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Short communication

High-level expression and characterization of two serine protease inhibitors from *Trichinella spiralis*

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ABSTRACT

Serine protease inhibitors (SPIs) play important roles in tissue homeostasis, cell survival, development, and host defense. So far, SPIs have been identified from various organisms, such as animals, plants, bacteria, poxviruses, and parasites. In this study, two SPIs (Tsp03044 and TspAd5) were identified from the genome of *Trichinella spiralis* and expressed in *Escherichia coli*. Sequence analysis revealed that these two SPIs contained essential structural motifs, which were well conserved within the tumor-infiltrating lymphocytes (TIL) and serpin superfamily. Based on protease inhibition assays, the recombinant Tsp03044 showed inhibitory effects on trypsin, α-chymotrypsin, and pepsin, while the recombinant TspAd5 could effectively inhibit the activities of α-chymotrypsin and pepsin. Both these inhibitors showed activity between 28 and 48 °C. The expression levels of the two SPIs were also determined at different developmental stages of the parasite with real-time PCR. Our results indicate that Tsp03044 and TspAd5 are functional serine protease inhibitors.

1. Introduction

Trichinellosis caused by the *Trichinella spiralis* is an important food-borne parasitic zoonosis (Pozio, 2000). *T. spiralis* can infect more than 100 animal species. Throughout its life cycle, the parasite is exposed to the digestive enzymes in the stomach, intestine, and body fluids. Evidence shows that the secretory protease inhibitors of many parasitic intestinal nematodes can neutralize the damage of these digestive proteases within the immediate environment of the nematodes, facilitate feeding, and regulate the immune response of the host (Bernard and Peanasky, 1993; Lu et al., 1998; Milstone et al., 2000). The serine protease inhibitors (SPIs) constitute the most important of these protease inhibitor families (Knox, 2007).

SPIs, which can inhibit one or more serine proteases, play important roles in the development, survival, and reproduction of animals, plants, parasites, insects, and viruses. Many SPIs have been isolated from various nematode genera including *Schistosoma* (Molehin et al., 2014), *Trichuris* (Rhoads et al., 2000), *Ancylostoma* (Milstone et al., 2000), *Taenia* (Nemeth and Juhasz, 1981), and other parasites. It has been suggested that SPIs play an important role in protecting nematodes from host digestive enzymes and interact with host cellular components involved in the regulation of inflammation (Ray et al., 1992). These SPIs can counteract the digestive activities of various target proteases including trypsin, chymotrypsin, pepsin, pancreatic elastase, and neutrophile lactase. According to the primary sequence, structural motifs and mechanism of binding, SPIs are divided into at least 18 families, such as kunitz, kazal, serpin, TAP, and tumor-infiltrating lymphocytes (TIL) family (Gettins, 2002).

The purpose of this study was to analyze the inhibitory activity of several SPIs derived from *T. spiralis* against different host proteases and at different stages of the life cycle. Briefly, we cloned two SPIs from *T. spiralis* and expressed them in *Escherichia coli*. The inhibitory effects of the two SPIs against various serine proteases and their respective temperature-dependent inhibitory activity were also determined. In addition, we determined the expression levels of two SPIs at different developmental stages in the life cycle of *T. spiralis*. The data reported here will considerably expand the existing knowledge of the roles of SPIs in *T. spiralis*.

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2. Materials and methods

2.1. Ethics statement

The experiments were approved by the Animal Ethics Committee of the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences (CAAS) and were performed in accordance with animal ethics guidelines and approved protocols (Animal Ethics Committee approval number SYXK [Hei] 20132035).

2.2. Parasite

The *T. spiralis* T1 strain (ISS3) used in this study was maintained via serial passages in Kunming mice. At ≥35 days post-infection, the infectious larvae were recovered from the muscles of Kunming mice by digestion with 1% pepsin in acidified water for 3 h at 37 °C. Adult fecunded female worms were collected from the intestines of mice at 5–7 days post infection.

2.3. Sequence analysis

The full-length cDNA sequence were obtained from GenBank and designated Tsp03044 and TspAd5 (XM_003379333 and EU263307, published by Mitreva and Liu) (Mitreva et al., 2011). The protein molecular weights were calculated using an online tool (http://www.bioinformatics.org/sms/prot_mw.html). The transmembrane helices and signal peptides were predicted using the SMART program (http://smart.embl-heidelberg.de/). The serine protease inhibition domains were analyzed with InterProScan EMBL-EBI Software (www.ebi.ac.uk/Tools/pfa/iprscan/). The homology analysis of multiple sequences was performed with ClustalX2, BioEdit, and MEGA6 software.

2.4. RNA isolation and cDNA synthesis

Total RNA was extracted from *T. spiralis* adult worms and larvae obtained from mouse muscle tissue using QIAGEN RNeasy Plus Mini Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. After verification of the RNA quality and concentration, 800 ng of total RNA was used as a template for the synthesis of first-stand cDNA using the ReverTra Ace® gPCR RT Master Mix (Toyobo Co., LTD., Japan). The samples were stored at −30 °C until use.

2.5. Expression and purification of Tsp03044 and TspAd5

Based on the sequences obtained from NCBI, specific primers were designed to amplify the open reading frames (ORF) of Tsp03044 and TspAd5 from the cDNA. The PCR products were cloned into a pET-30a vector (Novagen, Madison, WI) with a C-terminal His_6 tag, and then transformed into the *E. coli* strain BL21 (DE3) (TIANGEN CO., LTD., Beijing, China) for expression. The colonies were incubated in LB media at 37 °C until the optical density (OD_{600nm}) reached a value of 0.6. The cells were then incubated for an additional 16 h at 18 °C (Tsp03044) or 6 h at 37 °C (TspAd5) with 1 mM IPTG (Isopropyl-β-D-thiogalactoside), and then pelleted. Cell pellets were re-suspended in PBS (pH 7.4) and disrupted by sonication. The cell debris was pelleted at 12,000 g for 10 min at 4 °C, and the supernatant was loaded onto 1 mL of GE Healthcare Ni Sepharose™ 6 Fast Flow (GE Healthcare Bio-Sciences AB, Sweden). The resin was washed with binding buffer and eluted with elution buffer, and then desalted using PD-10 Desalting Columns (GE Healthcare Bio-Sciences AB, Sweden). The concentration and purity of the purified proteins were examined using PierceTM BCA Protein Assay Kit (3747N. Meridian Rd., Rockford, IL61101 U.S.A.) and SDS-PAGE analysis.

Table 1
Sequences of the primers used in real-time PCR.

Primers	Sequences (5′ to 3′)	Length of products
Tsp03044-F	CATTTGGGCGGCTGTGGACC	185
Tsp03044-R	TCAGCAATCATCAGGCATTA	bp
TspAd5-F	AGATTAATAAGTGGGTGAGCGA	80
TspAd5-R	GAGTATTTGCCTCAACTGTTCC	bp
18S rRNA-F	TTAAAGGAATTGACGGAAGG	136
18S rRNA-R	CTACCCACTGAATCAAGAAAGAG	bp

2.6. Protease inhibition assays

The analysis of protease inhibition against the serine proteases trypsin, α-chymotrypsin, and pepsin was performed according to a method described previously (Horita et al., 2010). Briefly, the kinetics parameters (K_m and K_{cat}) of the proteases were obtained using a reaction mixture containing 2 nM protease for trypsin or 3 nM protease for α-chymotrypsin and pepsin; 50, 57, 66, 80, 100, or 130 μM Peptidyl-MCA substrates (MCA substrate) (Peptide Institute, Osaka, Japan) or 5.0, 5.7, 6.6, 8.0, 10.0, or 13.0 μM MOCAc/Dnp type fluorescence-quenching substrates (MOCAc/Dnp substrate) (Peptide Institute, Osaka, Japan) in 10 mM Tris–HCl (pH 8.0) for trypsin and α-chymotrypsin; or 10 mM Tris–HCl (pH 1.5) for pepsin. The reaction mixture was analyzed at 37 °C using an EnSpire Multi-mode Plate Reader (PerkinElmer, Turku, Finland). The fluorescence intensity for MCA and MOCAc/Dnp substrates was measured at excitation and detection wavelengths of 380/460 nm and 328/393 nm respectively. The values of the inhibition constant (K_i)were determined using a reaction mixture containing the same proteases and substrates and 32 nM Tsp03044 or TspAd5. Prior to taking measurements, the reaction mixtures were incubated at 37 °C for 5 min. Each reaction was carried out in triplicate for statistical analysis. The acquired data were plotted on Lineweaver–Burk plots.

2.7. Temperature-dependent inhibitory assays

For experiments examining temperature-dependent inhibitory activity, the reaction mixture contained 2 nM trypsin or 3 nM α-chymotrypsin; 32 nM Tsp03044 or TspAd5; and 57, 66, 80, 100, or 130 μM MCA substrate in 10 mM Tris–HCl (pH 8.0). Prior to taking measurements, the reaction mixture was incubated at 28–48 °C for 5 min.

2.8. Quantitative real-time PCR

The total RNA of the adult worm and larvae obtained from mouse muscle tissue were extracted and cDNAs synthesized as described above. The gene-specific primers used in this study are listed in Table 1. The samples were analyzed using SYBR Green real-time PCR with a LightCycler480 II Real Time PCR System (Roche Diagnostics LTD., Rotkreuz, SWTZ). The cDNA of the 18S rRNA of the parasite was amplified as an internal control. Relative quantification was performed using the cycle threshold ($\Delta\Delta C_T$) method (Livak and Schmittgen, 2001). SYBR® Premix Ex *Taq* ™ II (Tli RNaseH Plus) with SYBR green I (TaKaRa Bio, Dalian, China) was used for the amplification.