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Identification and functional characterization of alpha-enolase from *Taenia pisiformis* metacestode

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ABSTRACT

Enolase belongs to glycolytic enzymes with moonlighting functions. The role of enolase in *Taenia* species is still poorly understood. In this study, the full length of cDNA encoding for *Taenia pisiformis* alpha-enolase (Tpeno) was cloned from larval parasites and soluble recombinant Tpeno protein (rTpeno) was produced. Western blot indicated that both rTpeno and the native protein in excretion–secretion antigens from the larvae were recognized by anti-rTpeno monoclonal antibodies (MAbs). The primary structure of Tpeno showed the presence of a highly conserved catalytic site for substrate binding and an enolase signature motif. rTpeno enzymatic activities of catalyzing the reversible dehydration of 2-phosphoglycerate (2-PGA) to phosphoenolpyruvate (PEP) and vice versa were shown to be 30.71 ± 2.15 U/mg (2-PGA to PEP) and 11.29 ± 2.38 U/mg (PEP to 2-PGA), respectively. Far-Western blotting showed that rTpeno could bind to plasminogen, however its binding ability was inhibited by ϵ-aminocaproic acid (ϵACA) in a competitive ELISA test. Plasminogen activation assay showed that plasminogen bound to rTpeno could be converted into active plasmin using host-derived activators. Immunohistochemistry and immunofluorescence indicated that Tpeno was distributed in the bladder wall of the metacestode and the periphery of calcareous corpuscles. In addition, a vaccine trial showed that the enzyme could produce a 36.4% protection rate in vaccinated rabbits against experimental challenges from *T. pisiformis* eggs. These results suggest that Tpeno with multiple functions may play significant roles in the migration, growth, development and adaptation of *T. pisiformis* for survival in the host environment.

1. Introduction

Taenia pisiformis is an ancient cestode parasite. The natural life cycle of this parasite includes canines as normal definitive hosts and lagomorphs as typical intermediate hosts (Loos-Frank, 2000). *Cysticercus pisiformis*, the larval stage of *T. pisiformis*, can cause socio-economic losses in rabbit breeding and serious health problems to the host, such as liver lesions, digestive disorders, growth retardation, weight loss, and even death (Rajasekariah et al., 1985; Sun and Cai, 2008). *T. pisiformis* is also regarded as an alternate experimental model for studying the protein functions of tapeworms in vaccine trials and in the evaluation of novel anti-cestode drugs (Toral-Bastida et al., 2011).

Similar to other parasitic cestodes, *T. pisiformis* lives in the digestive tract of the host and depends solely on its tegument for the acquisition of nutrition (Dalton et al., 2004). Under this condition, anaerobic glycolysis is considered as the principal way by which *T. pisiformis* obtains energy which is essential for certain activities of the parasite, such as growth, development, and survival in the host (Yang et al., 2012). Thus, further characterization of the glycolytic enzymes and selective inhibitors may boost the development of new potential therapeutic targets for treating parasitic infections.

Enolase (2-phospho-D-glycerate hydrolase, EC4.2.1.11) is a ubiquitous glycolytic enzyme involved in the glycolysis and gluconeogenesis pathways that catalyzes the reversible dehydration conversion of 2-phosphoglyceric acid (2-PGA) into phosphoenolpyruvate (PEP), which is an important metabolic intermediate

Abbreviations: TpM, *Taenia pisiformis* metacestodes; Tpeno, *Taenia pisiformis* enolase; RACE, rapid amplification of cDNA ends; UTR, untranslated region; MAb, monoclonal antibody; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; ϵACA, ϵ-aminocaproic acid; 2-PGA, 2-phospho-D-glycerate; PEP, phosphoenolpyruvate; TMB, 3,3′,5,5′-tetramethylbenzidine; DAB, 3,3′-diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; IHC, immunohistochemistry; IFA, indirect immunofluorescence assay.

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for the production of ATP and NADH (Pancholi, 2001; Rodriguez et al., 2006). Copley (2003) has stated that enolase acquires moonlighting functions in organisms when the protein changes its cellular localization. In addition to its innate glycolytic activity, enolase may play important roles in a variety of biological and pathophysiological processes by acting, for example, as a virulence factor (Li et al., 2013; Pancholi and Fischetti, 1998), hypoxic stress protein (Subramanian and Miller, 2000), and/or heat shock protein (Iida and Yahara, 1985).

Most of the information on enolase from parasites has been obtained from protozoa (Kibe et al., 2005; Navarro et al., 2007; Tovy et al., 2010) and helminthes (Liu et al., 2009; Ramajo-Hernandez et al., 2007). It is reported that enolase is involved not only in the regulation of gene transcription and expression during protozoa development (Ferguson et al., 2002; Holmes et al., 2010), but also in the encystation process (Chavez-Munguia et al., 2011; Segovia-Gamboa et al., 2010). In *Clonorchis sinensis*, the inhibition of enolase by excretory/secretory products could affect parasite growth (Wang et al., 2011). From this perspective, parasitic enolase, with its multiple biological functions, seems to be a suitable candidate for chemotherapeutic purposes.

There have been few similar studies on tapeworm enolase in the pathophysiological processes of parasitic invasion compared with other parasites. In this study, we identified and characterized the expression, enzymatic characteristics, interaction with the host plasminogen system, localization and protective efficacy in anticestode infections of the Tpeno protein.

2. Materials and methods

2.1. Animals and parasites

T. pisiformis metacestodes (TpM) used in this study were obtained from naturally infected rabbits at local abattoirs in Zhengzhou, Henan Province, China. The freshly separated metacestodes were prepared using the following procedure: (i) some metacestodes were immediately immersed in RNAlater (Qiagen, Germany) and stored at −70 °C for RNA extraction; (ii) other larvae were fixed in 10% neutral formalin and embedded in paraffin according to routine histological procedures for immunohistochemical analysis. Three *T. pisiformis* adult worms were recovered during necropsy from a hybrid dog raised in a rabbit farm in Zhengzhou, Henan Province, China. BALB/c mice and 4-month-old rabbits were prepared for the immunological trial at the Laboratory Animal Center of Lanzhou Veterinary Research Institute. The animal experimental protocol reported herein was carried out in strict accordance with the recommendations of The Animal Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

2.2. Calcareous corpuscles and excretion–secretion (E/S) antigens

The TpM were sterilely grinded on a metal screen with the plunger of a 5 ml syringe, followed by repeated centrifugation and rinsing of the pellets. Calcareous corpuscles were purified using Ficoll-paque plus (Sigma, USA), examined by light microscopy (Yang, 2000), and then immersed in a fixative solution (containing 60% acetone, 40% methanol) overnight at 4 °C for the immunofluorescence experiment. The E/S antigens from TpM were prepared according to the method described by Victor et al. (2012). After washing thoroughly in sterile saline, 68 cysts with intact bladder walls were incubated in 20 ml RPMI-1640 medium (containing 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% D-glucose, pH 7.2) for 6 h at 37 °C in an atmosphere of 5% CO_2 and their growth state was observed. The culture media were collected after 48 h and centrifuged at 3000 × *g* for 15 min, then concentrated to a final volume of about 0.8 ml with an Amicon Ultra-15 (3 kDa) centrifugal filter device (Millipore, USA).

2.3. Amplification of full-length cDNA encoding T. pisiformis enolase (Tpeno)

Total RNA was extracted from frozen TpM using Trizol reagent (Invitrogen, USA) and used immediately for cDNA synthesis using an MMLV first strand kit (Invitrogen, USA) according to the manufacturer's instructions.

The primers used in the initial amplification of the partial Tpeno cDNA fragment by RT-PCR were designed from the highly conserved regions of *Echinococcus granulosus* and *Taenia asiatica* enolase genes (GenBank accession numbers GU080332 and EF420377). All primers used in PCR amplifications are listed in Table 1. PCR reactions for the partial Tpeno cDNA fragment were performed in a 50 μl reaction mixture containing 25 μl 2× Master Mix (Biomiga, San Diego, CA, USA), 1 μl cDNA, 1 μl of each primer, and 12 μl H_2O. The optimal PCR program was as follows: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 45 s, extension at 72 °C for 1.5 min and finally incubation at 72 °C for 10 min. The PCR products were gel-purified, cloned into pGEM-T vector (Promega, Madison, WI, USA), and transformed into *Escherichia coli* DH5a cells (Tiangen, China). Finally, plasmid clones were sequenced by Shanghai Sangon Biotechnology Company.

Following the defined partial sequence of the Tpeno gene, the full-length cDNA was obtained by 5′- and 3′-RACE-PCRs using the SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). The gene-specific primers GSP1 and GSP2 are shown in Table 1. For 5′-RACE and 3′-RACE, the synthesized adaptor-ligated, oligo(dT)-primed, double-stranded cDNA and 10× universal primer mix (UPM) provided with the RACE kit were used for the RACE-PCR reaction according to the manufacturer's instructions. The enolase sequence analysis was conducted using online servers, including ProtParam, MEME, NetNGlyc 2.0, SignalP 4.0, and SecretomeP 2.0.

2.4. Expression and purification of recombinant Tpeno (rTpeno) in E. coli

The predicted open reading frame (ORF) region of Tpeno was PCR-amplified with Tp1 and Tp2 primers containing *Sac*I and *Xho*I restriction enzyme sites, respectively (Table 1). The specific PCR product was cloned into pET32a vector (Invitrogen, Grand Island, NY, USA) following enzyme digestion. The correct coding sequences containing ORF and the three upstream tags (Trx-tag, S-tag, His-tag) were confirmed by DNA sequencing, and then

Table 1
PCR primers used for *Taenia pisiformis* alpha-enolase analysis.

Primers	Sequence (5′–3′)
Cloning for partial sequence	
Tp F	5′-GGAAATCCTACTGTTGAGGTT-3′
Tp R	5′-TTACAAAGGATTGCGGAAGTG-3′
5′-RACE	
GSP1	5′-TCCAGGCGGGCCAGTCATC-3′
3′-RACE	
GSP2	5′-GTCCCTGGGTCGAAGCTGG-3′
10× UPM	5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′
Expression analysis	
Tp1	5′-C**GAGCTC** ATGTCAATCCAAAATATTCATGC-3′ *Sac*I
Tp2	5′-CCG **CTCGAG**TTACAAAGGATTGCGGAAGT-3′ *Xho*I

The underlined sequences are enzyme restriction sites.