Acta Tropica 116 (2010) 134–140

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Identification and characterization of a serine protease inhibitor of *Clonorchis sinensis*

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ARTICLE INFO

Article history:
Received 12 April 2010
Received in revised form 23 June 2010
Accepted 26 June 2010
Available online 9 July 2010

Keywords:
Clonorchis sinensis
Serine protease inhibitor
Serine protease
Intracellular serpin
Recombinant protein

ABSTRACT

A gene encoding a serine protease inhibitor of *Clonorchis sinensis* (CsSERPIN) was identified and characterized. CsSERPIN contained an open reading frame of 1158 bp that encoded 385 amino acid residues. Sequence analysis of the primary structure of CsSERPIN revealed that it had essential structural motifs including a reactive central loop (RCL), which well conserved in the serine protease inhibitor (serpin) superfamily. CsSERPIN was classified as a member of the ovalbumin-type serpin family on the basis of phylogenetic analysis and the absence of a classical N-terminal signal peptide. Recombinant CsSERPIN showed an inhibitory effect on chymotrypsin in a dose-dependent manner, but did not effectively inhibit trypsin, thrombin, elastases or cathepsin G. Optimal pH values of CsSERPIN were between 7.0 and 9.0, as evidenced by the rapid loss of inhibitory activity under acidic conditions. CsSERPIN was expressed at various developmental stages of the parasite, from eggs to adult worms, but its expression level was higher in eggs and adult worms than in metacercariae and juvenile worms. CsSERPIN was identified in the soluble extract of the parasite, but not in the excretory and secretory products (ESP) or insoluble extract of the parasite. Immunolocalization analysis of CsSERPIN showed that it mainly localized to the eggs and vitelline glands of the adult worm. These results suggest that intracellular CsSERPIN may be possibly involved in maintaining the physiology of eggs as well as in egg production of *C. sinensis* by regulating endogenous serine proteases.

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1. Introduction

Serine protease inhibitors (serpins) are members of a superfamily of proteins which had a characteristic well-conserved tertiary structure and found in a variety of organisms. Serpins are involved in a number of fundamental biological events, such as blood coagulation, fibrinolysis, angiogenesis, programmed cell death, development and inflammatory response (Ye and Goldsmith, 2001; Gettings, 2002; van Gent et al., 2003). Serpins were also identified in helminth parasites. The microfilariae of *Brugia malayi* produce a serpin (Bm-SNP-2) that specifically inhibits cathepsin G and elastase secreted by neutrophils (Zang et al., 1999; Maizels et al., 2001). This suggests that the functional role of Bm-SPN-2 is the neutralization of the immuno-stimulatory properties of cathepsin G, and thus Bm-SPN-2 contributes to the longevity of the parasite in the bloodstream. A serpin from *Onchocerca volvulus* (Ov-SPI-1) is likely to be involved in multifunctional biological processes such as molting, embryogenesis and spermatogenesis (Ford et al., 2005). A serpin of *Schistosoma haematobium*, which anchors in the membrane, has antitrypsin activity. The complex formed between the membrane-embedded serpin and human trypsin allows the parasite to reduce the immunogenicity of exposed serpin, thereby increasing its ability to evade host immune defenses (Huang et al., 1999). Immunization of *S. japonicum* serpin, which preferentially located in the tegument of adult worms, develops moderate protection against experimental infection in mice (Yan et al., 2005). An intracellular serpin of *Paragonimus westermani* effectively inhibits trypsin, chymotrypsin and thrombin and probably plays primary roles in regulating intracellular serine protease activity in the parasite (Hwang et al., 2009). These results collectively suggest that serpins of helminth parasites play important roles not only in parasite physiology but also in interactions with their hosts.

Clonorchis sinensis is a liver fluke that causes clonorchiasis in humans. Humans are usually infected with the parasite by eating raw or undercooked fish containing metacercariae. The metacercariae are eventually excysted in the duodenum whereupon they migrate into the bile duct and mature. The adult worms cause mechanical irritation and pathological changes in the epithelium

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doi:10.1016/j.actatropica.2010.06.007

of bile ducts. Heavy, long-standing infections can cause several hepatobiliary diseases including cholangitis, cholelithiasis, and cholangiectasis. A high correlation between *C. sinensis* infection and incidence as well as mortality of cholangiocarcinoma strongly suggests the parasite is associated with cholangiocarcinoma (Choi et al., 2004; Choi et al., 2006; Lim et al., 2006; Bouvard et al., 2009; Vennervald and Polman, 2009). Therefore, an in-depth understanding of the biology and pathogenesis of the parasite as well as that of host-parasite interactions is important. A serpin of *C. sinensis* (CsproSERPIN), which highly expressed in metacercaia of the parasite, has identified recently (Yang et al., 2009), but its biochemical property and biological function were not determined. In this study, we identified a novel gene encoding the second serpin of *C. sinensis* (CsSERPIN) and characterized the biochemical and functional properties of the protein.

2. Materials and methods

2.1. Parasite

C. sinensis metacercariae were collected from naturally infected *Pseudorasbora parva* obtained from Korea. Rats were infected by oral feeding with 100 metacercariae. The rats were sacrificed 6 weeks after experimental infection. Worms were collected from livers and washed five times with cold physiological saline to remove any contamination from the hosts. The collected parasites were stored at −70 °C until use or used immediately for RNA preparation.

2.2. Identification and cloning of the gene encoding CsSERPIN

The nucleotide sequence of CsSERPIN 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. 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). Seven clones, all of which had characteristic serpin motif and showed high levels (up to 32%) of sequence identity with presently known serpins including *P. westermani* (EU014295) and *S. japonicum* (AY815084), were identified and selected for further sequencing analysis using the T3 promoter primer. Finally, a full-length gene sequence encoding a CsSERPIN was identified. For cloning of the full-length gene of CsSERPIN, 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 the BD SMART™ RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. The full-length gene of CsSERPIN was amplified from the cDNA by polymerase chain reaction (PCR) using oligonucleotide primers flanking the open reading frame of the gene. The forward primer was 5′-ATGGCAAACCAGCTGGAGATCTACACTAGT-3′ and the reverse primer was 5′-TCAATTTTGCTCAGGCTCGACCACGTGACC-3′. The amplification reaction was performed using a thermal cycling profile of 94 °C for 4 min, 30 cycles at 94 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min followed by a 72 °C extension for 10 min. The PCR product was analyzed on a 1.2% agarose gel, gel-purified and ligated into the pGEM-T Easy vector (Promega). The ligation mixture was transformed into *Escherichia coli* DH5α competent cells, and positive clones were screened for the presence of plasmid with 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/). 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.

2.3. Expression and purification of recombinant CsSERPIN

To express the recombinant protein, the full-length gene of CsSERPIN was amplified using the following primers: 5′-GGATCCATGGCAAACCAGCTGGAGATC-3′, which contains a 5′ *Bam* HI site, and 5′-GTCGACTCAATTTTGCTCAGGCTCGAC-3′, which harbors a 5′ *Sal* I site. The purified PCR product was ligated into the pGEM-T Easy vector (Promega) followed by transformation into *E. coli* DH5α. The resulting plasmid DNA was digested with the appropriate restriction enzymes, ligated into the pQE-30 expression vector (Qiagen), and then transformed into *E. coli* M15 [pREP4] cells (Qiagen). Selected clones were grown and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). The bacteria were then suspended in native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), sonicated on ice and centrifuged at 4 °C for 20 min at 12,000 × *g*. The recombinant protein was purified by nickel-nitrilotriacetic acid (Ni-NTA) chromatography (Qiagen). The purification and purity of the recombinant protein was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.4. Production of polyclonal antisera for CsSERPIN (anti-CsSERPIN)

For production of polyclonal antisera for CsSERPIN, balb/c mice were immunized with purified CsSERPIN (50 μg) three times at 2-week intervals. Two weeks after the final boosting, the mice were sacrificed and the sera were collected. The specificity of the anti-CsSERPIN was tested by immunoblot analysis.

2.5. Inhibitory activity assay

The inhibitory effect of CsSERPIN on several serine proteases, trypsin from porcine pancreas (Sigma), chymotrypsin from bovine pancreas (Sigma), elastase from human leukocytes (Sigma), elastase from porcine pancreas (Sigma), thrombin from human plasma (Sigma), and cathepsin G from human leukocytes (Sigma), was determined by analyzing residual proteolytic activity after incubation of each enzyme with the CsSERPIN as previously described (Hwang et al., 2009). In brief, each individual serine protease (10 nM) was incubated with the same concentration of CsSERPIN or bovine serum albumin (negative control) in 50 mM phosphate-buffered saline (PBS, pH 7.4). After 20 min of incubation at room temperature, the substrate solution was added to the mixture, and the residual enzyme activity was measured by monitoring the release of fluorescence (excitation at 355 nm, emission at 460 nm) over 20 min at room temperature with a Fluoroskan Ascent FL (Thermo, Vantaa, Finland). The molar ratio was also calculated by taking into account the molecular weights of the tested enzymes and CsSERPIN. The substrate and incubation buffer for each enzyme were as follows. Porcine trypsin and human thrombin: 50 nM Boc-Glu-Ala-Arg-MCA, 50 mM Tris–HCl, 100 mM NaCl (pH 8.0); bovine chymotrypsin and human cathepsin G: 50 nM Suc-Ala-Ala-Pro-Phe-MCA, 100 mM Tris–HCl, 10 mM $CaCl_2$ (pH 7.4); human and porcine elastases: 50 nM Suc-Ala-Ala-Ala-MCA, 50 mM Tris–HCl (pH 7.4). All substrates were purchased from Peptide International (Osaka, Japan).

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