysis and used in this study.