Published in final edited form as:

Mol Biochem Parasitol. 2011 December ; 180(2): 115–119. doi:10.1016/j.molbiopara.2011.08.003.

TsAg5, a *Taenia solium* cysticercus protein with a marginal trypsin-like activity in the diagnosis of human neurocysticercosis

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Abstract

Neurocysticercosis is an endemic parasitic disease caused by *Taenia solium* larva. Although the mechanism of infection is not completely understood, it is likely driven by proteolytic activity that degrades the intestinal wall to facilitate oncosphere penetration and further infection. We analyzed the publicly available *Taenia solium* EST/DNA library and identified two contigs comprising a full-length cDNA fragment very similar to *E. granulosus* Ag5 protein. The *Taenia solium* cDNA sequence included a proteolytic trypsin-like-domain in the C-terminal region, and a thrombospondin type-1 adherence-domain in the N-terminal region. Both the trypsin-like and adherence domains were expressed independently as recombinant proteins in bacterial systems. TsAg5 showed marginal trypsin-like activity and high sequence similarity to Ag5. The purified antigens were tested in a Western immunoblot assay to diagnose human neurocysticercosis. The sensitivity of the trypsin-like-domain was 96.36% in patients infected with extraparenchymal cysts, 75.44% in patients infected with multiple cysts, and 39.62% in patients with a single cyst. Specificity was 76.70%. The thrombospondin type-1 adherence-domain was not specific for neurocysticercosis.

Keywords

trypsin; adherence; cysticercus; oncosphere; protease; diagnostics

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Taeniasis/cysticercosis caused by the cestode *Taenia solium* has been recognized as an increasing cause of human disease and its major complication, neurocysticercosis (NCC), is an important cause of morbidity accounting for more cases of epilepsy than any other pathology in these areas [1]. *T. solium* has a life cycle involving 2 hosts. Humans are the only known definitive host for the adult worm, and pigs are the usual intermediate hosts for the larval form. Pigs ingest complete proglottids or *T. solium* eggs through consumption of human feces or food contaminated with fecal matter, resulting in invasion of oncospheres and development of muscular and brain cysts. Similarly, ingestion of *T. solium* eggs by humans through fecal oral contamination causes human NCC.

The molecular mechanism by which *T. solium* oncospheres invade the intestinal epithelium is not known, however peptidase activity is widely used by many parasites to penetrate host tissue [2]. Oncospheres of helminths including *Taenia saginata* secrete serine-, cysteine-, metallo- and amino-peptidases [3]. Serine proteases, in particular chymotrypsin-like and trypsin-like peptidases, are found in the excretory/secretory antigens of *T. solium* and *T. saginata* oncospheres [4].

One well-characterized example of an excretory protein with trypsin-like activity is Antigen 5 (Ag5) of *Echinococcus granulosus*, one of the major antigens in and the causative agent of cystic hydatidosis. Ag5 is a thermolabile and highly immunogenic protein composed of 2 subunits of 38 kDa and 22 kDa [5]. The sequence of the 38 kDa subunit shows that it is closely related to serine proteases of the trypsin family. The 22 kDa subunit has heparan sulphate proteoglycans in the form of a glycosaminoglycan-binding adherence motif and calcium binding sites, which favors interactions with cell surfaces and the extracellular matrix [6].

In the present study, we characterize a *T. solium* homologue to the *E. granulosus* Ag5. This protein, dubbed TsAg5, is a trypsin-like protease linked to a thrombospondin type-I adherence-domain from the *T. solium* cysticercus. We produced this protein recombinantly, measured its proteolytic activity, identified its presence in the cyst and the oncosphere, and evaluated its antigenicity in human cysticercosis by Western immunoblot.

We obtained the cDNA sequence of Ag5 from the GenBank (ID: AAL14214), and used it as a query to identify its homologue in *T. solium*. A translated BLAST homology search against the *T. solium* partial genome database, publicly available (<http://132.248.32.30/karel/>), was performed locally. Two reported *T. solium* contigs including sequences of high similarity to Ag5, were aligned and assembled to provide the open reading frame (ORF) of the putative protein. The ORF of the assembly was determined using ORF-Finder [7]. In order to determine the cellular localization of the putative protein, the Phobius web server (<http://phobius.sbc.su.se>) was used to identify signal peptides and transmembrane segments [8], and the lipoP web server (<http://www.cbs.dtu.dk/services/LipoP>) was used to identify lipoprotein attachment sites [9]. Pfam search was performed to identify potential functional domains and/or motifs within the putative protein.

The assembled contigs encoded a novel protein from *T. solium* that is homologous to *E. granulosus* Ag5 (from now on referred to as TsAg5). A 1437 bp cDNA ORF for *T. solium* including a trypsin-like domain was predicted on the assembly and was deposited in GenBank as TsAg5 (ID: HQ393478). The translated amino acid sequence included a signal peptide in the first 21 amino acids (GenBank ID: ADP89566). Pfam analysis identified a conserved thrombospondin type-1 domain (TSP-1) in the N-terminal region, very similar to the glycosaminoglycan-binding adherence motif identified in the N-terminal region of Ag5. The C-terminal region contained a trypsin-like domain. A cleavage site was identified (Leu-

Lys dipeptide). Identity between TsAg5 and Ag5 DNA sequences was 84%; protein comparison showed 82% identity. Neither transmembrane regions nor lipoprotein attachment sites were detected; therefore TsAg5 is likely to be a secreted protein.

T. solium cysticerci were collected from naturally infected pigs. Cysts were freeze-dried for transport and were stored at -70°C until use. Total RNA was extracted from cysticerci using Trizol (Invitrogen™ Life Technologies, Carlsbad, CA) following the manufacturer's protocol. cDNA was produced from RNA by reverse transcription with oligo dT following the protocol from Applied Biosystems. The primer sequences for the adherence-domain were: forward, 5'-CTA GCT AGC CTC ACC CTC CAG CCG GAT G-3' and reverse, 5'-CGG GAT CCT CAG CTG TAT AGT GAC CTT TC-3' and for the trypsin-like domain were: forward, 5'-CTA GCT AGC ATT CTT AAC GGG AAA ATT GC-3' and reverse, 5'-CGG GAT CCT TAG ACT GTG TAG CGG TTG-3'. The amplified PCR products were purified and cloned in pET28a (Novagen) containing a six-histidine tag on the carboxy-terminal end. *Escherichia coli* Novablue cells (Novagen, San Diego, CA) were transformed directly from the ligation reaction according to the heat shock protocol. Plasmid DNA was extracted with the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and sequenced in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystem, Foster City, CA) in both directions. After verifying the sequence, the plasmids containing the adherence and trypsin-like domain sequences were sub-cloned in a bacterial expression system (*E. coli* BL21 (DE3) pLysS). Transformed cells were grown and lysed by repeated freeze-thaw cycles followed by sonication using a S3000 sonicator (Misonix, Farmingdale, NY). The insoluble fraction was loaded on a His-bind column (Pharmacia, Farmingdale, NJ) in buffer containing 5mM urea. The bound protein was eluted with different concentrations of imidazole ranging from 50 to 500 mM. The soluble fraction was treated similarly but without urea. Aliquots of the fractions obtained were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

According to the SDS-PAGE, the molecular weight of the recombinant adherence and trypsin-like domains were 24.1 kDa and 38.7 kDa respectively. These are 5–6 kDa higher than the theoretical values, probably due to post-translational modifications that are not reproduced in the *E. coli* expression system. In the expression, both domains appeared mainly in the inclusion bodies. However, evidence of the trypsin-like domain was observed in the soluble fraction. The insoluble trypsin-like domain that showed the highest purity according to the SDS-PAGE corresponded to the fraction eluted with 100 mM imidazole, while the soluble domain was eluted with 300 mM imidazole. The insoluble adherence-domain with the highest purity eluted with 100 mM imidazole. The insoluble fractions were solubilized with 8 M urea and purified at a final concentration of 1mg/ml. The soluble fraction of the trypsin-like domain was purified at a final concentration of 12.8 $\mu\text{g/ml}$. The presence of the adherence and trypsin-like domains was confirmed as a single band in a Coomassie blue stained SDS-PAGE (data not shown).

Proteolytic activity was measured in the soluble fraction of the purified TsAg5 trypsin-like domain that eluted with 300 mM imidazole. Trypsin-like activity was detected fluorometrically by the hydrolysis of Z-Arg-AMC (MP Biomedicals, Aurora, Ohio) [10] following the procedure described in our previous study [4]. The free AMC produced after the cleavage of the peptide was fluoresced, and the intensity of the fluorescence detected in a TD-700 fluorometer (Turner Designs, Sunnyvale, California) at 360 nm excitation and 460 nm emission wavelengths was used to determine the peptidase activity. Briefly the hydrolysis of Z-Arg-AMC was tested at pH 7.3 without DTT to reduce cysteine peptidase interference, thus detecting mainly trypsin-like activity, as confirmed by inhibition with chymostatin (pre-incubation of the purified protein with 0.1 mM chymostatin for 10 min at room temperature). The proteolytic activity was estimated as the concentration of

hydrolyzed substrate per hour, per amount of enzyme following the procedure previously reported [4].

As a double control, the trypsin-like fraction was tested for cysteine protease activity with the substrate Z-Phe-Arg-AMC and by inhibition with trans-Epoxy succinyl-L-Leucylamido-(4-Guanidino) Butane (E64) following the protocol described before [11]. As positive controls, commercial trypsin from Boehringer Mannheim GmbH (Mannheim, Germany) and purified cathepsin L-like protease [11], were tested.

The trypsin-like activity of the TsAg5 trypsin-like domain was estimated as $3.87 \times 10^{-6} \mu\text{M AMC} \times \text{h}^{-1} (\mu\text{g/ml protein})^{-1}$. TsAg5 did not show a significant trypsin-like activity after treatment with chymostatin and did not show any significant cysteine protease activity. The estimated activity of the commercial trypsin was $1.04 \times 10^{-2} \mu\text{M AMC} \times \text{h}^{-1} (\mu\text{g/ml protein})^{-1}$ and it was inhibited after treatment with chymostatin. The purified cathepsin L-like antigen (cysteine protease positive control) showed a high cysteine protease activity that was inhibited by E64.

TsAg5 was detected in the cyst fluid and in the excretory/secretory antigens of the cysticercus. Metacercariae of *T. solium* were individually collected from muscle tissue of naturally infected pigs. Cyst fluid was obtained by direct aspiration of the liquid content with a syringe. Cysts were cultivated for 72 hours in 12-well plates as reported previously [12]. After each 24-hour period, the supernatant of each well containing excretory/secretory (ES) antigens was harvested, collected and replaced. The collected ES antigens were resolved in an SDS-PAGE and revealed by Western immunoblot using pre-immune and post-immune anti-TsAg5 rabbit sera as previously described [13]. To confirm the presence of TsAg5 in the cyst, a complementary Western immunoblot was performed having the TsAg5 trypsin-like domain and the TsAg5 adherence domain as antigens, and revealed with rabbit anti-cysticercus sera (sera from a rabbit immunized with total cysticercus antigens). Pre-immune sera were used as a control.

The oncosphere total antigens and the ES antigens from the cysticercus showed positive reactive bands at 38 and 24 kDa in the Western immunoblot against rabbit anti-TsAg5 trypsin-like domain and anti-TsAg5 adherence domain sera, respectively (figures 1A, 1B). The TsAg5 fraction reacted positively in the Western immunoblot against rabbit anti-oncosphere and rabbit anti-cysticercus sera, showing reactive bands at 38 and 24 kDa (figures 1C, 1D). Additionally, the cysticercus fluid and the cysticercus ES antigens showed a positive trypsin-like activity by the hydrolysis of the fluorogenic Z-Arg-AMC substrate, and the inhibition with chymostatin, tested as previously described [4]. In a previous study, we demonstrated that the oncosphere antigens were able to hydrolyze the Z-Arg-AMC substrate, and were inhibited by chymostatin [4].

Additionally, TsAg5 was detected in the oncosphere. The total antigens from metabolically activated oncospheres were obtained following the procedure previously reported [14, 15]. Oncospheres were activated by incubation at 37°C for 45 min with artificial intestinal fluid [Sigma Chemical Co., St. Louis, MO], 200 mg Na₂CO₃, and 1 ml pig bile, following the protocol previously described [15]. The oncosphere total antigens were resolved in an SDS-PAGE and revealed by Western immunoblot using pre-immune and post-immune rabbit sera against the trypsin-like domain and the adherence domain. To confirm the presence of TsAg5 in the oncosphere, a complementary Western immunoblot was performed using the TsAg5 trypsin-like domain and the TsAg5 adherence domain as antigens, revealed with rabbit anti-oncosphere sera (sera from a rabbit immunized with total oncosphere antigens) (figures 1C, 1D, lane d). Pre-immune serum was used as a control.

The TsAg5 trypsin-like domain was tested in a Western immunoblot for human NCC. The adherence-like domain was not tested further because it was not specific in the checker board. The protein pattern in the Western immunoblot showed a specific band of 38.7 kDa when paired with strong positive and negative control sample sera (Figure 2). A nonspecific reaction was observed above this diagnostic band, probably due to contaminating antigens not eliminated during the purification. Since antibody reactivity on Western immunoblot varies according the type and stage of NCC, we used a homogeneous battery of 165 sera from patients with viable parenchymal NCC (1 to 20 viable brain cyst) and did not include patients with calcified disease or a single degenerating lesion, to allow sound comparison of our results with other works.

The immunodiagnostic performance was assessed in 361 sera samples from patients with a defined diagnosis of NCC, other parasitic infections, or healthy controls. Samples were selected from the archives of the Unit of Research in Infectious Diseases of the Universidad Peruana Cayetano Heredia, in Lima, Peru. To estimate sensitivity, we tested 165 positive sera from patients with NCC confirmed by detection of viable cysts in the brain with image-based diagnosis and LLGP-EITB positive tests. 53 samples corresponded to patients with a single intraparenchymal cyst; 57 samples corresponded to patients with 2 or more intraparenchymal cysts; and 55 samples corresponded to patients with extraparenchymal NCC. To estimate specificity, we tested 103 negative control sera corresponding to individuals from Iquitos, a town in the northern Peru, where *T. solium* is non-endemic but other helminth parasites are common.

The sensitivity of the trypsin-like domain against extraparenchymal NCC ranged from 94.55 – 96.36%. For multiple cysts ranged from 68.42 – 75.44%, and for single cysts ranged from 37.74 – 39.62%. The specificity ranged from 74.76 – 76.70%, (Table 1). The high sensitivity in the extraparenchymal NCC cases is due to the larger size and exposed surface, as well as the higher secretion of antigens in this stage of the parasite, which is metabolically more active due to the lack of contention tissue-barriers. Antigen levels are about 10 times higher in extraparenchymal NCC (particularly subarachnoid NCC) than in intraparenchymal NCC [16, 17]. The adherence-domain was not specific, and was not examined further. Based on three readings (the two readings from observer-1 and the reading from observer-2) of 93 sera samples from other parasites, patients with *E. granulosus* (1/16), *T. saginata* (1/10) and *H. nana* (6/28) cross-reacted in the Western immunoblot assay. No other sera tested showed a cross-reaction in the dot-ELISA. The inter-observer agreement for assessment of positive and negative sera between the first reading of observer-1 and the reading of observer-2 was 79.02% ($\kappa=0.6563$, $P<0.001$). The intra-observer agreement (estimated by comparing the first and second readings of observer-1) was 79.31% ($\kappa=0.68$, $P<0.001$).

In a previous study, we found that the excretory/secretory antigens of *T. solium* oncospheres are associated with cysteine-, serine-, and aminopeptidase proteolytic activity [4]. Although a wide pattern of proteolytic activity was observed, chymotrypsin-like and trypsin-like peptidases showed the highest activity, indicating that serine proteases are the most abundant secreted antigens in the oncosphere.

Previous studies using recombinant Ag5 from *E. granulosus* in Western immunoblot and ELISA assays to detect hydatidosis showed good sensitivity but a moderate specificity [6, 18]. Only 65% of the native-Ag5 positive hydatid disease serum samples tested positive with the recombinant complete Ag5, and just 21% gave positive results with the recombinant trypsin-like domain [18].

The recombinant trypsin-like domain of TsAg5 showed a moderate sensitivity and specificity in Western immunoblot assay for human cysticercosis on patients with multiple

cysts and extra-parenchymal NCC. These cases are considered the most serious, and are associated with the highest mortality [19] and account for about 70% of all NCC cases (H. García, personal communication).

The extreme levels of inter- and intra-observer variability (about 20%) are explained by the subjectivity of the Western immunoblot assay. Using an objective assay like an ELISA could prevent this limitation; however, for this it would be necessary to obtain the recombinant antigens with a higher purity.

The Western immunoblot pattern of the TsAg5 trypsin-like domain shows a nonspecific band just above the 38 kDa diagnostic band. However, this band is not observed in a Coomassie blue stained SDS-PAGE. This is probably a protein contaminant from *E. coli* that, although present in low concentration, is highly reactive in the Western immunoblot.

One limitation of the trypsin-like recombinant antigen includes a moderate cross-reaction in Western immunoblot: 6/28 (21.43%) cross-reacted against *H. nana*, which is a common parasite in developing countries; 1/16 (6.25%) cross-reacted with *E. granulosus*; and 1/10 (10%) cross-reacted with *T. saginata*. It should be stated, however, that *E. granulosus* and *T. saginata* are closely related to *T. solium* and have always shown high cross-reaction with immunodiagnostic tests for cysticercosis [20], and the cross-reaction with *H. nana*, the highest rate of cross-reaction in our study, has been previously shown in another study [21]. Although the endemic prevalence of *H. nana* is high [22], the impact of the diagnostic cross-reaction would not likely be significant since clinical symptoms can differentiate the two infections [23].

In conclusion, this study demonstrates the existence of a secreted protein from *T. solium* cysticercus, TsAg5, associated with a marginal trypsin-like activity that is homologous to Ag5 from *E. granulosus*. TsAg5 comprises a C-terminal trypsin-like domain of 38 kDa and a N-terminal thrombospondin type-1 adherence-domain of 24 kDa that is highly similar to the glycosaminoglycan-binding motif of Ag5.

Highlights

>TsAg5 was found in the excretory/secretory antigens of the cysticercus and in the oncosphere. >The trypsin-like domain of TsAg5 showed a marginal proteolytic activity. >The trypsin-like domain showed to be an important antigen for immunodiagnoses of multiple cyst and extra-parenchymal NCC. >The adherence domain of TsAg5 was not specific.

Acknowledgments

Financial support: This work was partially funded by research grants numbers CRP/PER08-02 from the ICGEB, P01 AI51976 TMRC, D43 TW001140 (Fogarty Training), and D43 TW007120 from the National Institutes of Health, USA. The sponsors had no role in the design or writing of this manuscript.

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Figure 1.

(A) Western immunoblot of oncosphere total antigens revealed with (a) rabbit anti-TsAg5-trypsin-like-domain sera, and (b) rabbit anti-TsAg5-adherence-domain sera. (B) Western immunoblot of cysticercus excretory/secretory antigens revealed with (a) rabbit anti-TsAg5-adherence-domain sera, (b) rabbit anti-TsAg5-trypsin and anti-TsAg5-adherence-domain sera, (c) rabbit anti-TsAg5-trypsin-like-domain sera and (d) rabbit pre-immune sera. (C) Western immunoblot of TsAg5 trypsin-like domain antigen revealed with: (a) pre-immune sera, (b): rabbit anti-TsAg5 trypsin-like domain sera, (c) rabbit anti-cysticercus sera, (d) rabbit anti-oncosphere sera. (D) Western immunoblot of TsAg5 adherence domain antigen revealed with: (a) pre-immune sera, (b): rabbit anti-TsAg5 adherence domain sera, (c) rabbit anti-cysticercus sera, (d) rabbit anti-oncosphere sera.

Rabbit anti-sera against the TsAg5 trypsin-like-domain and adherence-domain was obtained by immunization of six two-month old New Zealand white rabbits with the recombinant antigens with four doses given at intervals of four days. 75 µg of total antigen was administered. 0.5 ml of blood was sampled on the day prior to each immunization and two weeks after the last dose. Pre-immune and post-immune sera corresponding to the second and fourth dose were tested in a Western immunoblot using the trypsin-like-domain and adherence-domain as antigens.

Figure 2.

Control strips of the recombinant trypsin-like antigen. (a) Negative control. (b) Positive control. Resolved proteins were transferred onto a nitrocellulose membrane in a Transfer-Blot Cell (Bio-Rad, Hercules CA). After determining the best conditions in a checker board, nitrocellulose membranes were cut into small strips to be tested using individual sera. Strips were blocked with PBS pH 7.3, 0.3% Tween 20, and 5% dry milk for 10 min. Each serum at a final dilution 1:500 was incubated with a nitrocellulose strip overnight at 23°C. The proteins were visualized using a peroxidase-labeled anti-human IgG, followed by the addition of 3,3'-diaminobenzadine plus hydrogen peroxide as the substrate. Reactions were classified as positive or negative based on the presence or absence of band on the blot.

Table 1

Performance of the Western Immunoblot in the diagnosis of human neurocysticercosis (NCC): Sensitivity, specificity, and cross-reactions

	Serum donor	No. of sera	Observer	% (fraction)
Sensitivity	NCC case with single parenchymal cyst	53	1	37.74 (20/53)
			2	39.62 (21/53)
	NCC case with multiple parenchymal cysts	57	1	68.42 (39/57)
			2	75.44 (43/57)
	Extraparenchymal NCC	55	1	96.36 (53/55)
			2	94.55 (52/55)
Specificity	Healthy negative controls	103	1	76.70 (79/103)
			2	74.76 (77/103)
Cross-reactivity	Nematodes*	37	1 & 2	0 (0/37)
	<i>H. nana</i>	28	1 & 2	21.43 (6/28)
	<i>D. pacificum</i>	2	1 & 2	0 (0/2)
	<i>T. saginata</i>	10	1 & 2	10.00 (1/10)
	<i>E. granulosus</i>	16	1 & 2	6.25 (1/16)

The Western immunoblot strips were read blindly on the same day, by two independent observers (Observers 1 and 2), in order to estimate inter-observer variability. Sensitivities were estimated independently for each of the two readings and separately for each of the three groups of NCC cases: those with a single parenchymal cyst, multiple parenchymal cysts, and one or more extraparenchymal cysts. Specificity was estimated independently for each of the two readings using the negative control data. The 95% confidence intervals (CI) for the estimated sensitivities and specificities were calculated with the binomial distribution. Inter- and intra-observer variability was determined as percentage of agreement and by the kappa (κ) statistic.

To estimate sensitivity, 165 positive sera corresponded to patients with NCC confirmed by detection of viable cysts in the brain with image-based diagnosis and LLGP-EITB positive tests. 53 samples corresponded to patients with a single intraparenchymal cyst; 57 samples corresponded to patients with 2 or more intraparenchymal cysts; and 55 samples corresponded to patients with extraparenchymal NCC. To estimate specificity, 103 negative control sera corresponded to individuals from Iquitos, a town in the northern Peru, where *T. solium* is non-endemic but other helminth parasites are common. To estimate the level of cross-reaction with other parasites, 2 sera samples from patients infected with *Ascaris lumbricoides*, 8 samples from *Enterobius vermicularis*, 28 samples from *Hymenolepis nana*, 2 samples from *Diphyllobothrium pacificum*, 12 samples from *Strongyloides stercoralis*, 10 samples from *Taenia saginata*, 6 samples from *T. trichiura*, 9 samples from *Ancylostomidae*, and 16 samples from patients infected with *E. granulosus* were included in the validation.