Molecular characterization and diagnostic potential of serine proteinase inhibitors from *Taenia solium*

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**Abstract**

Serine protease inhibitors (serpins) play essential physiological roles in a wide range of biological processes. Serpins are researched limited in *Taenia solium*, although some are considered to participate in host immune responses. Tsserpins were identified as typical serpins due to the primary structure of characteristic features: the serpin motif, serpin signature and reaction centre loop (RCL). RCLs of four serpin genes (TsB6, Ts4848, Ts12383 and Ts570) contained the conserved sequences of inhibitory serpins, which may involve in immune regulation. TsEP45 differed greatly from the patterns of representative serpins, suggesting that TsEP45 may be non-inhibitory. The bioinformatic analyses were supposed that Tsserpins might be a potential antigen for diagnosis. The five recombinant Tsserpin proteins were expressed and identified reacting with *Cysticercus cellulosae*-positive serum samples. The indirect enzyme-linked immunosorbent assay (iELISAs) based on Tsserpins were developed and validated, one of the five Tsserpins, TsEP45, showed excellent diagnostic results with 93.33% sensitivity and 94.12% specificity, respectively. This performance was in perfect accordance with the results of the bioinformatic analysis. This study provided a comprehensive demonstration of sequences and structural-based analysis of Tsserpins. The iELISAs based on five Tsserpins were developed and compared. TsEP45 was the potential species-specific antigen for developing iELISA to detect porcine cysticercosis.

**Introduction**

Proteases play crucial roles in the life cycle of various helminthic parasites. Parasite proteases are likely to be participated in host–parasite interactions and parasite metabolism, for instance, host tissue invasion and evasion of host immune response (Na et al. 2006). Serine protease inhibitors (serpins) are members of a superfamily proteins identified in a broad range of living beings, including viruses, bacteria, animals and plants (Irving et al. 2000; Gettins, 2002), with a characteristic well-conserved tertiary structure (Roberts et al. 1995). Serpins participate in numerous fundamental biological functions such as blood coagulation (Debrock and Declerck, 1997; Sugino et al. 2003), signalling cascades (O’Donnell and Blackman, 2005), fibrinolysis (Mak et al. 1996), inflammation (Thorgersen et al. 2007), activation of the complement system (Congote, 2007) and in mechanisms associated with host immune modulation (Chopin et al. 1988). Numerous serpins have been identified in parasitic helminths that were involved in immune regulation and parasite survival via interference with the host immune-stimulatory signals (Molehin et al. 2012).

*Taenia solium* is an important zoonotic parasite that causes a great threat to public health and economic loss in developing countries (Fleury et al. 2011). Despite quite a few researches regarding the biology and immunology of *T. solium*, the mechanisms associated with host immune evasion or the roles of serpins in these processes were still unknown. In other organisms, serpins contributed to inhibiting blood coagulation, damaging host proteases, and escaping from host immune attacks. In parasitic helminths, serpins appeared to play the similar roles (Molehin et al. 2012). Additionally, serpins were of considerable value as a diagnostic tool. *Schistosoma mansoni* serpins, Sm-SERPIN and Sm-RP26, showed significantly high reactivity to sera from infected rats (Molehin et al. 2014), suggesting that the immunodiagnostics potential of the serpins stemmed from the important physiological roles in host–parasite interactions. In view of the immunodiagnostics potential of serpins, the possible diagnostic value of Tsserpins should be explored.
Through the analysis of previously published *T. solium* genome and transcriptome data (Tsai et al. 2013) and structure-to-function bioinformatics analysis of the Tserpins’ polypeptides, five Tserpins were produced as recombinant proteins in *Escherichia coli* system and identified as diagnostic candidates. The indirect enzyme-linked immunosorbent assays (iELISAs) were established based on the five recombinant proteins to explore a sensitive and specific assay for the diagnosis of porcine cysticercosis.

### Materials and methods

#### Ethics statement

This study did not involve the use of endangered or protected species, and was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All experiments were conducted maintaining current China laws. The protocol was approved by the Committee on the Ethics of Animal Experiments of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

#### Parasite materials and serum samples

Healthy pigs of 40–60 days aged without cysticercosis were purchased from a local area in Gaolan, Gansu Province, China, and all pig blood samples were collected as negative control by saphenous bleeding before infection. Thirty pigs were orally infected with 3000 eggs of *T. solium* that was collected from Dali, Yunnan Province, China. Blood samples were collected by saphenous bleeding 60 days post-infection (dpi) (pigs were confirmed by necropsy examination). *Trichinella spiralis*-positive sera were from six pigs orally infected with 2000 infective muscle larvae (ML) at 35 dpi (pigs were confirmed by necropsy examination). The sera against *Toxoplasma gondii* were obtained from six pigs that were inoculated peritoneally with 1000 oocysts. *T. gondii* infection was confirmed by polymerase chain reaction (PCR) as described previously (He et al. 2016) at 15 dpi.

*Cysticercus tenuicollis* positive sera (six samples) were collected from naturally infected pigs from local slaughterhouse in Yongdeng, Gansu Province, China. Pigs were provided with non-medicated feed and water *ad libitum* in the experiment, housed in ventilated pigsty and monitored every day. At the end of experiments, all pigs were humanely sacrificed, and sera samples were collected and stored at −20 °C until use. Meanwhile, the parasites were isolated and stored at −80 °C until use in other experiments.

#### Identification of Tserpins

Source sequences encoding Tserpins were analysed utilizing BLAST (Basic Local Alignment and Search Tool) with the BLASTP and BLASTN algorithm against the Taenia Genome Database (TGD) available at http://taenia.big.ac.cn/taenia/index.html and *T. solium* gene annotation (http://192.168.51.199/index.html). The serpin sequences were confirmed by inspecting the expected length range of 350–450 amino acids (Molehin et al. 2012) and the presence of two highly conserved motifs (NAVYFKG and DVNEEG) (Han et al. 2000).

#### Phylogenetic analysis

Amino acid sequences of Tserpins and the representative parasite serpins from GenBank (accession numbers in Fig. 2) were submitted to the ClustalW 1.83. The phylogenetic analyses were performed by MEGA6.0 for tree building and TreeView for tree drawing.

#### Structure modelling

The secondary structure of *T. solium* serpins were predicted using PredictProtein (http://www.predictprotein.org/). The three-dimensional (3D) structure of Tserpins was predicted by Swiss-Model program. QMENA was used to estimate model reliability and predict quality. The predicted structures were aligned and viewed using DeepView-SWISS pdbViewer v4.1.

To gain insight on probable functionality, the deduced *T. solium* serpins amino acid sequences were scanned against amino acid motif entries, PROSITE, ScanProsite, SignalP and TMHMM servers (ExPASy bioinformatics Resource Server; http://www.expasy.org/proteomics). Putative N-glycosylation sites were identified by NetNGly1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/) and phosphorylation sites were predicted by NetPhos2.0 server (http://www.cbs.dtu.dk/services/NetPhos/). The Hydrophilicity Plot-Kyte-Doolittle, Flexible Regions-Karpplus-Schulz, Antigenic Index-Jameson-Wolf, Surface Probability-Emini were analysed employing DNASTar 7.1 software (DNASTar, USA) to predict the potential linear B-cell epitopes of *T. solium* serpins.

#### Cloning and expression of Tserpins

The full-length coding sequences were amplified from *T. solium* cysticerci cDNA by PCR using Trizol reagent (Invitrogen, USA), according to the manufacturer protocol. All reagents for RNA extraction were prepared using diethylpyrocarbonate (DEPC) treated water. The concentration and integrity of extracted RNA samples were determined with a Nandrop spectrophotometer (Thermo Scientific). cDNA was synthesized from total RNA by One-step Reverse Transcription Kit (clontech) following the manufacturer’s instruction.

#### Total RNA extraction and cDNA synthesis

Total RNA was extracted from 30 mg *T. solium* cysticerci using Trizol reagent (Invitrogen, USA), according to the manufacturer protocol. All reagents for RNA extraction were prepared using diethylpyrocarbonate (DEPC) treated water. The concentration and integrity of extracted RNA samples were determined with a Nandrop spectrophotometer (Thermo Scientific). cDNA was synthesized from total RNA by One-step Reverse Transcription Kit (clontech) following the manufacturer’s instruction.
**Protein purification and immunoblotting**

Recombinant proteins were purified by a Ni2⁺ Sepharose™ 6 Fast Flow purification kit as the manufacturer’s instructions (GE, USA). Concentrations of purified protein samples were detected by Pierce™ BCA (bicinchonininc acid) protein assay kit (Thermo Scientific, USA) and identified by Western blotting. Briefly, the recombinant proteins were isolated by SDS–PAGE and transferred to nitrocellulose membrane, and then the membrane was incubated with the positive serum from porcine cysticercosis (1:200 dilution) and subsequently incubated with horseradish peroxidase (HRP)-conjugated rabbit anti-pig IgG (1:10 000 dilution, Sigma), the reactions were detected with DAB (diaminobenzidine).

**Development of iELISA based on Tsserpins**

Purified Tsserin were diluted in coating buffer (0·05 M carbonate-bicarbonate buffer, pH 9·6) to a final concentration of 5 µg mL⁻¹. The microtitre plates were coated with Tsserin protein (100 µL well⁻¹) and blocked with 1% (w/v) bovine serum albumin in PBST (Phosphate Buffered Saline with Tween-20) for 1 h at 37 °C. After three times of washing with PBST, the plates were incubated with diluted positive serum samples (1:200) from pigs infected with *T. solium* (*n* = 30), *C. tenuicollis* (*n* = 6), *T. gondii* (*n* = 6), *T. spiralis* (*n* = 6) and 51 negative control sera samples, respectively. The plates were washed three times and incubated with HRP-conjugated anti-pig IgG (1:10,000) for 30 min at 37 °C. After washing, the enzyme reaction was detected with TMB (3,3′,5,5′-tetramethylbenzidine) (Sigma, USA). The optical density (OD) value was measured at a wavelength of 450 nm with spectrophotometer (Bio-RAD).

**Statistical analysis**

Receiver operating characteristics (ROC) (Greiner et al. 2000) analysis was performed to assess the diagnostic value of antigens. One-way ANOVA tests were performed to compare all pairs of groups. Statistical significance was set at *P* < 0·05. Data analysis was showed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, USA).

**Results**

**Cloning and general characteristics of Tsserpins**

The cloned full-length cDNA and general characteristics of Tsserpins are listed in Table 1. According to the MEROPS (http://merops.sanger.ac.uk/inhibitors/index.shtml) classification of protease inhibitors (Rawlings et al. 2014), Tsserpins are members of inhibitor family 14 (Clan ID). Multiple sequence alignment of Tsserin amino acid sequences showed low overall identity (21–69%) with representative serpins from GenBank (Fig. 1). Analysis of Tsserin peptides indicated the presence of three conserved motifs, the serpin signature, serpin motif and the reactive centre loop (RCL) (Table 2). The cleavage sites of the RCL are located near the C-terminal at position 342 M-343C of TsB6, 344R-345C of Ts12383, 316R-317C of Ts4848, 364 M-365S of Ts570 and 383N-384Q of TsEP45, respectively. Analysis of Tsserin RCLs (Table 2) showed that the hinge region contained the typical consensus sequences, with a characteristic ‘P₁₇[E] – P₁₈[E/K/R] – P₁₉[G] – P₂₀[T/S] – P₂₁[X] – P₁₂–₉[AGSX] – P₉–₁[X] – P₁–₉′ common to all inhibitory serpins (Gettins, 2002), where the amino acid residues are defined as ...P₄–P₅–P₁( cleavage site).P₁ – P₂ – P₃–... and X is any amino acid. The RCL of TsB6, Ts4848 and Ts12383 were highly similar to typical pattern of RCL, while Ts570 was with residue variation only at position P₁₆ and P₁₇. Unexpectedly, TsEP45 differed greatly from the patterns of other serpins, which showed several variations: P₁₄ [I] and P₁₅–₉ [LLSV]. No signal peptide or transmembrane domain was found in the peptide sequences. Other post-translationally modification sites, such as CAMP- and cGMP-dependent protein kinase phosphorylation, casein kinase II phosphorylation, N-myristoylation, protein kinase C phosphorylation and tyrosine kinase phosphorylation sites were predicted to present in the five Tsserpins (Table 3S). According to the analysis of hydrophilicity, flexible regions, antigenic index and surface probability, potential B cell epitopes also might be present in all Tsserpins (Table 4S).

**Phylogenetic analysis**

Phylogenetic analysis showed that the Tsserpins were distributed on different evolutionary branches (Fig. 2). Tsserin all had the high-sequence homology to *Echinococcus granulosus* serpins: TsB6 shared 67% sequence identity with SerpinB9 (Accession number EU860253.1), Ts4848 and Ts12383 shared 79% to *E.granulosus* Serpin (Accession number CDS22753.1), Ts570 shared 75% with *E.granulosus* Serpin (Accession number EU63679.1). TsEP45 was closely related to *E.granulosus* Serpin (Accession number EU63680.1) with 81% sequence identity. Tsserin were distantly related to those of other helminths, especially the evolutionary status of TsEP45 and Ts570 were very ancient, far away from the serpins of several common porcine parasites, including *Ascaris suum* and *T. spiralis*.

**Structure-based alignment analysis of Tsserpins**

The potential tertiary structure of a protein reveals several specific biological functions. 3D protein structures of Tsserpins were predicted by homology modeling. Firstly, template structures were developed by SWISS-MODEL. The search results (Fig. 3) indicated that TsB6, Ts4848 and Ts12383 shared similar structure to SerpinB3 (2zv6, *Homo sapiens*, human), the structure of TsEP45 was similar to the serine protease inhibitor (3sto, *Schistosoma haematobium*), and Ts570 had a similar structure to antithrombin-III (2hij, *H. sapiens*, human). Ramachandran plotting indicated that the structure models were optimal (data not shown).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>GeneID</th>
<th>ORF(bp)</th>
<th>NO.of aa</th>
<th>MW</th>
<th>PI</th>
<th>SP</th>
<th>TMHMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>TsB6</td>
<td>KY045842</td>
<td>1131</td>
<td>376</td>
<td>42.3</td>
<td>5.89</td>
<td>No</td>
<td>No</td>
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<tr>
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<td>7.74</td>
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<td>No</td>
</tr>
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<td>5.7</td>
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</tr>
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<td>382</td>
<td>42.11</td>
<td>5.5</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

SP, Signal peptide; TMHMM, Transmembrane.
Expression and identification of Tsserpins

The PCR products were obtained (Fig. 1S) and inserted into pET-30a (+) vector. Then the recombinant plasmids pET-30a (+)-Tsserpins were confirmed by enzyme digestion (Fig. 2S) and DNA sequencing.

The recombinant pET-30a (+)-Tsserpins were expressed and analysed by Western blotting. SDS–PAGE analysis showed the recombinant proteins were mainly found in the pellet (Fig. 3S). The peak expression was observed at 37 °C when induced with IPTG (isopropyl-β-D-thiogalactoside) at 1.0 mM L\(^{-1}\), following 6 h induction. Tsserpins were purified by Ni\(^{2+}\)-affinity chromatography, and a single band for each of recombinant proteins in SDS–PAGE indicated that the recombinant serpins were free of obvious contaminants (Fig. 4). Immunoblotting results showed Tsserpins were reacted with the positive T.solium cysticercus sera (Fig. 5). And no reaction was observed between Tsserpins and the negative control sera from healthy animals.

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Evaluation of the serodiagnostic potential of Tsserpins

To evaluate the possibility of Tsserpin as antigens for diagnosis of cysticercosis, we compared the reactivity of the Tsserpin antigens and various serum samples from pigs experimentally infected Cysticercus cellulosae (n = 30) and negative samples with iELISA. The results showed that TsEP45, TsB6 and Ts570 were specifically reactive with sera from T. solium cysticercosis (Fig. 6). There was no cross-reactivity with positive samples of C. tenuicollis, T. gondii and T. spiralis. The remained two antigens, Ts4848 and Ts12383, detected without disease-specific reactivity (Fig. 6E and 6F), although diagnostic results of positive sera samples from infected pigs with C. cellulosae were higher than those of negative ones.

To validate of diagnostic performance of iELISAs based on TsEP45, TsB6 and Ts570, ROC analysis was employed for further evaluation. The cut-off values of TsEP45, TsB6 and Ts570 were calculated by sera from 51 uninfected pigs (negative control) and 30 C. cellulosae positive sera. The ROC curve and the corresponding statistics were summarized in Fig. 7. It can be concluded that TsEP45 showed the better diagnostic value than TsB6 and Ts570 and showed acceptable results to be a diagnostic antigen in iELISA.

Discussion

Tserpins were identified as typical serpins due to the presence of characteristic features such as the serpin motif, serpin signature and RCL (Rawlings et al. 2014). Additionally, secondary and tertiary structure prediction analyses indicate that Tserpins may contain 8–9 α-helices and three β-sheets, features were consistent with the known serpins (Huntington, 2006). The results of the predicted tertiary structure of Tserpins (Fig. 3) showed that the RCLs of native inhibitory serpins may be exposable and accessible to target proteases, which further strengthens the argument that Tserpins belong to the serpin superfamily (Rawlings et al. 2014). These observations allowed us to explore Tserpin biological function based on comparative modelling of known serpins. The conserved 20/21 residue peptide ‘P17[E]-P18[E/K/R]-P19[G]-P20[T/S]-P21[X]-P22[S/L]-P23[L/S]-P24[R/E]’ within the RCL determined whether the serpin was inhibitory or non-inhibitory (Irving et al. 2000). The primary structures of inhibitory and non-inhibitory serpins have different amino acids of the hinge region (P15 to P21), whereas they are well conserved in inhibitory serpins. In general, P15 is glycine, P16 threonine or serine, P12 to P16 are all alanine residues in most inhibitory serpins. Analysis of Tserpins showed that TsB6, Ts12383, Ts570 and Ts4848 have typical serpin structures, suggesting that they are functional inhibitory serpins. Further analysis of the hinge regions of TsB6, Ts12383, Ts570 and Ts4848 suggested that they are occupied predominantly by hydrophobic amino acid residues, which are deemed to be the construction of the skeleton conformation needed for the inhibitory activity (McCarty and Worrall, 1997). However, the corresponding regions of non-inhibitory serpins do not conform to this consensus sequence, for example, TsEP45 shows some variations: P14 [I] and P12 [I].

To our knowledge, the divergent functions or the specificity of target proteases mainly depends on the variety of RCLs. The target proteases mostly cleave the bonds between P1 and P2 in RCLs (Silverman et al. 2001), which suggested that serpins with different amino acid residues of P1 and P2 acted on diverse target proteases. According to the reference (Merrickbach and Ruppel, 2007) of the amino acid sequence of serpin<sub>1am</sub>, R<sub>34</sub> (arginine) can be identified as the P1 residue of Ts12383 and Ts4848, as well as cysteine is found at P1. Arginine at P1 could explain...
readily the inhibition of trypsin and pancreatic elastase (PE) since these proteinases cleave their substrates with high specificity after basic amino acids (Merckelbach and Ruppel, 2007). Likewise, the intracellular serpin B6 (human) inhibits trypsin via a P1-R (Riewald and Schleef, 1996). Therefore, we can envisage that Ts12383 and Ts4848 have potential to inhibit the activity of trypsin and PE. Based on the amino acid sequence of α-1-proteinase inhibitor (α1-antitrypsin), M (methionine) can be identified as the P1 residue of TsB6 and Ts570. Human neutrophil elastase (NE) was inhibited by α1-antitrypsin through P1-M. So, we can speculate that TsB6 and Ts570 have similar function to α1-antitrypsin inhibiting the activity of NE. In summary, P1-P1′ residues were very important in the RCL, and P1 played a crucial role in defining specificity (Potempa et al. 1994). Some serpins were specialized for single proteases, while others inhibited more than one substrate; further in vitro studies could provide evidence of the range of protease inhibition. The predictions

Fig. 3. Predicted tertiary structures of Tsserpins. 3A: TsEP45; 3B: Ts570; 3C: Ts4848; 3D: Ts12383; 3E: TsB6; The purple was represented the RCL. The green and red were represented the β sheet, the blue was represented the α helices. The figure shows the Tsserpins in their native conformation with the RCL being surface accessible by target proteases.

Fig. 4. Purification of recombinant Tsserpins proteins. M: Protein molecular weight Markers.

Fig. 5. Immunoblot analysis of recombinant T.solium serpins using the positive serum from porcine Cysticercosis. M: Protein molecular weight Markers, P: the positive serum from porcine Cysticercosis, Pc: Pig negative serum.
Fig. 6. Species-specific differential detection by Tsserpins. The iELISA was performed using samples from T. solium cysticercus (Ts, n = 30), C. tenuicollis (Ct, n = 6), T. spiralis (Tr, n = 6), and T. gondii (Tg, n = 6) experimentally infected pigs, respectively. Negative control samples (Nc, n = 51) from uninfected areas. The bar lines represent the median while the error bars represent the interquartile range. Vertical bars were SEM in panel A. Antigens used in this assay: (6A) the reactivity of the Tsserpins antigens in indirect ELISA; (6B) Ts570; (6C) TsEP45; (6D) TsB6; (6E) Ts4848; (6F) Ts12383. Each data point represents an average of three ELISA replicates. Statistical significance was set at $P < 0.05$ as depicted using asterisks: * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Fig. 7. ROC analysis for TsEP45, TsB6 and Ts570. ROC analysis was performed to determine the area under the curve (AUC), Likelihood ratio (LR), sensitivity and specificity as indicators of the diagnostic value of the TsEP45, TsB6 and Ts570. The TsEP45 showed relatively better diagnostic ability than TsB6 and Ts570. A: AUC, The AUC values close to 1.0 indicate an informative test; and close to 0.5 indicate an uninformative test (Hanley and McNeil, 1982). The value of LR greater than 10 (Jaeschke et al. 1994) practically confirmed the results of diagnosis.
above were contributed to a better understanding of *T. solium* in evading the host immune response. If the Tsserpins were secreted during the oncosphere infection phase, it might be able to block the proteolytic attack of host digestive enzymes, which could be the targets of the intestinal immune system and vaccine candidates. Phylogenetic analysis showed that Tsserpins are distributed on different evolutionary branches, suggesting the serpins had functional polymorphism and early evolutionary divergence. The Tsserpins in the same branch might share similar function, such as Ts12383, Ts4848 and serpin<sup>imm</sup> (*Echinococcus multilocularis*). In addition, the results of phylogenetic analysis showed that the evolutionary status of TsEP45 was very ancient, far away from the serpins of several common porcine parasites, including *A. suum*, *T. gondii* and *T. spiralis*, suggesting that TsEP45 was a special serpin in common parasites and has potential to act as a species-specific antigen for developing iELISA to detect porcine cysticercosis.

Even though Tsserpins do not contain signal peptide sequences by bioinformatics analysis, they would be released extracellularly through a special pathway independent of the proteolytic pathway. By collecting and analysing the data, and GXL performed all experiments and wrote the first draft of the paper. PHL and LJW contributed to laboratory work. LM, YDZ, SHZ, AJG, JLH and XNL critically reviewed the manuscript for the important intellectual content. All authors read and approved the final version of the manuscript.

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**Conflict of interest.** None.

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