Parasitology International 67 (2018) 415–424

Contents lists available at ScienceDirect

Parasitology International

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

Isolation and characterization of a novel serine protease inhibitor, SjSPI, from *Schistosoma japonicum*

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ARTICLE INFO

Keywords:
Schistosoma japonicum
Serine proteinase inhibitor
Exon-intron structure
Homology modeling
Chymotrypsin

ABSTRACT

Serine proteinase inhibitor (Serpin, SPI) is a vital superfamily of endogenous inhibitors that monitor proteolytic events active in a number of biological functions. In this study, we isolated a full length gene encoding a novel serine protease inhibitor of *Schistosoma japonicum* (*SjSPI*) and characterized its molecular properties. Our result showed that *SjSPI* contained an open reading frame of 1,218 bp, which encoded 405 amino acid residues. Chromosomal structure analysis showed that *SjSPI* gene was comprised of six exons separated by five introns. It had essential structural motifs which were well conserved among the Serpin superfamily and showed 17-33% sequence identities with Serpins from other helminthic parasites. Trematode Serpin diverged separately into two different subclades and that the SjSPI clustered Subclade I. Exon-intron structures of trematode Serpins were highly conserved, closely with cestode Serpins. No signal peptide but a strongly transmembrane domain was predicted to exist in SjSPI, suggesting that the protein might be a soluble membrane-associated protein. Homology modeling predicted in silico confirmed that the SjSPI structure also belonged to the Serpin superfamily, containing nine α-helices and a reactive central loop. The bacterially expressed recombinant GST-SjSPI protein effectively inhibited the activities of chymotrypsin, trypsin and thrombin. Expression of SjSPI was detected throughout various developmental stages of the parasite in host and reached its maximal levels at the adult and egg stages, which suggests that SjSPI may be possibly involved in maintaining the physiology of eggs by regulating endogenous serine proteases.

1. Introduction

Proteases, such as cysteine-, serine-, aspartic- and metallo- protease, play important roles within the life cycle of numerous medical protozoan and helminthic parasites. Parasite proteases might be participated in parasite metabolism between parasite and host interactions, such as host tissue invasion, anticoagulation, parasite nutrition, and evasion of host immune responses [1–4]. Besides, strict regulation of these protease activities is also essential to minimize excrescent damages in both the parasite and host. Although the narrow range of substrate specificity will partly minimize the collateral damage which may arises from unnormal protease activity, regulation is also achieved through a different class of protease inhibitors from both host and parasitic origin [5].

Serine protease inhibitors (Serpins) are members of an important protein superfamily of endogenous inhibitors which have key physiological and biological roles. The Serpins were identified in many organisms including plants, animals, bacteria, fungi and viruses with a typically features of about 350-410 amino acid residues. All Serpins have a highly conserved structure of eight or nine α-helices, a Serpin motif, a Serpin signature and a single reactive central loop (RCL) [6–8]. They function as serine protease inhibitors when specific residues (P1-P1′) in the RCL bind with the active site of a protease to form a covalent suicide complex [9]. Up to now, more than 1,500 members are identified within the Serpin superfamily [10]. Sixteen clades (clade a~clade p) were classified by a comprehensive phylogenetic analysis of numerous serpin sequences from Serpin superfamily [11]. The Serpin members from the invertebrate are typically organized in accordance with species diversity (with serpins of schistosome forming clade m, and serpins of nematode forming clade l), while Serpin members from the vertebrate are grouped into diverse clusters [11]. Till now, many proteins of Serpin superfamily from parasitic helminths are found and well characterized: trematodes such as blood fluke, liver fluke and lung fluke; cestodes such as *Echinococcus granulosus*; and nematodes such as *Ascaris lumbricoides* and *Brugia malayi* [12–19].

Schistosomiasis, one of the most widely distributed diseases in the world, especially in developing countries, is still a serious parasitic disease infecting not only human beings but also wild or domestic

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https://doi.org/10.1016/j.parint.2018.04.002
Received 20 October 2017; Received in revised form 2 April 2018; Accepted 7 April 2018
Available online 09 April 2018

animals [20]. It is caused by three major schistosome species: *Schistosoma mansoni*, *S. haematobium* and *S. japonicum* [21–23]. Schistosomiasis resulted from *S. japonicum* is one of the most serious endemic diseases in Asia country. Nearly 1 million people in China are presently afflicted with the disease, and 30 million people are at risk of infection [24]. Paired adult worms reside in mesenteric veins via the hepatic portal system. Several hundreds or even thousands of eggs per day are deposited. Many eggs are trapped in the fine venules of intestine wall, others are carried into the liver by the portal venous system in which they become trapped in the portal triad. The most serious pathologic consequences of schistosomiasis are caused by inflammatory responses around the eggs [25]. Adult schistosome worms survive for several years in the host's mesenteric veins draining the intestines or the vesicular veins draining the urinary bladder, in which the parasites do not stimulate clotting [26]. They are seemingly invisible to the complement system [27,28] and inhibit neutrophil attack [29]. Meanwhile, the worm might be continuously exposed to proteases from both host and worm. In order to manage those protease activities and to minimize superfluous damages, the parasite is equipped with the anti-protease enzyme system, such as Serpins and Cysteine Protease Inhibitors [30]. In *Schistosoma* species, Serpins control the homeostasis of serine proteases in both the parasites themselves and their mammalian hosts [30], which suggests that Serpins of *Schistosoma* parasites play very important roles not only in the parasite physiology but also in the interactions with their hosts.

Parasite-host interaction has been reported in *S. haematobium* (ShSPI) [14]. In *S. mansoni*, one of the Serpins regulates endogenous cercarial elastase instead of host serine proteases [31]. Another *S. mansoni* serpin is located on the tegument of adult males and females, which may indicate its role in inhibiting host proteolytic enzymes [12]. A third *S. mansoni* serpin (SmSPI), which was highly expressed in schistosomules, predominantly in the head gland, and in adult male and female with intensive accumulation on the spines, suggests that it may facilitate intradermal and intravenous survival. The SmSPI specifically inhibits chymotrypsin but not trypsin and elastase [32]. Knowledge of serpins in *S. japonicum* is relatively limited. To date, only two Serpins have been characterized: the Serpin (SjB10) inhibiting human pancreatic elastase in a dose-dependent manner [13]; and another membrane-embedded serpin allowing the parasite to reduce the immunogenicity of its exposed serpin to possibly evade the immune defenses [33]. During our series of BLAST searches, at least 5 serpins have been found in the genome of *S. japonicum*. Other *S. japonicum* Serpins should also be investigated. In this study, a novel gene encoding Serpin from the trematode platyhelminth *S. japonicum* (SjSPI) was isolated, and its sequence properties, including other parasitic helminthes Serpins, were characterized by silico analysis. The bacterially expressed recombinant SjSPI protein effectively inhibited chymotrypsin. The expression pattern suggested that the SjSPI molecule might play primary roles in regulating the activities of cytoplasmic serine proteases of the parasite egg physiology.

2. Materials and methods

2.1. Parasite

Positive *Oncomelania hupensis* snails infected with *S. japonicum* were obtained from the Institute of Parasitic Disease Control and Prevention, Jiangxi Province, China. *S. japonicum* (Chinese mainland strain) was maintained in *O. hupensis*. The 10-week-old specific pathogen free (SPF) female BALB/c mice, which were provided by the Wuhan University A3 Laboratory Animal Center, were infected percutaneously through shaven abdomen with 30 cercariae of *S. japonicum* by an adaptation of the ring method. The mice were killed on the 42th day after infection, adult worms were recovered by perfusion of the hepatic portal veins. Then, adults were washed several times to remove host cells and debris with physiological saline. Some samples were either frozen and kept in liquid nitrogen, or immediately used for nucleic acid extraction. For total RNA isolation, the worms were washed with diethypyrocarbonate (DEPC)–treated 0.9% NaCl solution twice and preserved in TRIZOL Reagent (Invitrogen, NY, CA) at −80 °C. The use of animals was approved by the Animal Ethics Committee of Wuhan University.

2.2. Cloning of partial SjSPI cDNA sequence

The non-redundant GenBank database at National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.njh.gov) was screened with the nucleotide sequence of *S. mansoni* serine protease inhibitor (accession no. CCD74817) used as query in the BLAST searches. A clone from *S. japonicum* database (accession no. AY808321) encoding a partial serine protease inhibitor protein was selected based on BLAST algorithms.

Total RNA of adult *S. japonicum* worms was extracted with Trizol reagents (Invitrogen, NY, USA), and was treated with RNase-free DNase (GIBCO, MD, USA). The first strand cDNAs were synthesized from 1 μg total RNA by the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada) following the manufacture's instruction. Specific primers were designed based on the partial *SjSPI* cDNA sequence. The primer sequences were *SjSPI*-F, 5′-TCATCCTAACCATTTT ACTCAG-3′ and *SjSPI*-R, 5′-GTATTCATCAGTGCTATTCGTG-3′. PCR was performed by using the above specific primers with the first strand cDNA as a template. The PCR was conducted with a thermal cycling profile of 94 °C for 3 min, 35 cycles of 1 min at 94 °C, 1 min at 50 °C, 1 min at 72 °C and a final extension of 10 min at 72 °C. The amplified products were resolved by 2% agarose gel electrophoresis, visualized by ethidium bromide (EtBr) staining, and recovered by using an Agarose Gel DNA Purification Kit (TaKaRa, Shiga, Japan). The purified products were then cloned into the pGEM-T Easy Vector (Promega, Madison, USA), and transformed into the competent *Escherichia coli* DH5α cells. The positive clones were screened and identified by PCR and sequencing.

2.3. Rapid amplification of cDNA ends (RACE)

RACE-PCR procedures were performed with the 3′-Full RACE Kit and 5′-Full RACE Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. The total RNA from adult worms was used as the template for both RACE-PCRs. 3′-RACE and 5′-RACE were performed with gene-specific primers (3′-end outer primer 5′-TTAACTGCTCTGTT GGGATC-3′ and 3′-end inner primer 5′-ACTTCAGAGCGAGTCTTGGT-3′ for 3′-RACE; 5′-end outer primer 5′-GTTACCAAGACTCGCTCTGAAG-3′ and 5′-end inner primer 5′-TAGCCGTGTTTTGACGAATCCC-3′ for 5′-RACE), which were designed based on the partial *SjSPI* sequence. The cycling conditions for 3′-RACE outer/inner PCRs were 94 °C for 3 min, 25/30 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and a final extension of 10 min at 72 °C. The cycling conditions for 5′-RACE outer/inner PCRs were 94 °C for 3 min, 20/25 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and a final extension of 10 min at 72 °C. After the identification by cloning and sequencing, the two specific PCR products were merged into a full *SjSPI* sequence. To amplify the entire putative open reading frame (ORF), specific primers (*SjSPI*-F, 5′-ATGTTTGACG ATCAATTAGTAAT-3′ and *SjSPI*-R, 5′-TTACCAGTAAGAAGTTATTT CGA-3′) were designed with the amplification conditions of an initial denaturation at 94 °C for 3 min followed by 35 cycles of 30 s at 94 °C, 30 s at 47 °C, 90 s at 72 °C and a final extension of 10 min at 72 °C. The amplified product was then cloned into the pGEM-T Easy vector (Promega) for sequencing.

2.4. Determination of SjSPI chromosomal structure

Genomic DNA was isolated from adult *S. japonicum* by the Wizard Genomic DNA Purification Kit (Promega, Madison, USA) following the manufacturer's instruction. The chromosomal segment corresponding