

An antigenic recombinant serine protease from *Trichinella spiralis* induces protective immunity in BALB/c mice

Xin Li · Jian-ping Yao · Ai-hua Pan · Wei Liu ·
Xu-chu Hu · Zhong-dao Wu · Xing-wang Zhou

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Abstract In this study, we report the cloning and characterization of a cDNA encoding a *Trichinella* serine protease gene (TspSP-1.3) from GenBank. The recombinant TspSP-1.3 protein (rTspSP-1.3) was expressed in an *Escherichia coli* expression system and purified with Ni-affinity chromatography. Real-time quantitative PCR analysis revealed that TspSP-1.3 was expressed at significantly higher levels in muscle larvae and adult worms than in newborn larvae. TspSP-1.3 was detected in excretory–secretory proteins of *Trichinella spiralis* with western blotting. Immunization with the rTspSP-1.3 antigen induced humoral immune responses, which manifested as elevated specific anti-rTspSP-1.3 IgG and IgE antibodies and a mixed Th1/Th2 response. To determine whether purified rTspSP-1.3 had good antigenicity and could be a vaccine candidate for the control of *T. spiralis* infection, we immunized BALB/c mice with rTspSP-1.3 and subsequently challenged the mice with *T. spiralis* larvae. The results showed that mice vaccinated with rTspSP-1.3 exhibited an average reduction in the muscle larvae burden of 39 % relative to the control group. These results suggest that TspSP-1.3 could be a novel vaccine candidate for controlling *Trichinella* infection.

Introduction

Trichinella spiralis, a major species of the *Trichinella* genus, is an intracellular parasitic nematode of vertebrates and one of the most widespread zoonotic pathogens in the world (Dupouy-Camet 2000). The entire life cycle of the parasite takes place in a single host. There are three major antigenic stages for *T. spiralis*: muscle larvae (ML), adult worms (Ad), and newborn larvae (NBL). Trichinellosis is not only a public health hazard but also represents an economic problem in porcine animal production and food safety (Gottstein et al. 2009). Human trichinellosis outbreaks affect as many as 11 million people worldwide, and it is regarded as a re-emerging infectious disease (Dupouy-Camet 2009; Hernandez-Bello et al. 2008), especially in developing countries, such as China, Argentina, and some eastern European countries (Gottstein et al. 2009), where cooking habits, along with poverty, poor sanitary conditions, and lack of veterinary controls facilitate the occurrence of human trichinellosis. For example, 15 outbreaks of meat-borne human trichinellosis, consisting of 1,387 cases and four deaths from 2004–2009, have been reported in southwestern China (Cui et al. 2011).

The wide distribution of domestic and wild animal reservoirs and sources of human infection make the prevention and control of trichinellosis difficult. Currently, a lack of reliable early diagnostic methods for *Trichinella* infection favors the late-stage establishment of mature and encapsulated *Trichinella* muscle larvae, which are resistant to drug treatment (Gottstein et al. 2009). Recently, strong effort has been directed toward the development of effective *Trichinella* vaccines to prevent parasite infection and eradicate its transmission.

The development of effective *Trichinella* vaccines to prevent parasite infection has been reported in *Trichinella* animal models based on *Trichinella* crude larval extracts (Aucouturier et al. 2001; Deville et al. 2005), total excretory–secretory (ES) antigens (Dea-Ayuela et al. 2006; Quan et al. 2004), recombinant proteins (Wang et al. 2006; Yang

Xin Li and Jian-ping Yao contributed equally to this work.

X. Li · A.-h. Pan · X.-c. Hu · Z.-d. Wu · X.-w. Zhou (✉)
Sun Yat-sen University Zhongshan School of Medicine,
Key Laboratory of Tropical Disease Control
(Sun Yat-Sen University), Ministry of Education, 74 Zhongshan
2nd Road, Guangzhou 510080, China
e-mail: zhouxw2@mail.sysu.edu.cn

J.-p. Yao
1st Affiliated Hospital of Sun Yat-Sen University, Guangzhou,
China 510080

W. Liu
3rd affiliated Hospital of Sun Yat-Sen University, Guangzhou,
China 510080

et al. 2008), DNA (Tang et al. 2012; Yang et al. 2010), and peptides (McGuire et al. 2002). Although partial protection has been achieved with the above vaccination strategies, a vaccine with adequate protection against *Trichinella* has not yet been developed to the point of commercialization (Gottstein et al. 2009).

Proteins are usually the first choice as candidates for vaccine antigens because they are easier to identify and modify than other compounds and can also be produced economically in large quantities using modern biotechnology (Hein and Harrison 2005). *Trichinella* ES products, which are directly exposed to the host immune system, play a primordial role in modulating host immune responses and parasitism (Bai et al. 2012; Bruschi and Chiumiento 2012). The ES products of some parasitic nematodes also contain allergens and IgE-potentiating factors (Stromberg 1979, 1980). Therefore, *Trichinella* ES proteins have recently attracted a great deal of attention in studies on the host–parasite interaction and offer targets for vaccine development.

Parasite proteases, especially secretory proteases, are thought to have important functions in modulating the interactions between parasites and hosts because of their particular roles in the invasion of host tissues, parasite nutrition, and evasion of host immune responses (Fishelson et al. 1992; McKerrow 1989; Trap et al. 2006).

The roles of parasite serine proteases, a major trypsin-like serine protease, have been covered by a review (McKerrow 1989) and established in studies of *Onchocerca lienalis* (Lackey et al. 1989), *Schistosoma mansoni* (McKerrow et al. 1985; Newport et al. 1988), *Necator americanus* (Brown et al. 1995), and *T. spiralis* (Abbas et al. 1996; Todorova et al. 1995). A trypsin-like serine protease could be involved in host immune suppression by influencing the relationship between the parasite and host and may contribute to parasite infection and survival (Balasubramanian et al. 2010). Secreted proteinases have also been found to be the targets of significant antibody responses in a number of nematodiasis (Kennedy and Qureshi 1986). Therefore, immunogenic parasite serine proteases have emerged as promising serodiagnostic markers and vaccine targets (Dalton et al. 2003; Dixit et al. 2008). The complete sequence and molecular characterization of *T. spiralis* serine protease have been described in some reports (Nagano et al. 2003; Robinson and Connolly 2005). Specifically, the recent molecular cloning and expression of *T. spiralis* serine protease TspSP-1 (Romaris et al. 2002) and TspSP-1.2 (Wang et al. 2012) have been reported. However, the protective immunity of *T. spiralis* serine proteases remains poorly understood.

In this paper, we cloned and analyzed a cDNA encoding a serine protease from *T. spiralis* (TspSP-1.3, GenBank accession number EU302801) and identified it as a component of ES products of the parasite. Furthermore, we investigated the immunogenic antigenicity and vaccine development potential

of TspSP-1.3. Our finding demonstrates that mice vaccinated with recombinant TspSP-1.3 show marked immune protection against *T. spiralis* larvae challenge. The results suggest that TspSP-1.3 could be a novel vaccine candidate for controlling *Trichinella* infection.

Materials and methods

Preparation of the parasites

The *T. spiralis* (ISS 533) parasites used in this study were originally isolated from a swine source and routinely maintained through serial passages in Kunming mice in the laboratory of the department of parasitology at Sun Yat-sen University Medical School.

Adult worms were collected from the intestines of rats at 6 days post-infection. The muscle of infected mice was digested to recover ML using a standard pepsin–hydrochloric acid digestion method as described by Dennis et al. (1970). NBL were obtained from fertile female adult worms cultured in vitro for 36 h at 37 °C. ES products were prepared with conventional methods (Todorova et al. 1995).

Animals

BALB/c mice and Wistar rats aged 6–8 weeks and free of specific pathogens were purchased from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China), and the animals were raised carefully in accordance with the National Institutes of Health guidelines on animal care and the ethical guidelines. All experimental procedures were approved by the Animal Care and Use Committee of Sun Yat-sen University.

RNA isolation, reverse transcription, cDNA synthesis, and real-time quantitative PCR

Total RNA of *T. spiralis* adult worms, newborn larva, and 35-day-old muscle larva was isolated and purified using TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription and cDNA synthesis were performed with reverse transcriptase (M-MLV RT, Promega) according to the manufacturer's recommendations. Real-time quantitative polymerase chain reaction (PCR) was performed to evaluate gene transcription levels in different *Trichinella* developmental stages using SYBR® Premix Ex Taq™ (TAKARA Biotechnology, Dalian, China) with a LightCycler480 real-time PCR System (Roche Diagnostics Inc.). Each reaction contained 10 µl of 2× SYBR green Master Mix, 0.4 µl of 20-µM primers, and 1 µl of cDNA to a total volume of 20 µl. The thermal cycling conditions included an initial denaturation step at 95 °C for 2 min, followed by 40 cycles at 95 °C for 15 s, annealing at

60 °C for 15 s, and extension at 72 °C for 15 s. At the end of the PCR cycles, the specificities of the amplification products were controlled via dissociation curve analysis. All data were analyzed using the LightCycler480 software, and Ct was calculated using the $2^{-\Delta\Delta C_t}$ method. A constitutively expressed *Trichinella* gene (18S rRNA, GenBank accession No: U60231) was used as an internal control. The primers for the TspSP-1.3 gene (GenBank accession No: EU302801) were as follows: 5'-TGGATCTCAGACAGGCAAAG-3' (forward) and 5'-GAGAATCTGGAGGTGGTGGT-3' (reverse). The primers for 18S rRNA were 5'-TAAAGGAATTGACGGAA GGG-3' (forward) and 5'-ATGCCACTACCCACTGAA-3' (reverse). Three independent experiments were performed, and two repeats were measured for each sample.

Sequence analysis of the TspSP-1.3 gene

The full-length cDNA sequence of the *T. spiralis* TspSP-1.3 gene was obtained from GenBank (EU302801). The catalytic sites of the deduced protein (TspSP-1.3) were predicted using ScanProsite (www.expasy.ch/tools/scanprosite). The theoretical protein molecular weight was determined with ProtParam (<http://tw.expasy.org/tools/protparam.html>). The signal peptide was predicted by Prop1.0 Server (www.cbs.dtu.dk/services/Prop). The domain analysis of the serine protease was performed using InterProScan EMBL-EBI Software (www.ebi.ac.uk/Tools/pfa/iprscan/). Linear B cell epitopes were predicted using BepiPred (Larsen et al. 2006) (<http://www.cbs.dtu.dk/services/Bepipred/>), and the score threshold for epitope assignment was set at 0.35.

Cloning, expression, and purification of recombinant mature TspSP-1.3

The coding region containing the predicted mature serine proteinase of the TspSP-1.3 gene (1,185 bp corresponding to amino acid residues 28–421) was cloned with PCR using synthesized cDNA from 35-day post-infection (PI) muscle larvae. *Bam*HI and *Xho*I restriction enzyme sites were added to the primers (underlined) to assist cloning into the expression vector in frame. The primers used for constructing the recombinant expression vectors were as follows: forward (5'-GACGGATCCAGTATTGTGGAAAT CC-3', with an in-frame *Bam*HI restriction site) and reverse (5'-GGGCTCGAGTCAGTAAAAAGAGTCAAAATTTTG-3', with an in-frame *Xho*I restriction site). After PCR amplification, the PCR products were fractionated on a 1 % agarose gel, and the band corresponding to PCR products (1,182 bp) was purified from agarose gel slices using the QIAquick Gel extraction kit (QIAGEN, USA), digested with *Bam*HI and *Xho*I (Fermentas), and cloned into a linearized pET-28a(+) expression vector.

The purified PCR products were subcloned into the pET-28a(+) expression vector with the His-tag at N-terminus (Novagen). The recombinant plasmid (pET28a/TspSP-1.3) was transformed into competent *Escherichia coli* DH5 α cells. Positive clones were picked and confirmed with DNA sequencing (Invitrogen). The expression of the recombinant proteins was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM at 37 °C for 4 h according to a previous report (Zhou et al. 2007). The pellets of the bacterial culture were harvested following the induced incubation and disrupted by sonication in 50-mM Tris-HCl/0.5-mM NaCl buffer (pH 8.5). Recombinant proteins expressed in inclusion bodies were completely solubilized with 8 M urea in 50-mM Tris-HCl/0.5-mM NaCl buffer (pH 8.0). The recombinant TspSP-1.3 protein (rTspSP-1.3) was purified under denaturing conditions with Ni-affinity chromatography (Qiagen, USA) according to the manufacturer's instructions. The purity of the recombinant protein was determined with SDS-PAGE. Endotoxin was removed from rTspSP-1.3 using a two-phase extraction method as described in a previous report (Liu et al. 1997), resulting in <0.25 endotoxin units/ml, as measured by the limulus amoebocyte lysate assay (GenMed) according to the manufacturer's protocol.

Infected sera and antisera

Sera were obtained from BALB/c mice orally infected with 500 larvae of *T. spiralis* at 1, 7, 13, 19, 25, 31, and 37 days PI. A polyclonal antibody against the rTspSP-1.3 protein was produced in immunized BALB/c mice, which were injected subcutaneously with 20 μ g of the purified recombinant proteins and complete Freund's adjuvant (FCA, Sigma, USA). This initial treatment was followed by three booster injections of 20 μ g of the proteins mixed with incomplete Freund's adjuvant at 2-week intervals. The collected antisera were affinity purified using the AminoLink Immobilization kit (Thermo) according to the manufacturer's instructions. Affinity-purified antibodies were tested for efficacy and specificity with enzyme-linked immunosorbent assay (ELISA) and western blotting, respectively.

Western blotting

Test samples, including purified recombinant protein and ES products of *T. spiralis*, were separated by 10 % SDS-PAGE. Then, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, USA) using a Trans-Blot apparatus (Bio-Rad, USA). After being blocked with 5 % (w/v) skim milk in Tris-buffered saline with Tween 20 (TBST; 100-mM Tris, pH 8.0, 0.9 % NaCl, and 0.1 % Tween 20), the membrane was incubated with a His-tagged monoclonal antibody (1:5,000 dilution) from Bio-Rad (USA), *T. spiralis*-

infected mouse sera (1:500 dilution), or mouse anti-rTspSP-1.3 polyclonal antibody (1:200 dilution). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG used as the second antibody (Jackson, USA) (dilution 1:10,000) was added after washing. Western blot detection was performed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, USA).

ELISA measurement of antibody response

Serum samples were analyzed for recombinant protein-specific IgG, IgG1, IgG2a, and IgE antibodies with a standard ELISA. Briefly, flat-bottomed 96-well microtiter plates (Greiner, Germany) were coated for 2 h at 37 °C with 100 µl of the recombinant protein at a concentration of 5 µg/ml in 0.05-M bicarbonate buffer (pH 9.6). After the microplates were washed three times with PBS-0.05 % Tween-20 buffer (PBST), they were blocked with 1 % bovine serum albumin/5 % (w/v) skim milk in 100 µl of PBST for 1 h at 37 °C. After three more washes with PBST, the microplates were probed with 1:100 immune sera (100 µl/well) for 1 h at 37 °C. After washing, 100 µl of HRP-conjugated monoclonal goat anti-mouse IgG, IgG1, IgG2a, and IgE (Bethyl, USA) antisera diluted 1:10,000 in PBST was added to each well, and the plates were incubated for 1 h at 37 °C. Following a final wash step, 100 µl of 3, 3', 5, 5'-tetramethylbenzidine substrate was added to each well, and the reaction was terminated by adding 50 µl of 2-M H₂SO₄. The optical density at 450 nm was measured with a microplate absorbance reader (Tecan, Switzerland). A negative control (without primary antibody antisera) was present on each plate. All samples were run in triplicate, and the values were expressed as the mean ± standard deviation (SD).

Immunization, challenge infection, and protective immunity

Mice were divided into two groups of 15 animals each (pre-immune sera were collected by tail bleeding 2 days prior to the first injection). The experimental group of mice was subcutaneously inoculated with 20 µg of the recombinant protein TspSP-1.3 per mouse emulsified with FCA (Sigma, USA). The control group of mice was given ×1 PBS buffer mixed with FCA. Subsequently, the experimental group of mice was boosted three times with the recombinant protein (20 µg/mouse) emulsified with incomplete Freund's adjuvant at intervals of 2 weeks, whereas the control group was boosted with incomplete Freund's adjuvant in PBS buffer only. Mouse blood samples were collected every 7 days.

Three weeks after the final boost, the mice were challenged orally with 400 *T. spiralis* infective muscle larvae per mouse. At 42 days PI, five mice from each group were sacrificed to evaluate the worm burden reduction by counting the number of muscle larvae recovered from the mice. The rate of muscle

larval burden reduction was calculated according to the recovered larvae per gram muscle according to a previous report (Yang et al. 2010).

The protective immunity was evaluated based on the mean number of muscle larvae collected from the vaccinated group compared with the control group. The reduction rate was calculated as follows: muscle larvae reduction (%) = (1—mean number of larvae per gram muscle in vaccinated mice/mean number of larvae per gram muscle in control mice) × 100 %.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad InStat Software, San Diego, California, USA) and SPSS 13.0 software. The data were expressed as the means ± SD. Differences among groups were evaluated with a one-way ANOVA and student *t* test, and *p* values were denoted as follows: **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 (*p* < 0.05 was regarded as statistically significant).

Results

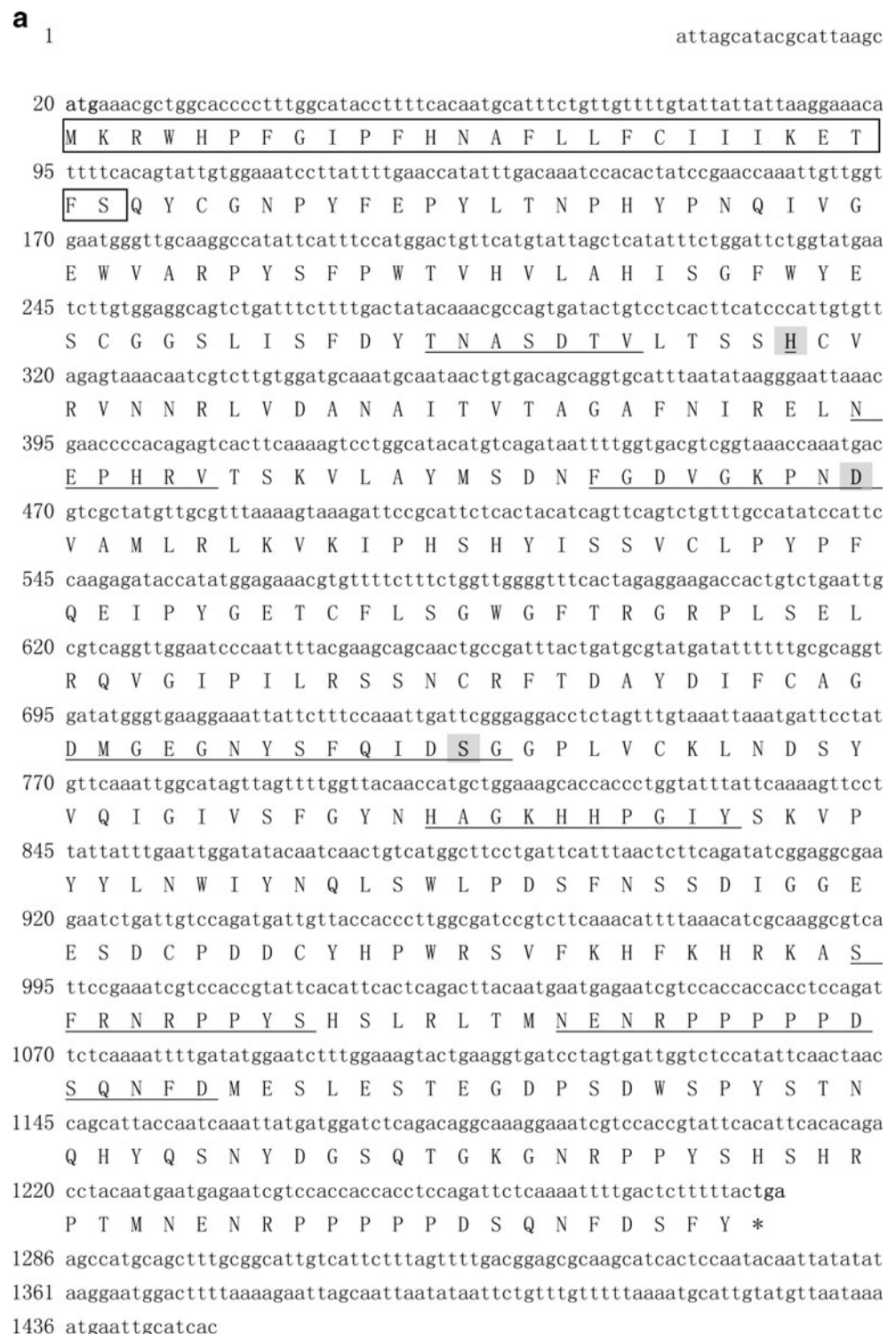
Sequence analysis of TspSP-1.3

The full-length cDNA sequence of the *T. spiralis* TspSP-1.3 gene was 1,449 bp (CDS: 20–1,285 bp). The cDNA contained a 1,266 bp open reading frame (ORF) encoding a protein of 421 amino acids, which was composed of a 27-residue signal sequence and a 394-residue mature protein. The predicted molecular weight of the mature protein was calculated as 44.8 kDa (394 amino acids and pI 6.08). In Fig. 1a, the open boxes indicate the probable signal peptide (20–100 bp) predicted with the Signal P program (Bendtsen et al. 2004). Based on the prediction of catalytic sites of the deduced protein, the active site residues “histidine, aspartic acid, and serine” of the catalytic triad that is characteristic of the serine protease family are indicated in boldface and underlined in the shaded boxes (Fig. 1a). Bepipred results predicted seven linear B cell epitopes, and the distribution was scattered (Fig. 1b). The highest score of B cell epitope prediction for the TspSP-1.3 protein was associated with amino acids residues 341–355, with predicted antigenicity (Fig. 1b). Overall, the bioinformatic analysis suggested that TspSP-1.3 efficiently induced B cell responses to trigger antibodies.

Cloning, expression, and purification of recombinant TspSP-1.3 and western validation

To obtain large quantities of recombinant TspSP-1.3 protein for immunological and vaccination studies, the coding region of TspSP-1.3 was successfully cloned into the pET28a(+)

Fig. 1 Analysis of the full-length cDNA sequence of TspSP-1.3 and linear B cell epitope prediction. **a** Full-length cDNA sequence of TspSP-1.3 and the amino acid sequence encoded by the ORF. The amino acid sequence in the *open boxes* indicates the putative signal peptides of TspSP-1.3; the initiation codon (*atg*) and termination codon (*tga*) are in *boldface*; the active sites of the serine protease is in *boldface* and *underlined* in the *shaded boxes*; the predicted linear B cell epitopes are *underlined*. **b** Graphical result of Bepipred prediction of linear B cell epitopes. Bepipred linear epitope prediction of B cell recognition sites, with a bar indicating the position of the highlighted epitope. Higher scores above the threshold denote linear B cell epitopes. The horizontal line is the threshold



expression vector. The recombinant TspSP-1.3 (101–1,282 bp) with the His-tag was expressed in *E. coli* BL21 through induction with IPTG. After purification with Ni-affinity chromatography, the recombinant protein was expressed in inclusion bodies in *E. coli* and showed a single band on SDS-PAGE after purification with Ni-NTA agarose (Fig. 2a). Western blotting was performed with the anti-His peptide antibody

(Fig. 2b), and the molecular mass of recombinant TspSP-1.3 protein was approximately 47 kDa, which was consistent with the expected size.

To determine whether rTspSP-1.3 had good antigenicity, western blotting was performed using the serum of *T. spiralis*-infected mice (1, 7, 13, 19, 25, 31, and 37 days after infection) and showed that rTspSP-1.3 was strongly recognized

Fig. 1 (continued)

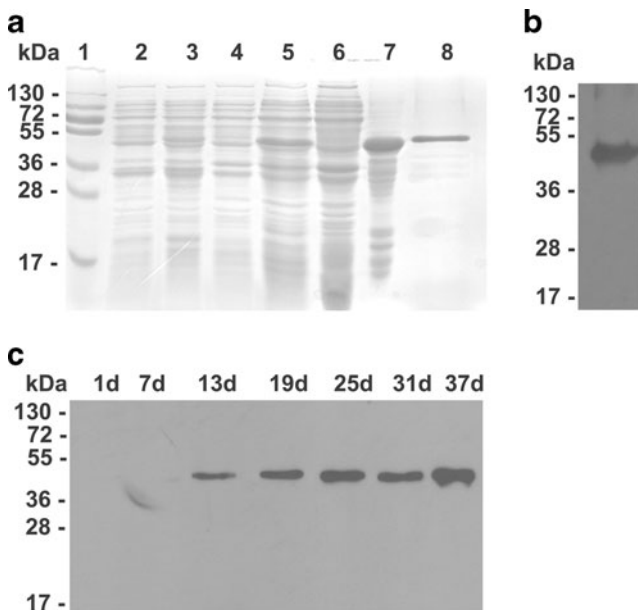
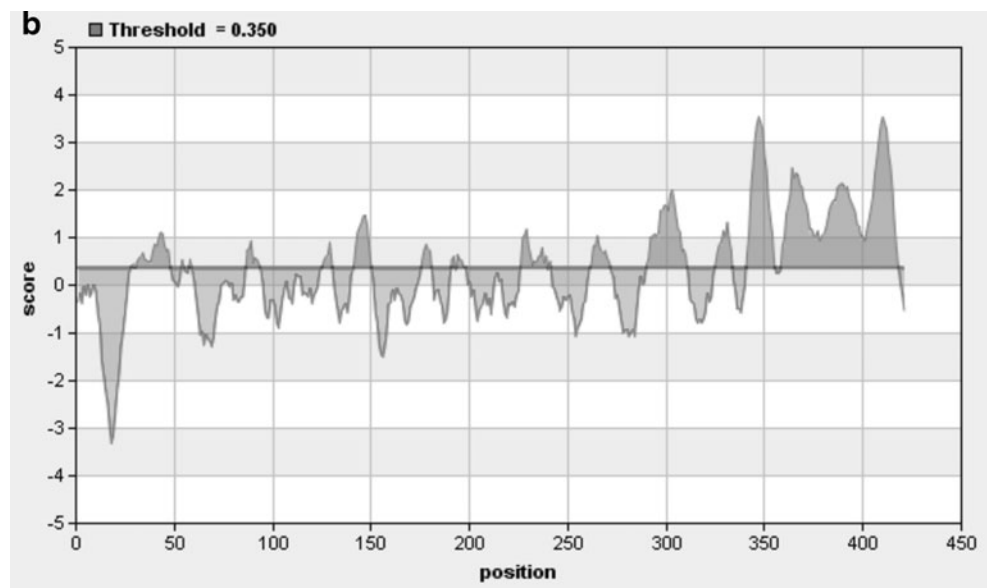


Fig. 2 Analysis of the expression products and purified products of recombinant TspSP-1.3. **a** SDS-PAGE was performed to separate purified rTspSP-1.3 from an *E. Coli* expression. Proteins were separated on 10 % SDS-PAGE gels. Lane 1, standard marker; lanes 2 and 3, empty vector pET28a(+) without/with IPTG induction; lanes 4 and 5, recombinant plasmid pET28a(+)-TspSP-1.3 without/with IPTG induction; lanes 6 and 7, supernatant and precipitate of lysate of pET28a(+)-TspSP-1.3 transformant *E. coli* with IPTG induction; lane 8 purified rTspSP-1.3. **b** Western Blot validation of purified his-tagged rTspSP-1.3. The recombinant protein was separated on a 10 % SDS-PAGE gel and electro-transferred to a PVDF membrane. Western blot analysis was performed using a mouse anti-His monoclonal antibody, followed by incubation with the secondary goat anti-mouse IgG (lane 1). rTspSP-1.3 migrates at approximately 47 kDa. **c** Western blot analysis of recombinant pET-28a(+)-TspSP-1.3 with the infected serum of Balb/c mice. The proteins were separated on a 10 % SDS-PAGE gel, transferred to a PVDF membrane and analyzed with western blotting using infected serum as the primary antibody and goat anti-mouse IgG as the secondary antibody (infection sera obtained at different time intervals (1, 7, 13, 19, 25, 31, and 37 days post-infection))

by the *T. spiralis*-infected sera beginning at 13 days and then increased slowly until the end of the study (Fig. 2c). This result indicates that rTspSP-1.3 is an immunodominant antigen that can be recognized by the host immune system during nature infection. These results suggested that the recombinant protein had good antigenicity similar to naturally expressed gene TspSP-1.3, consistent with the previous prediction of its linear B cell epitopes.

Analysis of stage-specific expression levels of the TspSP-1.3 gene

Real-time quantitative PCR was performed to determine the transcription level of TspSP-1.3 in different developmental stages of *T. spiralis* (including adult worms, newborn larvae, and muscle larvae), (Fig. 3a). The fold change of TspSP-1.3 gene expression levels in muscle larvae and adult worms was calculated relative to that in newborn larvae. The expression levels of the TspSP-1.3 gene rapidly increased by 2.28- and 2.40-fold in muscle larvae and adult worms, respectively.

To determine whether TspSP-1.3 was expressed as a secretory product, Western blotting was performed using mouse antiserum against rTspSP-1.3 on total ES products from muscle larvae. As shown in Fig. 3b, ES antigens were recognized by the mouse anti-rTspSP-1.3 polyclonal antibody, and a single Western band migrated between 37 and 55 kDa as we expected. These results suggest that TspSP-1.3 could be a *Trichinella* secretory protein.

Serum antibody responses to recombinant TspSP-1.3

To assess the potential of rTspSP-1.3 in eliciting host-protective antibodies, we measured rTspSP-1.3-specific total IgG in protected mouse sera with ELISA (Abbas et al. 1996). A high titer of specific antibodies (IgG) was elicited following

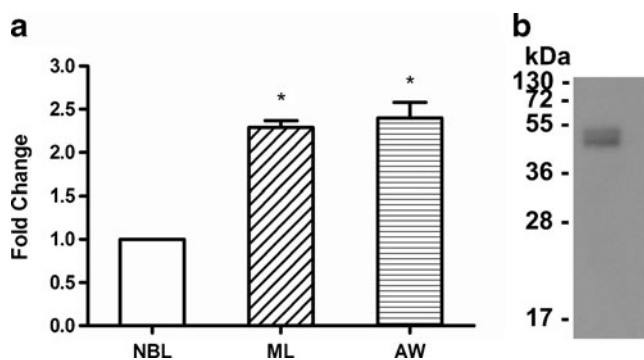


Fig. 3 Analysis of stage-specific expression levels and secretory expression of TspSP-1.3 **a** Stage-specific expression levels of the TspSP-1.3 gene assessed with real-time quantitative PCR. Total RNA was extracted from newborn larvae (NBL), muscle larvae (ML), and adult worms (AW) of *T. spiralis*. 18S rRNA was employed as the internal control. The gene expression levels of TspSP-1.3 were significantly increased ($p < 0.05$) in ML and AW compared with NBL. **b** Excretory-secretory antigens were recognized with mouse antisera to rTspSP-1.3. The excretory-secretory antigens from muscle larvae were separated on a 10 % SDS-PAGE gel, transferred to a PVDF membrane and analyzed with Western blotting using mouse antisera to rTspSP-1.3 (1:200 dilution) as the primary antibody and goat anti-mouse IgG as the secondary antibody

a boost with rTspSP-1.3 in all immunized mice (Fig. 4a). Next, to further assess the efficacy of rTspSP-1.3 in inducing a Th1 or Th2 response in vivo, IgG subclass antibody levels were measured using the same method. The predominant subclass antibody appeared to be IgG1 beginning at 14 days (Fig. 4b), and there was also a significant level of IgG2a beginning at

21 days (Fig. 4c). This increase was not apparent in the control group immunized with PBS. The results suggest that rTspSP-1.3 vaccination appears to induce a mixed pattern of IgG1 (Th2) ($p < 0.001$) and IgG2a (Th1) ($p < 0.01$), which may be important in host-protective immunity against *T. spiralis*.

In addition, antigen-specific IgE responses were strong in the sera of mice immunized with adjuvant rTspSP-1.3, indicating that rTspSP-1.3 could be an allergenic molecule for the host (Fig. 4d).

Protective immunity

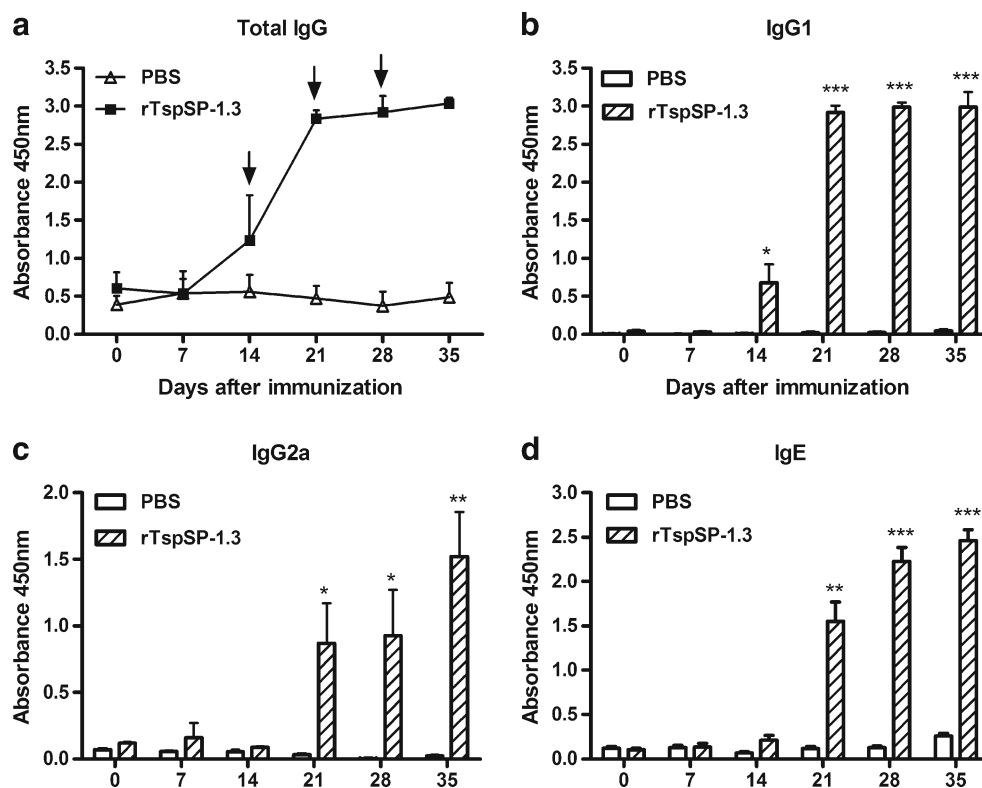
To determine whether rTspSP-1.3 could be a vaccine candidate for the control of *T. spiralis* infection, we immunized BALB/c mice with purified rTspSP-1.3 and subsequently challenged the mice with *T. spiralis* larvae. Mice immunized with rTspSP-1.3 exhibited a 39 % reduction in muscle larva burden compared with the control group (Fig. 5) and these differences were statistically significant ($p < 0.05$). The data showed that rTspSP-1.3 possessed marked protective immune effects against *T. spiralis* larval challenge in mice.

Discussion

A variety of proteases with different functions have been identified and characterized from parasitic nematodes. *T. spiralis* produces many serine proteinases (Criado-Fornelio

Fig. 4 Dynamic analysis of serum responses, including total IgG, IgG1/IgG2a subclasses, and IgE, in mice vaccinated with rTspSP-1.3. **a** The total IgG levels in the sera of immunized mice (rTspSP-1.3+FA (Freund's adjuvant)) or control mice (PBS + FA) were measured with ELISA. **b** and **c** The IgG1/IgG2a subclass responses were detected at different time points. **d** IgE levels in the sera of immunized mice were measured. The boost time points are indicated with arrows. The values are expressed as the mean $\pm$ SD.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



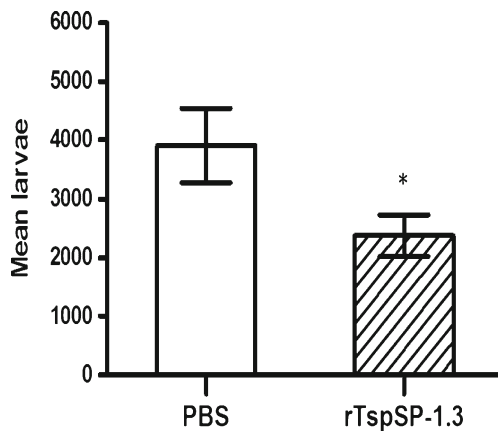


Fig. 5 Muscle larva reduction in vaccinated mice after challenge with *T. spiralis* larvae BALB/c mice were immunized with rTspSP-1.3 (rTspSP-1.3+FA) or PBS control (PBS + FA). FA, Freund's adjuvant. The number of larvae per gram muscle is presented as the mean $\pm$ S.D. The asterisk (*) indicates statistically significant differences in larval burden between the groups ($p < 0.05$)

et al. 1992) (Todorova et al. 1995). Serine proteases are assumed to play important roles in *Trichinella* development related to the tissue penetration process (Yamamoto et al. 1994), invasion and migration of the parasite because most of these classes of antigens are found in Ad and NBL stages (Wu et al. 2009). Interestingly, in this study, our real-time quantitative PCR results showed that TspSP-1.3 gene expression (a secretory serine protease of *T. spiralis*) in adult worms and muscle larvae was significantly higher than that of newborn larvae. This result implies that TspSP-1.3 may have important functions in the adult worm and muscle larvae stages (Todorova and Stoyanov 2000; Wu et al. 2009).

The TspSP-1.3 gene we cloned in this paper encodes a predicted mature protein of 421 amino acids residues, which is similar to a serine protease. Sequence analysis demonstrated that TspSP-1.3 contained an IVGE motif, which is a characteristic motif of serine proteinases in vertebrate and invertebrate species (Nagano et al. 2003). The sequence showed identity to homologues from other organisms. The recombinant protein encoded by the TspSP-1.3 clone was successfully expressed in the *E. coli* expression system in this study, and the recombinant protein was used to identify characteristics of the native gene product, including immunogenicity, preparation of antibodies, and protective immunity. However, we only obtained the inclusion body protein and could not activate it successfully, despite numerous efforts. To better characterize the functions of the serine protease, a eukaryotic expression system, such as yeast or baculovirus expression, should be used to obtain native TspSP-1.3 protease in the future.

TspSP-1.3 harbors several potential linear B cell epitopes predicted by bioinformatics, which showed that the serine protease could most likely induce B cell responses efficiently to trigger an antibody response. Western blotting analysis

showed the presence of antigenic epitopes of TspSP-1.3 recombinant protein in the infected serum of BALB/c mice, which were detected beginning in the early days post-infection, suggesting that TspSP-1.3 proteins might be secreted at early infection stage by the parasite into the host cell, inducing an early and highly antigenic response continuing to the muscle larvae stage. Furthermore, protective immunity results showed that the vaccination of mice with rTspSP-1.3 led to a significant reduction in the muscle larva burden, which was likely antibody dependent.

The immunoprotective effects of rTspSP-1.3 in this paper are similar to those reported from studies of a recombinant serine protease vaccine candidate (rTs-Adsp) in *T. spiralis* (Feng et al. 2013). The report (Feng et al. 2013) even showed that mice vaccinated with rTs-Adsp exhibited an average reduction in the muscle larvae burden of 46.5 % relative to the control group. However, the gene sequences of these two *Trichinella* serine proteases are totally different (Li et al. 2008; Wu et al., 2009). The rTsp-1.3 gene in this study was cloned with PCR using synthesized cDNA from 35-day PI muscle larvae, while the serine protease gene in Feng et al.'s paper (Feng et al. 2013) was obtained from the cDNA library of 3-day adult worms. In addition, other characteristics of these two serine protease genes are also distinct (Feng et al. 2013). Nonetheless, the fact that *Trichinella* has at least several serine protease genes, might indicate that a vaccine against one of the proteases will not give a very high protective immunity. Therefore, using a hybrid immunity strategy in the future would make the effect of immune protection better, according to the report that multiple antigens can elicit better immune responses (Lustigman et al. 2002).

Protective immunity against *Trichinella* infection is strongly associated with the production of IgG (Yang et al. 2008). IgG1 antibodies reflect a Th2 response, and IgG2a antibodies reflect Th1 cell activation (Else and Finkelman 1998). The strong antibody responses showed that immunization with rTspSP-1.3 significantly induced not only the Th1-like antibody response but also Th2-associated antibodies. We also analyzed the expression of IgE, and elevated levels of IgE antibodies may be associated with the clearance of *T. spiralis* worms at the intestinal mucosa level (Watanabe et al. 2005). The preliminary analysis suggests that the vaccination induced a mixed IgG2a/IgG1 (Th1/Th2) antibody response, with IgG1 (Th2) being relatively predominant. Our results are in agreement with some previous reports that demonstrated that combined Th1 and Th2 immune responses were important for immunity against *T. spiralis* infection, but Th2-type antibodies appear to be more important (Deville et al. 2005; Kołodziej-Sobocińska et al. 2006; Yang et al. 2010). Additionally, because we used a polyclonal anti-rTspSP-1.3 antibody in this study, the strongest epitope of TspSP-1.3 in producing protective immunity needs to be further confirmed in future studies to better understand the immunity effects of TspSP-1.3.

In conclusion, our results clearly demonstrate the characteristics of the serine protease TspSP-1.3 of *T. spiralis* with regard to molecular data, antigenic recognition and immunoprotective effects. The secretory serine protease TspSP-1.3 of *T. spiralis* induces strong humoral immune responses. The vaccinated mice showed significant protection against challenge with *T. spiralis* larvae. Collectively, these findings provide a valuable component for future vaccine development against trichinellosis. Although the mechanisms of action of the protective immunity of the serine protease TspSP-1.3 need to be better understood, our work provides a solid foundation for vaccine development in the future that will exploit a better scheme to improve protection or be combined with other effective stage-specific antigens to enhance the protective response.

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References

- Abbas AK, Murphy KM, Sher A (1996) Functional diversity of helper T lymphocytes. *Nature* 383:787–793
- Aucouturier J, Deville S, Perret C, Vallee I, Boireau P (2001) Assessment of efficacy and safety of various adjuvant formulations with a total soluble extract of *Trichinella spiralis*. *Parasite* 8(2 Suppl):S126–S132
- Bai X, Wu X, Wang X et al (2012) Inhibition of mammalian muscle differentiation by excretory secretory products of muscle larvae of *Trichinella spiralis* in vitro. *Parasitol Res* 110:2481–2490
- Balasubramanian N, Toubarro D, Simoes N (2010) Biochemical study and in vitro insect immune suppression by a trypsin-like secreted protease from the nematode *Steinernema carpocapsae*. *Parasite Immunol* 32(3):165–175
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S (2004) Improved prediction of signal peptides: SignalP 3.0. *J Mol Biol* 340:783–795
- Brown A, Burleigh J, Billett E, Pritchard D (1995) An initial characterization of the proteolytic enzymes secreted by the adult stage of the human hookworm *Necator americanus*. *Parasitology* 110:555–563
- Bruschi F, Chiumiento L (2012) Immunomodulation in trichinellosis: does *Trichinella* really escape the host immune system? *Endocr Metab Immune Disord Drug Targets* 12:4–15
- Criado-Fornelio A, de Armas-Serra C, Gimenez-Pardo C, Casado-Escribano N, Jimenez-Gonzalez A, Rodriguez-Caabeiro F (1992) Proteolytic enzymes from *Trichinella spiralis* larvae. *Vet Parasitol* 45:133–140
- Cui J, Wang Z, Xu B (2011) The epidemiology of human trichinellosis in China during 2004–2009. *Acta Trop* 118:1–5
- Dalton JP, Brindley PJ, Knox DP et al (2003) Helminth vaccines: from mining genomic information for vaccine targets to systems used for protein expression. *Int J Parasitol* 33:621–640
- Dea-Ayuela MA, Rama-Iniguez S, Bolas-Fernandez F (2006) Vaccination of mice against intestinal *Trichinella spiralis* infections by oral administration of antigens microencapsulated in methacrylic acid copolymers. *Vaccine* 24:2772–2780
- Dennis DT, Despommier DD, Davis N (1970) Infectivity of the newborn larva of *Trichinella spiralis* in the rat. *J Parasitol* 56:974–977
- Deville S, Pooter AD, Aucouturier J et al (2005) Influence of adjuvant formulation on the induced protection of mice immunized with total soluble antigen of *Trichinella spiralis*. *Vet Parasitol* 132:75–80
- Dixit AK, Dixit P, Sharma RL (2008) Immunodiagnostic/protective role of cathepsin L cysteine proteinases secreted by *Fasciola* species. *Vet Parasitol* 154:177–184
- Dupouy-Camet J (2000) Trichinellosis: a worldwide zoonosis. *Vet Parasitol* 93:191–200
- Dupouy-Camet J (2009) Presidential address of ICT12 Conference. “*Trichinella* and trichinellosis—a never ending story”. *Vet Parasitol* 159:194–196
- Else K, Finkelman FD (1998) Invited review intestinal nematode parasites, cytokines and effector mechanisms. *Int J Parasitol* 28:1145–1158
- Feng S, Wu X, Wang X et al (2013) Vaccination of mice with an antigenic serine protease-like protein elicits a protective immune response against *Trichinella spiralis* infection. *J Parasitol* 99(3):426–432
- Fishelson Z, Amiri P, Friend DS et al (1992) *Schistosoma mansoni*: Cell-specific expression and secretion of a serine protease during development of cercariae. *Exp Parasitol* 75:87–98
- Gottstein B, Pozio E, Nöckler K (2009) Epidemiology, diagnosis, treatment, and control of trichinellosis. *Clin Microbiol Rev* 22:127–145
- Hein W, Harrison G (2005) Vaccines against veterinary helminths. *Vet Parasitol* 132:217–222
- Hernandez-Bello R, Bermúdez-Cruz RM, Fonseca-Lián R et al (2008) Identification, molecular characterisation and differential expression of caveolin-1 in *Trichinella spiralis* maturing oocytes and embryos. *Int J Parasitol* 38:191–202
- Kennedy M, Qureshi F (1986) Stage-specific secreted antigens of the parasitic larval stages of the nematode *Ascaris*. *Immunology* 58:515–522
- Kołodziej-Sobocińska M, Dvorožnakova E, Dziemian E (2006) *Trichinella spiralis*: macrophage activity and antibody response in chronic murine infection. *Exp Parasitol* 112:52–62
- Lackey A, James ER, Sakanari JA et al (1989) Extracellular proteases of *Onchocerca*. *Exp Parasitol* 68:176–185
- Larsen JEP, Lund O, Nielsen M (2006) Improved method for predicting linear B-cell epitopes. *Immunome Res* 2:2
- Li LR, Fu BQ, Lu Q et al (2008) Cloning and analysis of serine protease gene of *Trichinella spiralis*. *Chin Vet Sci* 38:200–205
- Liu S, Tobias R, McClure S, Styba G, Shi Q, Jackowski G (1997) Removal of endotoxin from recombinant protein preparations. *Clin Biochem* 30:455–463
- Lustigman S, James ER, Tawe W, Abraham D (2002) Towards a recombinant antigen vaccine against *Onchocerca volvulus*. *Trends Parasitol* 18:135–141
- McGuire C, Chan WC, Wakelin D (2002) Nasal immunization with homogenate and peptide antigens induces protective immunity against *Trichinella spiralis*. *Infect Immun* 70:7149–7152
- McKerrow J, Pino-Heiss S, Lindquist R, Werb Z (1985) Purification and characterization of an elastolytic proteinase secreted by cercariae of *Schistosoma mansoni*. *J Biol Chem* 260:3703–3707
- McKerrow JH (1989) Parasite proteases. *Exp Parasitol* 68:111
- Nagano I, Wu Z, Nakada T, Boonmars T, Takahashi Y (2003) Molecular cloning and characterization of a serine proteinase gene of *Trichinella spiralis*. *J Parasitol* 89:92–98
- Newport GR, McKerrow JH, Hedstrom R et al (1988) Cloning of the proteinase that facilitates infection by *Schistosoma* parasites. *J Biol Chem* 263:13179–13184
- Quan FS, Matusmoto T, Lee JB et al (2004) Immunization with *Trichinella spiralis* Korean isolate larval excretory-secretory antigen induces protection and lymphocyte subset changes in rats. *Immunol Invest* 33:15–26
- Robison MW, Connolly B (2005) Proteomic analysis of the excretory-secretory proteins of the *Trichinella Spiralis* L1 larva, a nematode parasite of skeletal muscle. *Proteomics* 5:4525–4532

- Romaris F, North SJ, Gagliardo LF et al (2002) A putative serine protease among the excretory–secretory glycoproteins of L1 *Trichinella spiralis*. *Mol Biochem Parasitol* 122:149–160
- Stromberg B (1979) IgE and IgG1 antibody production by a soluble product of *Ascaris suum* in the guinea-pig. *Immunology* 38:489–495
- Stromberg BE (1980) Potentiation of the reaginic (IgE) antibody response to ovalbumin in the guinea pig with a soluble metabolic product from *Ascaris suum*. *J Immunol* 125:833–836
- Tang F, Xu L, Yan R, Song X, Li X (2012) Evaluation of the immune response induced by DNA vaccines expressing MIF and MCD-1 genes of *Trichinella spiralis* in BALB/c mice. *J Helminthol* 86:430–439
- Todorova V, Knox D, Kennedy M (1995) Proteinases in the excretory/secretory products (ES) of adult *Trichinella spiralis*. *Parasitology* 111:201–208
- Todorova VK, Stoyanov DI (2000) Partial characterization of serine proteinases secreted by adult *Trichinella spiralis*. *Parasitol Res* 86:684–687
- Trap C, Fu B, Le Guerhier F et al (2006) Cloning and analysis of a cDNA encoding a putative serine protease comprising two trypsin-like domains of *Trichinella spiralis*. *Parasitol Res* 98:288–294
- Wang B, Wang ZQ, Cui J (2012) Immunodiagnostic value of the recombinant 35.5 ku protein of *Trichinella spiralis*. *J Pathog Biol (China)* 7:602–605
- Wang Z, Cui J, Wei H, Han H, Zhang H, Li Y (2006) Vaccination of mice with DNA vaccine induces the immune response and partial protection against *T. spiralis* infection. *Vaccine* 24:1205–1212
- Watanabe N, Bruschi F, Korenaga M (2005) IgE: a question of protective immunity in *Trichinella spiralis* infection. *Trends Parasitol* 21:175–178
- Wu X, Fu BQ, Wang XL et al (2009) Identification of antigenic genes in *Trichinella spiralis* by immunoscreening of cDNA libraries. *Vet Parasitol* 159:272–275
- Yamamoto M, Sawaya R, Mohanam S et al (1994) Activities, localizations, and roles of serine proteases and their inhibitors in human brain tumor progression. *J Neurooncol* 22:139–151
- Yang J, Yang YP, Gu Y, Li Q, Wei JF, Wang SH, Boireau P, Zhu XP (2008) Identification and characterization of a full-length cDNA encoding paramyosin of *Trichinella spiralis*. *Biochem Biophys Res Commun* 365(3):528–533
- Yang Y, Zhang Z, Yang J, Chen X, Cui S, Zhu X (2010) Oral vaccination with Ts87 DNA vaccine delivered by attenuated *Salmonella typhimurium* elicits a protective immune response against *Trichinella spiralis* larval challenge. *Vaccine* 28:2735–2742
- Zhou XW, Mudannayake M, Green M et al (2007) Proteomic studies of PP2A-B56gamma1 phosphatase complexes reveal phosphorylation-regulated partners in cardiac local signaling. *J Proteome Res* 6:3433–3442