

Molecular identification, immunolocalization, and characterization of *Clonorchis sinensis* triosephosphate isomerase

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Abstract *Clonorchis sinensis* triosephosphate isomerase (CsTIM) is a key regulatory enzyme of glycolysis and gluconeogenesis, which catalyzes the interconversion of glyceraldehyde 3-phosphate to dihydroxyacetone phosphate. In this study, the biochemical characterizations of CsTIM have been examined. A full-length complementary DNA (cDNA; Cs105350) sequence encoding CsTIM was obtained from our *C. sinensis* cDNA library. The open reading frame of CsTIM contains 759 bp which encodes 252 amino acids. The amino acid sequence of CsTIM shares 60–65 % identity with other species. Western blot analysis displayed that recombinant CsTIM (rCsTIM) can be probed by anti-rCsTIM rat serum and anti-*C. sinensis* excretory/secretory products (anti-CsESPs) rat serum. Quantitative reverse transcription (RT)-PCR and western blotting analysis revealed that CsTIM messenger RNA (mRNA) and protein were

differentially expressed in development cycle stages of the parasite, including adult worm, metacercaria, excysted metacercaria, and egg. In addition, immunolocalization assay showed that CsTIM was located in the seminal vesicle, eggs, and testicle. Moreover, rCsTIM exhibited active enzyme activity in catalytic reactions. The Michaelis constant (K_m) of rCsTIM was 0.33 mM, when using glyceraldehyde 3-phosphate as the substrate. The optimal temperature and pH of CsTIM were 37 °C and 7.5–9.5, respectively. Collectively, these results suggest that CsTIM is an important protein involved in glycometabolism, and CsTIM possibly take part in many biological functions in the growth and development of *C. sinensis*.

Keywords *Clonorchis sinensis* · Triosephosphate isomerase · Excretory/secretory products · Enzyme activity

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Introduction

Clonorchis sinensis (*Cl. sinensis*) causes clonorchiasis, which is an important food-borne parasite disease distributed in Southeast Asia, Korea, and Vietnam. It is estimated to infect approximately 35 million people worldwide, among which 15 million people are in China (Lun et al. 2005; Young et al. 2010). People are infected by *Cl. sinensis* by eating raw or undercooked freshwater fish containing infective *Cl. sinensis* metacercariae. After entering the duodenum, *Cl. sinensis* metacercariae become juvenile flukes and then grow into adult worms (Keiser and Utzinger 2009). *Cl. sinensis* infection causes clonorchiasis with the outcomes of cholecystitis, cholangectasis, cholelithiasis, and hepatic fibrosis (Choi et al. 2004; Lim et al. 2006) and is suggested to be associated with promotion of cholangiocarcinoma (Shin et al. 2010).

However, there are still few effective measures to prevent this neglected tropical disease. Much more attention has renewed interest in targeting metabolic enzymes in the treatment of infectious diseases (Zhou et al. 2013).

During the life cycle of *Cl. sinensis*, adult worms can take in external glucose and then get energy supply through glycolysis pathway (Kang et al. 1969). Triosephosphate isomerase (TIM), a key regulatory enzyme of glycolysis and gluconeogenesis, plays an essential role in metabolism and development of most organisms (Knowles 1991). TIM catalyzes the interconversion of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate can be further processed to pyruvate permitting the generation of ATP and NADH. Thus, TIM enables these three carbon atoms to be processed in the glycolytic pathway. Without this reaction, no ATP would be produced by glycolysis. In humans, hereditary deficiency of TIM is associated with a form of hemolytic anemia (Orosz et al. 2009). The loss of enzymatic activity most likely results from misfolding of the enzyme or its failure to dimerize (Daar et al. 1986; Seigle et al. 2008 and Ralser et al. 2006). Furthermore, the TIM knockout can lead to metabolic diseases and neurological dysfunction (Velur Selvamani et al. 2014; Roland et al. 2013; Eanes et al. 2006).

Giardia depends on glycolysis as its major ATP source (Adam 2001). TIM from *Schistosoma* species was considered as a potential drug and vaccine target (Chen and Wen 2011 and Zinsser et al. 2013a, b). *Clonorchis sinensis* triosephosphate isomerase (CsTIM) has been identified as a component of *Cl. sinensis* excretory/secretory products (CsESPs) (Zheng et al. 2011), which are generally believed to play key roles in host–parasite interaction in previous studies (Mulvenna et al. 2010 and Xu et al. 2013). However, the biochemical characterizations of CsTIM remain obscure. In this study, the expression, structures, and biochemical properties of CsTIM were characterized.

Materials and methods

Bioinformatics analysis of CsTIM

A full-length complementary DNA (cDNA; clone number csin105350) sequence encoding CsTIM was obtained from our *Cl. sinensis* cDNA library (Wang et al. 2011). The open reading frame (ORF) was found with ORF finder tool in NCBI web site (<http://www.ncbi.nlm.nih.gov/>). Proteomics tools in ExPaSy web site (<http://www.expasy.org/>) were used to analyze the physicochemical parameters, characteristic motifs, and functional domain of the deduced amino acids. The homology of the deduced amino acids was analyzed by Vector NTI suite 8.0.

Cloning, expression, and purification of the recombinant CsTIM

Sense primer (5'-CAGGATCCATGCCTACGGACAGAAAG-3') introducing *BamHI* and restriction sites and antisense primer (5'-ATCTCGAGCTAGGCATTCGCATTGCAAATTC-3') harboring *XhoI* restriction sites were used to amplify ORF of CsTIM from the template isolated from the cDNA of adult *Cl. sinensis*. The amplification was performed by a procedure of 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. The last extension of 5 min was performed at 72 °C before storing the samples at 4 °C. The obtained PCR products were purified and digested with *BamHI* and *XhoI* enzymes and then cloned into prokaryotic expression vector pET30a (+) that was predigested with the same enzymes. The recombinant plasmid was sequenced to ensure fidelity and then transformed into *Escherichia coli* BL21 (*E. coli*, Promega, USA). The transformed bacteria were grown and induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) at 30 °C for 4 h in Luria-Bertani medium (LB). After inductions, the bacteria were harvested by centrifugation at 8000 \times g for 15 min at 4 °C and suspended in native lysis buffer (0.5 M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 8.0), sonicated on ice, and centrifuged at 12,000 \times g for 15 min at 4 °C.

The supernatant contained the recombinant fusion protein. Purification was performed with His Bind Purification kit (Novagen, USA) according to the user manual. The recombinant protein was dialyzed in 0.15 mol/l phosphate-buffered saline (PBS, pH 7.4) at 4 °C. Protein samples were analyzed by sodium dodecyl sulfide–polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein concentration was measured by BCA protein assay kit (Novagen, USA).

Parasites and parasite proteins preparation

Adult worms, metacercariae, excysted metacercariae, and eggs were obtained as described previously (Na et al. 2008). Some adult worms, metacercariae, excysted metacercariae, and eggs were used to collect total proteins as described (Na et al. 2008; Alirahmi et al. 2010); others were kept at –80 °C for extraction total RNA. The excretory–secretory products (ESPs) were harvested as described (Zhou et al. 2013). The concentration of ESPs and total proteins was measured by BCA protein assay kit (Novagen, USA). After adding 10 mM phenylmethyl sulfonyl fluoride (PMSF), all proteins were kept at –80 °C.

Anti-sera preparation

The purified rCsTIM (200 μ g) and ESPs (200 μ g) were respectively emulsified with equal volume of Freund's complete adjuvant (Sigma, USA) and subcutaneously injected into

Sprague-Dawley (SD) rats and followed by two booster injections of 100 µg purified rCsTIM and ESPs mixtures with equal volume Freud's incomplete adjuvant at 2-week interval. Meanwhile, the pre-immune serum was taken before injection. The immune sera were collected at the 6th week. The titer of anti-sera was determined using enzyme-linked immunosorbent assay (ELISA) and stored at -80 °C.

Identification of CsTIM by western blotting

The purified rCsTIM (5 µg) and CsESPs (30 µg) were resolved on 12 % SDS-PAGE and then immobilized onto PVDF membrane (Millipore, USA). The PVDF membranes were blocked with 5 % (w/v) skimmed milk in PBS (pH 7.4) for 2 h at room temperature (RT) and then probed with rat anti-CsESPs (1:500 dilutions), anti-rCsTIM (1:1000 dilutions), and rat pre-immune serum (1:500 dilutions) for overnight at 4 °C, respectively. Excess of antibody was removed by washing the membrane five times for 5 min with PBS-0.05 % Tween 20. Membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-Rat IgG (1:4000 dilutions, ProteinTech Group, USA) for 2 h at RT. After washing the membrane five times, detection was then done with enhanced chemiluminescence (ECL) method.

Quantitative RT-PCR and western blotting analysis of CsTIM life stages of *Cl. sinensis*

Total RNA from adult worms, metacercariae, excysted metacercariae, and egg was respectively extracted using TRIZOL reagent (Invitrogen, USA) according to manufacturer's protocol. Nucleic acid/protein analyzer (Beckman Coulter, USA) and agarose gel electrophoresis were used to test the quality and concentration of total RNA. First-strand cDNA was synthesized with reverse transcriptase (AMV, TaKaRa, Japan) with oligo (dT) primer using total RNA as template. Quantitative reverse transcription (RT)-PCR reactions were done on Bio-Rad iQ5 instrument (Bio-Rad, USA) using SYBR Premix ExTaq Kit (TaKaRa, Japan). The forward and reverse primers for CsTIM were 5'-TTCTTTGTTGGTGGAACTGGA-3' and 5'-GGATCAATCTTGGCATGGTA-3'. *C. sinensis* β-actin (No. EU109284) was used as an internal control. The forward and reverse primers for *Cl. sinensis* β-actin were 5'-ACCGTGAGAAGATGACGCAG A-3' and 5'-GCCAAGTCCAAACGAAGAATT-3'. The 20-µl PCR reactions contained 2 µl cDNA, 10 µl SYBR Premix ExTaq (2×), 0.4 µl each primer (10 µM), and 7.2 µl RNase-free distilled H₂O. The quantitative RT-PCR was 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 20 s, with an incremental increase of 0.1 °C/s from 60 to 95 °C. The data was analyzed by using the 2^{-ΔΔCt} method (Pfaffl 2001). Thirty micrograms of total proteins from adult worms, metacercariae, excysted metacercariae, and eggs were

subjected to 12 % SDS-PAGE and then immobilized onto PVDF membrane (Millipore, USA). The PVDF membranes were probed with rat anti-CsTIM (1:1000 dilutions) and rat pre-immune serum (1:1000 dilutions) and then (HRP)-conjugated with goat anti-Rat IgG (1:4,000 dilutions). Detection was then done by enhanced chemiluminescence (ECL) method.

Immunohistochemical localization of CsTIM

The collected *Cl. sinensis* adult worms were fixed with formalin, embedded with paraffin wax, and sliced into 4–5 µm. Sectioned worms in paraffin wax were deparaffinized in xylene and hydrated in a series of graded ethanol. Then, they were blocked with normal goat serum for 2 h at RT and incubated with anti-rCsTIM rat serum (diluted 1:400 with 0.1 % BSA in PBS). Pre-immune rat serum was used as a negative control. After being washed three times with PBS-0.05 % Tween 20, the sections were Cy3 dye-conjugated with goat anti-rat IgG (1:400 dilutions, Molecular Probe, USA) for 1 h in the dark at RT and quenched for 15 min. The sections were washed again and subsequently imaged with fluorescent microscope (Zeiss, Germany).

Enzyme activity assay of rCsTIM

Triosephosphate isomerase activity was measured using glyceraldehyde 3-phosphate (Sigma, USA) as the substrate according to the description (Ostoa-Saloma et al. 1997) with slight modifications by continuously monitoring nicotinamide-adenine dinucleotide hydride (NADH) utilization at 340 nm with a multifunctional microplate reader (SpectraMax M5). The standard assay mixture contained 100 mM triethanolamine and 10 mM EDTA, pH 7.4, 0.5 mM NADH, 1 mM glyceraldehyde 3-phosphate, and 1 U sn-glycerol 3-phosphate dehydrogenase (Sigma, USA) in a final volume of 150 µl. Reaction was initiated by the addition of rCsTIM. The change of NADH was determined by using an absorption coefficient ($\epsilon=6222 \text{ M}^{-1} \text{ cm}^{-1}$, 340 nm). One enzyme unit (U) is defined as the amount of enzyme which can oxidize 1 µmol NADH per minute. The effects of temperature and pH on enzyme activity were analyzed. All experiments were adapted to using Michaelis-Menten equation $V=V_{\max}[S]/(K_m+[S])$.

Results

Sequence analysis of CsTIM

The ORF of CsTIM contained 759 bp encoding 252 amino acids with a predicted molecular weight of 27.7 kDa. Its isoelectric point was 6.66. No signal peptide or transmembrane

region was found. The triosephosphate isomerase active site was found in the position of aa_{167–177}. Blastx analysis showed that the deduced amino acid sequence was homologous with TIM of *Caenorhabditis elegans*, *Schistosoma japonicum*, *Schistosoma mansoni*, *Drosophila melanogaster*, and *Homo sapiens* with 61, 63, 65, 60, and 62 % (Fig. 1).

Expression, and purification of the recombinant CsTIM

The recombinant pET30a (+) plasmid containing CsTIM coding region was confirmed by sequencing. The recombinant CsTIM was expressed as a fusion protein with a 6× His-tag in *E.coli* with 0.5 mM IPTG induction at 37 °C for 4 h. The molecular mass of the purified protein was approximately 32.0 kDa (containing His-tag). The final concentration was about 450 µg/ml in PBS (Fig. 2).

Identification of CsTIM as a component of Cl. sinensis ESPs by western blotting

A specific band was visible when CsESPs were probed with anti-rCsTIM rat serum, and rCsTIM was recognized by anti-CsESPs rat serum, while no band was detected with naive rat serum (Fig. 3).

Quantitative RT-PCR and western blotting analysis of CsTIM at different developmental stages of Cl. sinensis

The transcripts of CsTIM were detected at the *Cl. sinensis* life cycle stages (adult worms, metacercariae, excysted metacercariae, and eggs) with *Cl. sinensis* β-actin messenger

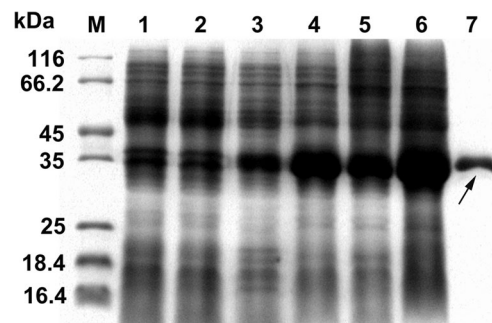


Fig. 2 Expression and purification of rCsTIM by 12 % SDS-PAGE. Protein molecular weight markers (M); the lysate of *E. coli* with pET-30a vector before IPTG induction (1) and after IPTG induction (2); the lysate of *E. coli* with pET-30a-CsTIM before IPTG induction (3) and after IPTG induction (4); supernatant of the lysate of *E. coli* with pET-30a-CsTIM after IPTG induction (5) and sediment (6); and purified 27.7 kDa rCsTIM protein (7)

RNA (mRNA) as the internal control (Fig. 4a). Compared with adult worms, the expression of CsTIM mRNA was higher in both metacercariae and excysted metacercariae stages. Specific bands were detected with anti-rCsTIM rat serum by western blotting assay (Fig. 4b). Expression of CsTIM protein was highest in adult worms, followed by metacercariae, excysted metacercariae, and eggs, while no band was detected with naive rat serum.

Immunolocalization of CsTIM in the Cl. sinensis adult worm

The native CsTIM was detected in paraffin-embedded *Cl. sinensis* adult worm sections by immunolocalization. The

Fig. 1 Multiple alignment of the amino acid sequence of triosephosphate isomerase from *Clonorchis sinensis* (C.s), *Caenorhabditis elegans*, *Schistosoma japonicum*, *Schistosoma mansoni*, *Drosophila melanogaster*, and *Homo sapiens*. *Caenorhabditis elegans* triosephosphate isomerase (C.e, AAA79846.1); *S. japonicum* (S.j, AAC47855.1); *S. mansoni* (S.m, XP_002571861.1); *Drosophila melanogaster* (D.m, CAA40804.1); *Homo sapiens* (H.s, AAH17917.1). Asterisk = active site, number sign = substrate binding site

		1	#	#	65
C. e	(1)	--MTRKFFVGGNWKMGNDYASVDGIVTFLNASADNSSVDVWVAPPAPYLAYAKSKLKAGVLVAAQ			
C. s	(1)	MPTDRKFFVGGNWKMGNSKKENDKLIEMLTHAKIDPNTVEVLVAPPALYLPSVREKLDKRFHVAQA			
S. j	(1)	MSSSRKFFVGGNWKMGNSRADNKVLIHSLSEAHFCGDTEILIAAPFVYLNEVRQSLAKEIHVAAQ			
S. m	(1)	MSGSRKFFVGGNWKMGNSRDDNDKLLKLSSEAHFDNDTEVLIAAPFVYLNEVRQSLAKEIHVAAQ			
D. m	(1)	--MSRKFVGGNWKMGNDQKSIABEAKTLSSAALDPNTEVVIWGPATYLMYARNLLPCELGLAGQ			
H. s	(1)	MAFSRKFFVGGNWKMGNRKQSLGELIGTLNAAKVPADTEVVCAPPTAYIDFARQKLDPKIAVAQA			
		66	#	#	130
C. e	(64)	NCYKVPKGAFTGEISFAMIKDLGLEWVILGHSERRHVFGESDALIAEKTVHALEAGIKVVFCTIGE			
C. s	(66)	NCYKVPKGAFTGEVSPAMIKDVGCDWVILGHSERRHILLETDLVGEKTNHATSAGVNVFIACIGE			
S. j	(66)	NCYKVPKGAFTGEISFAMIKDVGCDWVILGHSERRSIFNESDELVAEKVQHALAGGLSVVACIGE			
S. m	(66)	NCYKVPKGAFTGEISFAMIKDVGCDWVILGHSERRNIFGESDELIAEKVQHALAEGLSVFIACIGE			
D. m	(64)	NAYKVAKGAFTGEISFAMIKDIGADWVILGHSERRAIFGESDALIAEKAHALAELGKVIACIGE			
H. s	(66)	NCYKVTNGAFTGEISFGMIKDCGATWVVLGHSERRHVFGESDELIGQKVAHALAELGLVIACIGE			
		131	#	#	195
C. e	(129)	KLEEREAGTKDVFNRQLQAIYVDEKGV---SWENIVIAAYEPVWAIGTGKTASGEQAQEVHWEWTRAF			
C. s	(131)	KLEEREAGKTEVVCFRQMEAIRTKKS---AWRRRLVSLP-----QSPFRGT			
S. j	(131)	TLSERESGKTEEVVCRQLNATANKIKSIDWKR/VVAYEPVWAIGTGKVASPQQAQEVHNFRLRW			
S. m	(131)	TLSERESNKTTEVVCVRLKATANKIKSADEWKR/VVAYEPVWAIGTGKVASPQQAQEVHNFRLRW			
D. m	(129)	TLEEREAGKTEVVARQMCAYAQKTK---DWKNVVVAYEPVWAIGTGKATPDAQAQEVHASLRQW			
H. s	(131)	KLDEREAGITEKVVFEQTKVIADNVK---DWSKVVLAYEPVWAIGTGKATPDAQAQEVHKEKLRW			
		196	#	###	253
C. e	(191)	LKEKVSFVAVADATRIIYGGSVTADNAADVGGKPDIDGFLVGGASLKFDFVKIINARS-			
C. s	(184)	YASRMSSGQSG-WKLLTSGSVTAANCRTLAKQPDVDGFLVGGASLKFDFVIEICNANA-			
S. j	(196)	FRTNTPSGVDQRIIYGGSVTAANCKELAQHQHDVDGFLVGGASLKFDFEIDICKARQ-			
S. m	(196)	FRTNAPNGVDEKIRIYGGSVTAANCKELAQHQHDVDGFLVGGASLKFDFEITCKARQR			
D. m	(191)	LSDNISKEVSAASLRIQYGGSVTAANAKELAKKPDIDGFLVGGASLKFDFLDIINARQ-			
H. s	(193)	LRSNVSDAVAQSTRIIYGGSVTGATCKELASQPDVDGFLVGGASLKFDFVDIINARQ-			



Fig. 3 Western blotting analysis of rCsTIM. rCsTIM was conformed to one component of ESPs. ESPs probed with naïve rat serum (1); ESPs probed with anti rCsTIM rat serum (2); rCsTIM probed with naïve rat serum (3); rCsTIM probed with anti-ESP rat serum (4)

protein was localized on the seminal vesicle, eggs, and testicle (Fig. 5), while the naïve rat serum showed no specific fluorescence in all tissues.

Enzymatic characteristics of rCsTIM

The enzymatic activity of rCsTIM was more than 80 % under 25–55 °C, and the optimal temperature was 37 °C (Fig. 6a). The enzymatic activity of rCsTIM was more than 70 % at pH 7.0–10.0, and the optimal pH was 7.5–9.5 (Fig. 6b). The K_m value of rCsTIM was 0.33 mM (Fig. 6c) when using glyceraldehyde 3-phosphate as the substrate.

Discussion

In this study, a full-length gene encoding TIM from *Cl. sinensis* was identified, cloned, and overexpressed in *E. coli*.

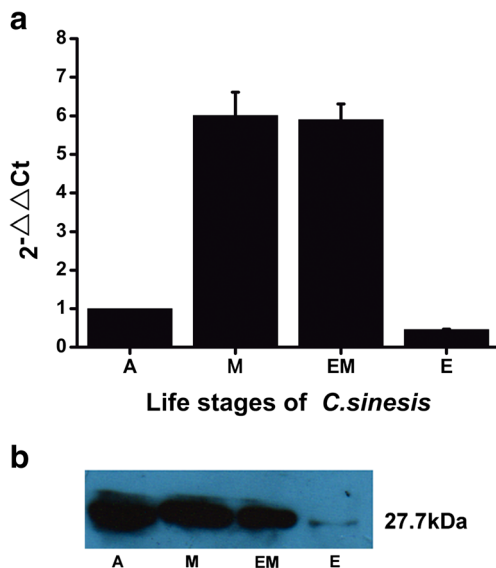


Fig. 4 **a** *CsTIM* mRNA expression level at different life cycle stages of *C. sinensis* was detected by Quantitative RT-PCR. The stages included adult worms (A), metacercariae (M), excysted metacercariae (EM), and egg (E). No difference was detected at the stages of adult worm and egg ($p > 0.05$). *CsTIM* showed a higher expression level at the stage of metacercaria than adult worm (6.01-fold, $p < 0.05$) and egg (13.15-fold, $p < 0.05$). No difference was detected at the stages of excysted metacercariae and metacercaria ($p > 0.05$). Expression of *CsTIM* gene was higher in excysted metacercariae than in adult worms (5.90-fold, $p < 0.05$) and egg (12.92-fold, $p < 0.05$). **b** Western blotting analysis of *CsTIM* at different stages of *C. sinensis* visualized using ECL method

The enzymatic characteristics of *CsTIM* were also investigated. TIM is a well-known glycometabolism enzyme in almost species, and its distinct properties have been described in parasitic infections such as *Trichomonas vaginalis* (Figueroa-Angulo et al. 2012), *S. japonicum* (Zhu et al. 2006), *Fasciola hepatica* (Zinsser et al. 2013a, b), and *Taenia solium* (Sanabria-Ayala et al. 2015). *CsTIM* showed high homology with other species (Fig. 1), indicative of similar functions between them. TIM had been shown conserved during evolution across species (Ostoa-Saloma et al. 1997). Analysis of qRT-PCR and western blotting indicated that *CsTIM* was expressed in all developmental stages of *Cl. sinensis*, i.e., adult worms, metacercariae, excysted metacercariae, and eggs. These imply the essential roles of *CsTIM* in glucose metabolism throughout the life stages. Interestingly, *CsTIM* exhibited higher mRNA and protein expression level in the adult worms, metacercariae, and excysted metacercariae stages (Fig. 4). *CsTIM* could serve as energy provider of *C. sinensis* adult worms creeping and transporting solutes and nutrients. When *Cl. sinensis* invade the definitive host and then transform into juvenile flukes and adults, they also need abundant energy through glycometabolism. Other research studies have shown that most of the glucose was degraded in the freshly excysted metacercariae of *F. hepatica* (Tielens et al. 1987). *CsTIM* was also expressed in vesicle, eggs, and testicle of adult worm (Fig. 5). These suggest that *CsTIM* as the glycometabolism enzyme could take part in energy generation in these organs for the growth and reproduction of the parasite. In addition, TIM was also detected in the cercariae and eggs of *S. mansoni* (Curwen et al. 2006; Cass et al. 2007).

Enzymatic analysis of rCsTIM showed that its enzymatic activity was more than 80 % of the optimal activity under 25–55 °C, and 70 % at the pH 7.0–10.0. The range of temperature and pH is responsible for catalytic activities for different host environments. The optimal temperature and pH range were close to other species, i.e., human (Dabrowska et al. 1978) and *Leishmania donovani*. The life cycle of *Cl. sinensis* involves many hosts (Wang 1983), and the temperature and pH of the hosts are different. Therefore, *CsTIM* is responsible for catalytic activities under host environment, suggesting that *CsTIM* plays an important role in generating energy and metabolic intermediates for growth and development of *Cl. sinensis*. The K_m value *CsTIM* is similar to those records for the enzyme for other species. *F. hepatica* has a K_m for glyceraldehyde 3P of 0.66 mM (Zinsser et al. 2013a, b), just double the value for *CsTIM*. For *S. mansoni*, this value is 1.11 mM (Zinsser et al. 2013a, b), which is more than threefold the value for *CsTIM*. The purified human skeletal enzyme has a K_m value of 0.34 mM for glyceraldehyde 3P (Dabrowska et al. 1978).

rCsTIM was recognized by anti-*CsESP* rat serum, and *CsESP*s also can be probed by anti-rCsTIM rat serum by western blotting assay (Fig. 3). Moreover, *CsTIM* was

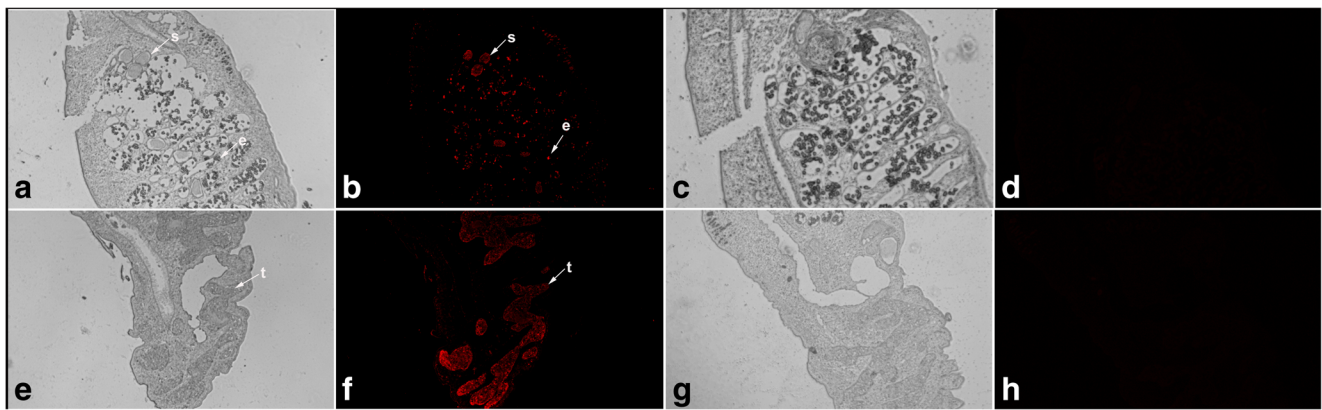


Fig. 5 Immunolocalization of *CsTIM* in the *C. sinensis* adult worm using sections of paraffin-embedded worms. Rat anti-*CsTIM* serum was used as primary antibody and goat anti-rat IgG labeled with red-fluorescent

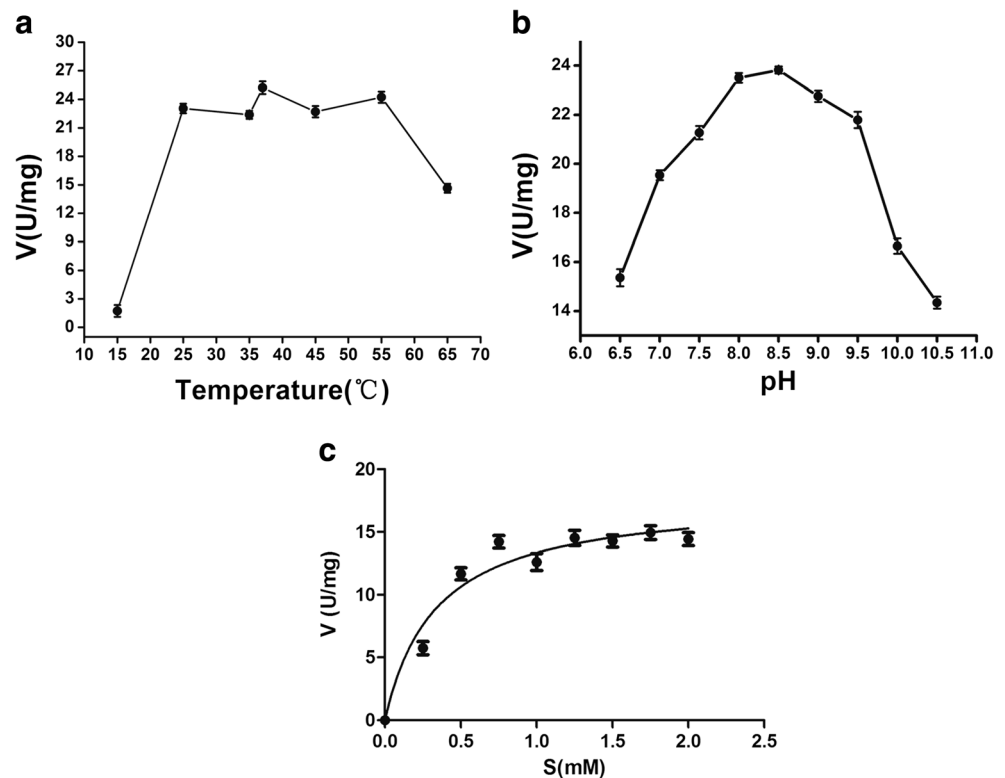
Cy3 as secondary antibody. In the adult worm, *CsTIM* is localized on the *s* seminal vesicle, *e* eggs (panel b), and *t* testicle (panel f). The images are magnified at $\times 100$

identified as a component of *Cs*ESPs by LC-MS/MS (Zheng et al. 2011). How *CsTIM* is secreted, in the absence of a signal peptide, remains unclear. *CsTIM* as a component of *Cs*ESPs is probably due to an unknown secretory mechanism (Lorenzatto et al. 2012; Gomez-Arreaza et al. 2014). On the other hand, TIM has been detected in the ESPs of other flukes including *Opisthorchis viverrini* (Mulvenna et al. 2010), *F. hepatica* (Jefferies et al. 2001), *S. mansoni* (Guillou et al. 2007; Wilson 2012), and *S. japonicum* (Liu et al. 2009). What is more, it can take part in the recognition of cell surface and extracellular matrix glycoproteins in some pathogens

(Karkowska-Kuleta et al. 2011; Furuya and Ikeda 2011; Furuya and Ikeda 2009; Pereira et al. 2007). Therefore, *CsTIM* may participate in host–parasite interaction.

Taken together, *CsTIM* is an important protein involved in glycometabolism. The protein also takes part in many biological functions in the growth and development of *Cl. sinensis*, such as generating energy, metabolic intermediates, and reproduction. What is more, *CsTIM* takes part in host–parasite interaction. Our study will be the cornerstone for better understanding of biological characterization of *CsTIM* and the role of host–parasite interplay.

Fig. 6 Enzymatic characteristics of r*CsTIM*. **a** Optimal temperature of r*CsTIM* was detected as ranging from 15 to 65 °C. **b** Optimal pH value of r*CsTIM* was detected as ranging from 6.5 to 11.5. **c** K_m value was measured using glyceraldehyde 3-phosphate as the substrate. All assays were conducted in triplicate



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