RESEARCH ARTICLE

Molecular cloning and characterization of a novel aspartyl aminopeptidase from *Trichinella spiralis*

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ABSTRACT

Trichinellosis is an important zoonotic parasitic disease worldwide and is principally caused by ingesting animal meat containing *Trichinella* infective larvae. Aspartyl aminopeptidase is an intracytoplasmic metalloproteinase that specifically hydrolyzes the N-terminus of polypeptides free of acidic amino acids (aspartic acid and glutamate), and plays an important role in the metabolism, growth and development of organisms. In this study, a novel *T. spiralis* aspartyl aminopeptidase (TsAAP) was cloned and expressed, and its biological properties and roles in worm growth and development were investigated. The results revealed that TsAAP transcription and expression in diverse *T. spiralis* stages were detected by RT-PCR and Western blotting, and primarily localized at cuticle, stichosome and intrauterine embryos of this nematode by immunofluorescence test. rTsAAP has the enzymatic activity of native AAP to hydrolyze the substrate H-Glu-pNA. There was a specific binding between rTsAAP and murine erythrocyte, and the binding site was localized in erythrocyte membrane proteins. Silencing of TsAAP gene by specific dsRNA significantly reduced the TsAAP expression, enzymatic activity, intestinal worm burdens and female fecundity. The results demonstrated that TsAAP participates in the growth, development and fecundity of *T. spiralis* and it might be a potential target molecule for anti-*Trichinella* vaccines.

Keywords: *Trichinella spiralis*; aspartyl aminopeptidase; enzyme activity; RNA interference.

INTRODUCTION

Trichinella infects humans and more than 150 other mammals (such as pigs, horses, reptiles) and birds (Pozio, 2005). Trichinellosis is a food-borne zoonotic parasitic disease mainly resulted from ingestion of undercooked meat containing *Trichinella* larvae (Cui *et al.*, 2011). The pork of domestic pigs is the principal infectious source of human trichinellosis in most developing countries (Cui *et al.*, 2013a; Jiang *et al.*, 2016; Rostami, 2017). But it is hard to interdict *Trichinella* infection in animals because of its broad distribution of animal hosts (Liu *et al.*, 2015b). Anti-*Trichinella* vaccines need to be developed for blocking and eliminating *Trichinella* infection in food animals (Bai *et al.*, 2017). The current development of anti-*Trichinella* vaccines is mainly focused on identification of new protective antigens and combined vaccination. Vaccination with an individual recombinant *Trichinella* protein only induced about 50% immune protection against *Trichinella* challenge infection (Yue *et al.*, 2020). Vaccination of mice with two recombinant *Trichinella* proteins or protein combined with DNA vaccine conferred a more significant reduction of larval burden than a single protein or DNA vaccine (Zhang *et al.*, 2018; Xu *et al.*, 2020a). Hence, it is requisite to characterize novel *Trichinella*

proteins as the candidate target molecules of preventive vaccines against *Trichinella* infection (Ren *et al.*, 2019, 2021).

After being ingested, the *T. spiralis* muscle larvae (ML) contained in infected meat are released under the action of the host's gastric juice and activated to intestinal infective larvae (IIL), the IIL intrude into intestinal mucosal epithelium where they develop into adult worms (AW). After mating, the female worms produce newborn larvae (NBL) that migrate throughout the host's body and invade the skeletal muscles to become the encapsulated ML (Campbell, 1983; Ren *et al.*, 2011). Muscle larvae survive in the host for several years until it is ingested by a new host, thus starting a new round of life cycle (Li *et al.*, 2015). However, the mechanism of survival and immune escape of this parasitic nematode is not completely elucidated.

Peptidases are divided into three categories according to their substrates, namely aminopeptidase, carboxypeptidase and endopeptidase. Aminopeptidase hydrolyzes the amino acid at the amino end of the peptide chain (Walling, 2006). Aspartyl aminopeptidase (AAP) is assigned to the M18 family of peptidases, widely distributed in bacteria and eukaryotes, and is a highly conserved cytoplasmic enzyme. AAP is an intracytoplasmic metalloproteinase that specifically hydrolyzes the N-terminus of polypeptides free of acidic amino acids

(aspartic acid and glutamate). The AAP has strong selectivity for N-terminal aspartic acid and glutamic acid residues (Wilk *et al.*, 1998). Current research on parasite AAP is mainly focused on protozoa. Previous studies have found that *Toxoplasma gondii* AAP (TgAAP) in the cytosol was essential for its blood stage replication, TgAAP gene knockout inhibited the *T. gondii* invasion and growth, and this enzyme may be a promising candidate target for anti-*Toxoplasma* drugs (Zheng *et al.*, 2016). *Plasmodium falciparum* AAP (PfM18AAP) performs various functions in host red blood cells, such as hemoglobin digestion, red blood cell invasion, parasite growth and parasite escape from host cells (Kumari *et al.*, 2016). *P. falciparum* requires a variety of metal aminopeptidases. PfM1MAA (membrane alanine aminopeptidase), PfM17LAP (leucine aminopeptidase) and PfM18AAP (aspartyl amino-peptidase) synergistically degrade host hemoglobin to generate free amino acids, which can be used as a source of amino acids to synthesize their own proteins. *Plasmodium* mutated Cin the M18AAP enzyme cannot survive, indicating that it plays a key role in the survival of *P. falciparum* and can serve as an important molecular target for development of potential therapeutic agents for the control of malaria infections (Lauterbach & Coetzer, 2008). *P. vivax* AAP is a single gene copy present in *Plasmodium*, the AAP acts in the final stage of host hemoglobin and completes the hydrolysis process (Rout & Mahapatra, 2019).

In previous studies, a *T. spiralis* aminopeptidase (TsAP) has been identified in proteases generated by the IIL larvae after co-culture with intestinal epithelial cells (IEC). More-over, TsAP was also identified in AW excretion/secretion (ES) protein (Wang *et al.*, 2013c, 2017). TsAP participates in *T. spiralis* invasion, development and fecundity (Guo *et al.*, 2020). But TsAP did not include *T. spiralis* AAP (TsAPP), the TsAPP was not identified and characterized before.

In the present study, a novel *T. spiralis* TsAAP (TsAAP, GenBank: KRY29491.1) is retrieved from the draft genome of this parasite (Mitreva *et al.*, 2011), but its biological functions in *T. spiralis* lifecycle was not reported in the literatures. The aim of this study was to investigate the biological properties and roles of TsAAP in growth and development of *T. spiralis*.

MATERIALS AND METHODS

Parasites, animals and antigens
Trichinella spiralis isolate (ISS534) was acquired from domestic pigs naturally infected in central China (Wang *et al.*, 2012). The 4-6 week old female Kunming mice was purchased from Henan Experimental Animal Center (Zhengzhou, China). The ML was collected from infected murine carcasses at 40 days post infection (dpi) by artificial digestion method (Jiang *et al.*, 2012). The IIL at 6 hours post infection (hpi), and AW at 3 and 6 days post infection (dpi) were recovered from infected murine small intestine (Liu *et al.*, 2015d; Sun *et al.*, 2015). Adult females at 6 dpi were cultured in RPMI-1640 with 10% fetal bovine serum (FBS; Gibco) at 37°C, 5% CO_2 for 24 h, and NBL were collected (Zhang *et al.*, 2013; Wu *et al.*, 2016). The somatic crude proteins and ES proteins of various *T. spiralis* stage worms (ML, IIL, 3 d AW, 6 d AW and NBL) were prepared as previously reported (Yang *et al.*, 2015).

Bioinformatics analysis of TsAAP
The structural domain and enzyme active sites of TsAAP (GenBank: KRY29491.1) were predicted and analyzed by using online webtool and CN3D software (Song *et al.*, 2018b). The amino acid sequence of TsAAP gene was compared with AAP from different species /genotypes of *Trichinella* by using Clustal X (Zhang *et al.*, 2020b). The GenBank accession numbers of AAP from *Trichinella* spp. and other organisms were as follows: *T. spiralis* (KRY29491.1), *T. nelsoni* (KRX25887.1), *T. britovi* (KRY46829.1), T9 (KRX51999.1), T6 (KRX76744.1), *T. nativa* (KRZ55718.1), *T. murrelli* (KRX35979.1), *T. patagoniensis* (KRY14399.1), T8 (KRZ82592.1), *T. papuae* (KRZ70603.1), *T. zimbabwebsis* (KRZ06548.1), *T. pseudospiralis* (KRX94200.1), *Homo sapiens* (NP_001306047.1) and *Mus musculus* (AAD01212.1). At present, based on the presence or absence of a collagen capsule that surrounds the ML in the muscle cell (i.e., nurse cell), the genus *Trichinella* is classified into two major clades. Members of the encapsulated clade, which infect only mammals, are *T. spiralis*, *T. nativa, T. britovi, T. murrelli, T. nelsoni, T. patagoniensis*, and three other genotypes *Trichinella* T6, T8 and T9. The other non-encapsulated clade, which infects mammals, reptiles and/or birds, consists of *T. pseudospiralis*, *T. papuae* and *T. zimbabwensis* (Zarlenga *et al.*, 2020). The phylogenetic analysis was performed in MEGA 7.0 based on the Neighbor-joining (NJ) method as described previously (Zhang *et al.*, 2013; Qi *et al.*, 2018b).

Cloning, expression and identification of TsAAP
Total RNA was isolated from the ML using Trizol (Invitrogen, USA). The full-length cDNA sequence of TsAAP was amplified by PCR using specific primers with restriction enzyme sites *BamH* I and *Pst* I (underlined) (5'-AGCC<u>GGATCC</u>ATGAATGGAAAAT GTTGTTC-3'; 5'-CGG<u>CTGCAG</u>TTATAATTTTTTGTCGACTTCGG-3'). The PCR products were cloned into the expression vector pQE-80L, then the recombinant pQE-80L/TsAAP was transformed into *Escherichia coli* BL21 (DE3) (Novagen, USA). The rTsAAP was expressed by the induction using 1 mM IPTG for 8 h at 30°C (Xu *et al.*, 2018, 2020b), subsequently purified using Ni-NTA-Sefinose resin (Sangon Biotech Co, Shanghai, China) (Liu *et al.*, 2017). The concentrations of the purified rTsAAP proteins were measured by Bradford method, and separated and identified on SDS-PAGE and Western blotting (Wang *et al.*, 2017).

Murine immunization and polyclonal anti-rTsAAP antibody assay
Twenty mice were subcutaneously immunized with 20 μg rTsAAP emulsified with complete Freund's adjuvant. Three boosts were performed with 20 μg rTsAAP emulsified by incomplete Freund's adjuvant at a 2-week-interval (Sun *et al.*, 2018b; Hu *et al.*, 2020a). One week after the last immunization, tail blood was collected and anti-rTsAAP serum was separated (Cui *et al.*, 2013b). Serum anti-rTsAAP antibody IgG in serum samples of immunized mice was assayed by using ELISA (Xu *et al.*, 2021b). Briefly, the ELISA plate was coated with 2.5 μg/ml rTsAAP at 4°C overnight. After washes using phosphate buffered saline (pH 7.4) containing 0.05% Tween-20 (PBST), the plate was blocked with 5% skimmed milk at 37°C for 2 h, probed with 1:100 dilutions of murine immune sera at 37°C for 2 h, followed by the incubation of HRP-conjugated anti-mouse IgG (1:10000; Sigma) for 1 h at 37°C. Plates were developed with OPD (Sigma; USA) plus H_2O_2, the reaction was finished by addition of 2 M H_2SO_4. The OD values at 492 nm were assayed by a microplate reader (Tecan, Schweiz, Switzerland) (Liu *et al.*, 2015a; Lei *et al.*, 2020).

Western blotting and RT-PCR
Western blotting analysis was conducted as previously reported (Liu *et al.*, 2016; Yang *et al.*, 2020a). Somatic crude proteins from various *T. spiralis* stage worms and rTsAAP were separated by SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, USA) at 18 V 35 min. The membranes were blocked with 5% skimmed milk in TBST. The membrane was cut into strips which were probed with 1:100 dilutions of anti-TsAAP serum at 37°C for 2 h. After

washes using Tris buffered saline (pH 7.4) containing 0.05% Tween-20 (TBST), the membranes were further incubated with HRP-conjugated goat anti-mouse IgG (1:10 000; Southern Biotech). Finally, the membranes were developed with DAB (Sigma), and the coloration was stopped with deionized water.

RT-PCR analysis of TsAAP transcription was performed as described before (Wang *et al*., 2013a; Xu *et al*., 2021a). Total RNAs from diverse *T. spiralis* stages (ML, IIL, AW and NBL) was extracted with TRIzol reagent (Invitrogen). *T. spiralis* GAPDH (GenBank: AF452239) was also amplified and used as a housekeeping gene control (Long *et al*., 2015). PBS was used as a negative control in all PCRs.

Indirect immunofluorescence (IIF)
The IIF test was carried out with fresh complete worms of diverse *T. spiralis* phases (ML, IIL, AW and NBL) as reported before (Li *et al*., 2018; Liu *et al*., 2018). The intact worms were fixed with 4% formaldehyde for 30 min, and then re-fixed with cold acetone for 20 min. Moreover, worms were embedded in paraffin and cut into 2 μm thick cross-sections with a microtome. The intact whole nematodes and cross-sections were blocked with 5% normal goat serum in PBS, and then probed at 37°C for 1 h with a 1:10 dilution of various sera (anti-rTsAAP serum, infection serum or pre-immune serum). After washing with PBS, they were incubated with FITC-conjugated goat anti-mouse IgG (1:100; Santa Cruz, USA). After washing again, the whole worms and cross-sections were observed under a fluorescence microscopy (Olympus, Japan) (Cui *et al*., 2019; Zhang *et al*., 2020a).

Enzymatic activity assay of rTsAAP
To determine the enzymatic activity of rTsAAP, the diluted rTsAAP (0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.06, 0.07 μg/μl) was pre-incubated at 37°C for 10 min in various pH buffer (0.05 M Tris-HCl buffer is used at pH 7.0-9.0, and 0.1 mol/L citric acid-sodium citrate buffer is used at pH 6.0 and 6.5), subsequently the AAP substrate 0.1 mM H-Glu-P-nitroaniline (H-Glu-pNA, Yuanye, Shanghai, China) was added into the reaction mixture and incubated at different temperatures (17-47°C) for 30 min, and the absorbance at 410 nm was measured with a spectro-photometer (Lauterbach & Coetzer, 2008). In order to verify whether the enzymatic activity of rTsAAP depends on metal ions, the common auxiliary metal ions (Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+}) were added into the reaction system at the same concentration (0.1 mM) to analyze their effect on enzyme activity (Park *et al*., 2017; Guo *et al*., 2020). Effects of different enzyme inhibitors (1 mM PMSF, 5 μM E64, 10 mM EDTA and 1 mM AEBSF) on rTsAAP activity were also determined (Cui *et al*., 2015a). Standard curve was calculated according to rTsAAP hydrolysis of different concentrations of substrate H-Glu-pNA (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 mM), and obtained Km on the basis of Michaelis-Menten curve and Lineweaver-Burk curve value (Long *et al*., 2015).

IIF assessment of binding of rTsAAP and erythrocytes
Normal murine blood samples were first diluted with isosmotic PBS buffer (pH 7.4). Blood smear was prepared on slides, fixed with formaldehyde for 15 min, then washed with PBS, and blocked with 5% goat serum at 37°C for 2 h. After washing with PBS, the slides were incubated at 37°C for 2 h with different proteins (rTsAAP, AW crude proteins, maltose binding protein (MBP), and rTsAAP treated by EDTA). The slides were probed with different sera (1:10; anti-rTsAAP serum, anti-MBP serum, infection serum, and normal serum) at 37°C for 2 h. After washing, FITC-conjugated anti-mouse IgG (1:100, Santa Cruz) was incubated at 37°C for 1 h, and examined under fluorescence microscopy (Cui *et al*., 2015b; Yan *et al*., 2021).

Binding of rTsAAP and erythrocyte membrane proteins determined by Far Western
The *in vitro* binding between rTsAAP and erythrocyte membrane proteins was analyzed by Far-western analysis (Hu *et al*., 2020b). Erythrocytes were hemolysed, centrifuged at of 15000 *g* for 10 min. Supernatant was discarded, sediment was supplemented with 5 mmol·L^{-1} Tris-HCl (pH 7.4) and centrifuged again to obtain erythrocyte membrane (Coetzer and Palek, 1986). Erythrocyte membrane was boiled and membrane proteins were separated by SDS-PAGE with 12% separation gel (Wang *et al*., 2013b). After being blotted, the membrane cut into strips, blocked using 5% skimmed milk in TBST at 37°C for 2 h, and incubated with rTsAAP for 2 h at 37°C. The strips were probed anti-rTsAAP serum, anti-mouse IgG-HRP conjugate (1:10 000; Southern Biotech), and developed using substrate DAB (sigma) (Han *et al*., 2020).

RNA interference
TsAAP-specific dsRNA target sequence of 555~1176 bp of full length TsAAP cDNA sequence was selected to design the primers (Table 1). To verified the specificity of TsAAP-dsRNA, a *T. spiralis* cathepsin L gene (TsCL, GenBank: XM_003375022.1) was used as a specific control. Besides, green fluorescent protein (GFP) dsRNA was prepared for a negative control. The 5' end of the upstream and downstream PCR primer to amplify TsAAP gene was added with the T7 promoter sequence (Zhang *et al*., 2016). dsRNA was introduced into *T. spiralis* larvae by electroporation and cultured in RPMI 1640 medium at 37°C for 3 days. qPCR and Western blot analysis was performed to determine the expression level of TsAAP in ML (Liu *et al*., 2013; Han *et al*., 2020), and the expression level of GAPDH protein was used as a housekeeping gene control (Yang *et al*., 2019). The enzyme activity of natural TsAAP in crude proteins of dsRNA-TsAAP treated ML was analyzed by the above-described method and compared with the untreated ML.

Table 1. TsAAP-specific primers flanked by T7 RNA polymerase promoter sequences

Primer	Sequences
T7-F-TsAAP	5'-GATCAC<u>TAATACGACTCACTATAGGG</u>CACGTTCACCTCCAATACGAC-3'
T7-R-TsAAP	5'-GATCAC<u>TAATACGACTCACTATAGGG</u>TGTAGATCCGCATCCGATGTC-3'
T7-F-GFP	5'-GATCAC<u>TAATACGACTCACTATAGGG</u>AGTGCTTCAGCCGCTACC-3'
T7-R-GFP	5'-GATCAC<u>TAATACGACTCACTATAGGG</u>GCTTCTCGTTGGGGTCTT-3'

The underlined portion is T7 promoter sequence.

Challenge infection of mice with dsRNA-TsAAP transfected *T. spiralis* larvae

In order to investigate the larval development in host intestine after dsRNA-TsAAP interference, 30 mice were equally divided into 3 groups for larval challenge experiment. Each mouse was infected orally with 500 ML treated with 60 ng/μl dsRNA-TsAAP, Control dsRNA or PBS. At 3 and 6 dpi, intestinal adult worms were collected from 5 mice of each group, and intestinal worm burdens were ascertained and adult morphology were observed under microscopy (Liu *et al.*, 2015c). Fifty female adult worms at 6 dpi from each group of infected mice were cultivated, and female reproduction capacity (fecundity) was assessed according to the production of NBL deposited by each female within 72 h (Qi *et al.*, 2018a; Sun *et al.*, 2019).

Statistical analysis

All data were analyzed using SPSS 21.0 software, and the data were expressed as mean ± standard deviation (SD). Chi-square test was used to analyze the relative transcription and expression levels of TsAAP among various groups, and One-way ANOVA was used to analyze the differences of adult burdens, NBL production and parasite length. $P<0.05$ is considered statistically significant.

RESULTS

Bioinformatics analysis of TsAAP

The full-length cDNA sequence of TsAAP gene is 1404 bp encoding 467 amino acids, with a molecular weight (MW) of 51.76 kDa and isoelectric point (pI) of 6.24. TsAAP had no signal peptide site. The homology of TsAAP amino acid sequences was compared with AAP of other species or genotypes of the genus *Trichinella*. The amino acid sequences of the TsAAP had an identity of 94-99% with AAP of the 8 encapsulated *Trichinella* species, and an identity of 91-92% with AAP of three non-encapsulated *Trichinella* species.

SMART analysis showed that TsAAP contained a Peptidase_M18 domain, which has the function of catalysis of the hydrolysis of N-terminal amino acid residues. In the three-dimensional model, TsAAP has fourteen enzymatic activity sites (3 histidines, 2 glutamic acids, 2 aspartic acids, asparagine, serine, glycine, tyrosine, lysine, valine, methionine). Phylogenetic analysis results of TsAAP with AAP from *Trichinella* spp. showed that the monophyletic groups of 11 species/genotypes of *Trichinella* are well supported, and there are two distinct clades, one was the encapsulated clade (including *T. spiralis*, *T. nelsoni*, T6, *T. nativa*, *T. patagoniensis*, *T. murrelli*, *T. britovi*, T9 and T8), the other was the non-encapsulated clade (*T. pseudospiralis*, *T. zimbabwensis* and *T. papuae*). Compared with other *Trichinella* species/ genotypes, TsAAP is closely related to *T. nelsoni.*

Western blotting identification of rTsAAP antigenicity

The results of SDS-PAGE analysis showed that pQE-80L/TsAAP BL21 expressed a 51.76 kDa protein band. After purification with Ni-NTA Sefinose Column, a clear rTsAAP band with 51.76 kDa was observed, and the rTsAAP MW was consistent with its predicted size (51.76 kDa). Western blot analysis showed that lysates of recombinant pQE-80L/TsAAP prior to induction were not detected by infection serum. rTsAAP was recognized by anti-rTsAAP immune serum and anti-his tag monoclonal antibody (McAb), but not by pre-immune serum (Figure 1).

Transcription and expression of TsAAP in different worm stages

The RT-PCR results showed that TsAAP gene was transcribed in different *T. spiralis* phase worm (ML, IIL, 3 and 6 d AW, and NBL), and the GAPDH internal control gene also produced
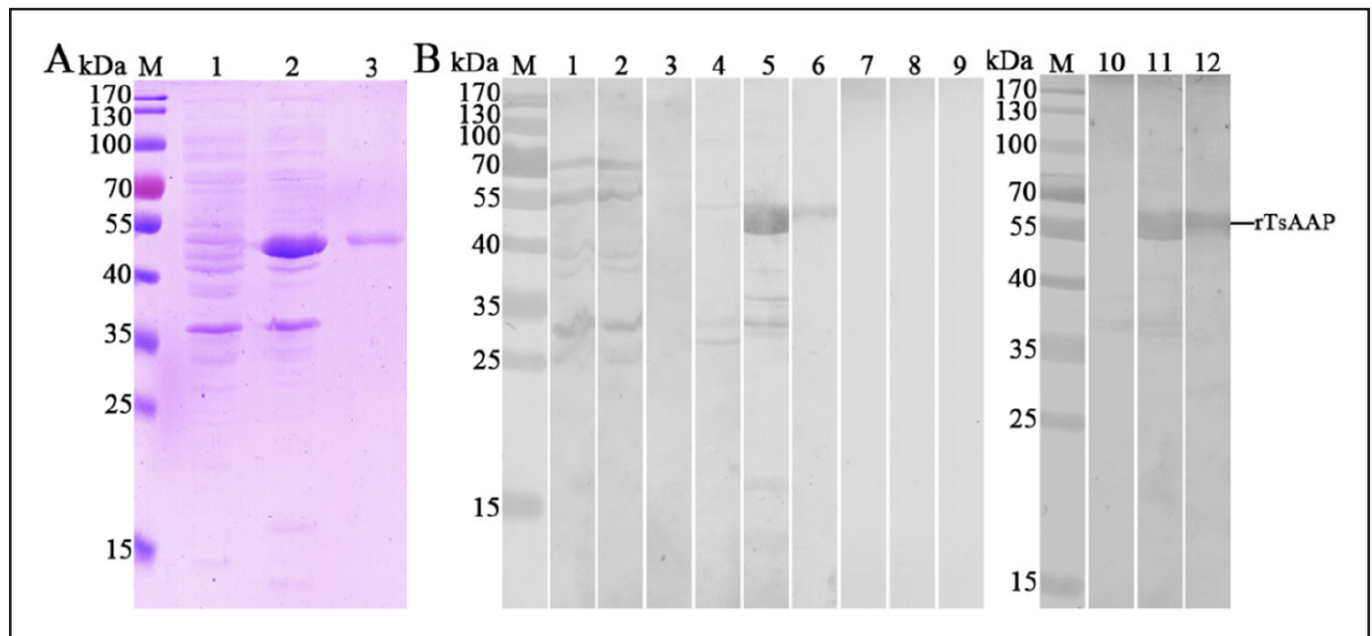
Figure 1. Antigenicity of rTsAAP protein. A: SDS-PAGE analysis of rTsAAP. Lane M: Protein marker; lane 1: lysate of recombinant *E. coli* incorporating pQE-80L/TsAAP prior to induction; lane 2: lysate of recombinant *E. coli* carrying pQE-80L/TsAAP following induction; Lane 3: purified rTsAAP. B: Western blotting of rTsAAP. Lysates of pQE-80L/TsAAP prior to induction (Lane 1, 4, 7 and 10) were not detected by infection serum (lane 1), anti-rTsAAP serum (Lane 4), pre-immune serum (Lane 7) and anti-his McAb (Lane 10). The lysates of pQE-80L/TsAAP following induction (Lane 2, 5, 8 and 11) and purified rTsAAP (Lane 3, 6, 9 and 12) were probed by anti-rTsAAP serum (Lane 5 and 6) and anti-his McAb (Lane 11and 12), but not by *T. spiralis* infected serum (lane 2 and 3) and pre-immune serum (Lane 8 and 9).

the expected size (570 bp) in all developmental phases (Figure 2). The natural TsAAP with 51.76 kDa in worm crude proteins of various phases (ML, IIL, 3 and 6 d AW and NBL) was identified by anti-rTsAAP serum on Western blotting analysis.

Expression and worm tissue location of native TsAAP in diverse *T. spiralis* stage

The results of IIF with whole parasites revealed that green immunostaining on the epicuticle of IIL, AW and NBL other than ML was observed using anti-rTsAAP serum and infection serum (Figure 3). When worm cross-sections were probed by anti-rTsAAP serum, the green fluorescence was located at the cuticle and stichosome of ML and IIL, and intrauterine embryos of female adults (Figure 4). No worm tissue components of the nematode were identified by pre-immune serum.

Enzyme activity of rTsAAP

The enzyme activity of rTsAAP increased with elevation of rTsAAP protein concentration, and stabilized when the rTsAAP concentration was 0.06 µg/µl (Figure 5). rTsAAP enzyme activity is the highest at 37°C and pH 7.5. The metal ions Co^{2+}, Mn^{2+}, Mg^{2+}, and Ni^{2+} enhanced the activity of rTsAAP enzyme, the effect size is $Co^{2+}>Mn^{2+}>Ni^{2+}>Mg^{2+}$, and Ca^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} have obvious inhibitory effect on rTsAAP enzyme activity. EDTA inhibited the rTsAAP enzymatic activity. The hydrolysis of H-Glu-pNA by rTsAAP conformed to Miman's kinetics. The kinetic parameter Vmax is 194.9 $\mu M \cdot min^{-1} \cdot mg^{-1}$, and the Km is 1.05 mM.

Binding of rTsAAP and erythrocyte by IIF

The IIF results showed that when anti-rTsAAP serum was used, green fluorescence was observed on the surface of erythrocytes pre-incubated with rTsAAP, but no fluorescence

staining was observed on surface of erythrocytes detected by normal serum and PBS (Figure 6). The erythrocytes pre-incubated by heat-inactivated rTsAAP and EDTA-treated rTsAAP did not exhibit any green fluorescence. The results showed that rTsAAP specifically combined to the surface of erythrocyte membrane.

Binding of rTsAAP and erythrocyte membrane proteins determined by Far Western

SDS-PAGE results showed that erythrocyte membrane proteins had 6 bands of 42-240 kDa. Far-western blotting revealed that five bands (42, 76, 80, 220 and 240 kDa) of them pre-incubated with rTsAAP were recognized by anti-rTsAAP serum, but not by normal mouse serum (Figure 7). Four bands (76, 80, 220 and 240 kDa) of them pre-incubated with rTsAAP were also recognized by infection serum. One band (114.4 kDa) pre-incubated with rTsAAP was not recognized by infection serum. Additionally, erythrocyte membrane proteins pre-incubated with MBP were not probed by anti-MBP serum. The results suggested that rTsAAP specifically was bound to erythrocyte membrane proteins (240 kDa α-spectrin, 220 kDa β-spectrin, 80 kDa protein 4.1, 76 kDa protein 4.2, and 42 kDa actin).

**Suppression of TsAAP expression and enzymatic activity by dsRNA-TsAAP**

dsRNA was introduced into the ML by electroporation and larval survival was observed after being cultured for 3 d. The results showed that larval survival rates of dsRNA-TsAAP, dsRNA-GFP and PBS group were 99.46, 99.49 and 98.78%, respectively ($\chi^2 = 0.276$, *P*>0.05), suggesting that electroporation has no obvious effect on larval survival. When the ML were transfected with different dose of dsRNA-TsAAP of (15, 30, 45, 60 ng/µl) and cultured for 3 days, compared with the PBS group, TsAAP mRNA expression levels were reduced
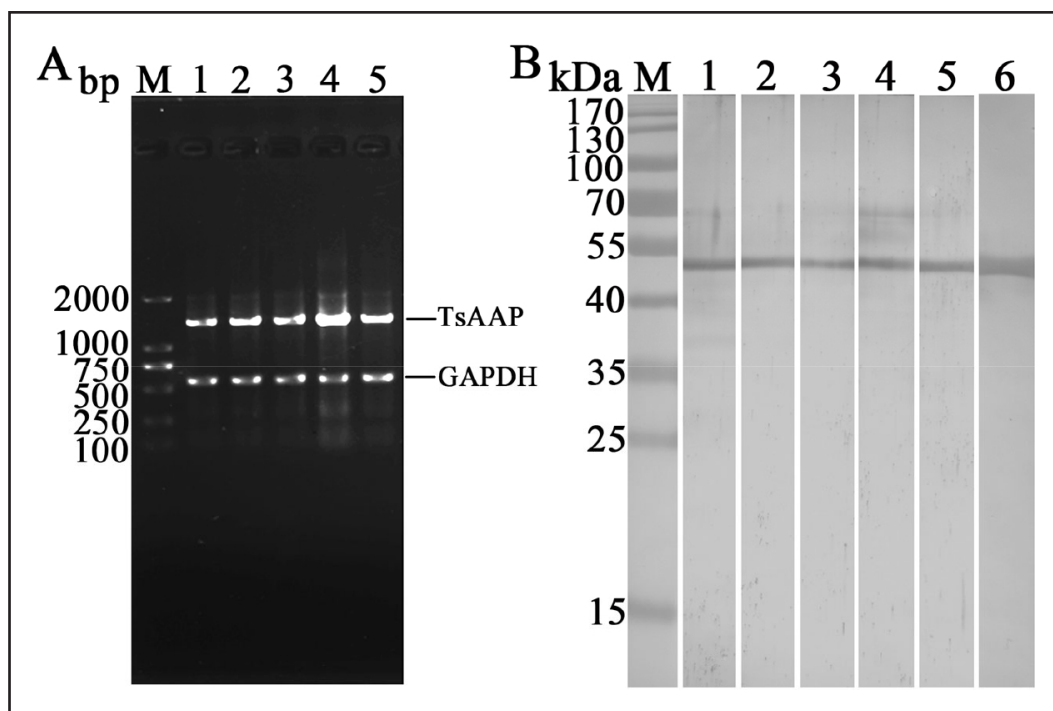

Figure 2. Transcription and expression of TsAAP in diverse *T. spiralis* stages. A. RT-PCR analysis of TsAAP gene transcription in different stages. M: DNA marker; 1: ML; 2: 6 h IIL; 3: 3 d AW; 4: 6 d AW; 5: NBL. B: Identification of native TsAAP in crude proteins of various *T. spiralis* stages on Western blot analysis with anti-rTsAAP serum. Lane 1: ML; lane 2: IIL; lane 3: 3 d AW; lane 4: 6 d AW; lane 5: NBL and rTsAAP (lane 6) probed by anti-rTsAAP serum was served as positive control; M: Protein marker.
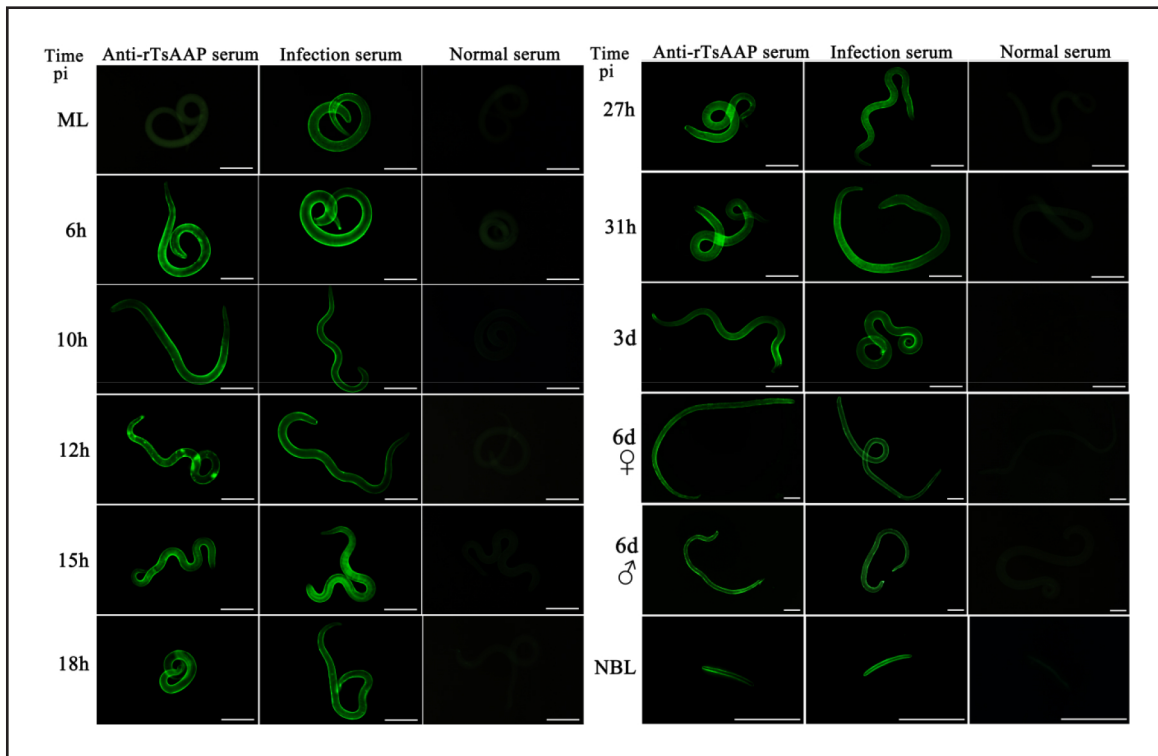
Figure 3. Expression of TsAAP at epicuticle of various *T. spiralis* stages by IIF. The whole worms were probed by anti-rTsAAP serum, and immunostaining was observed at the epicuticle of 6-31 h IIL, AW and NBL, but not at ML epicuticle. Normal serum did not recognize any worm components of the parasite. Scale bars of ML, IIL and AW = 100 μm; Scale bars of NBL = 50 μm.
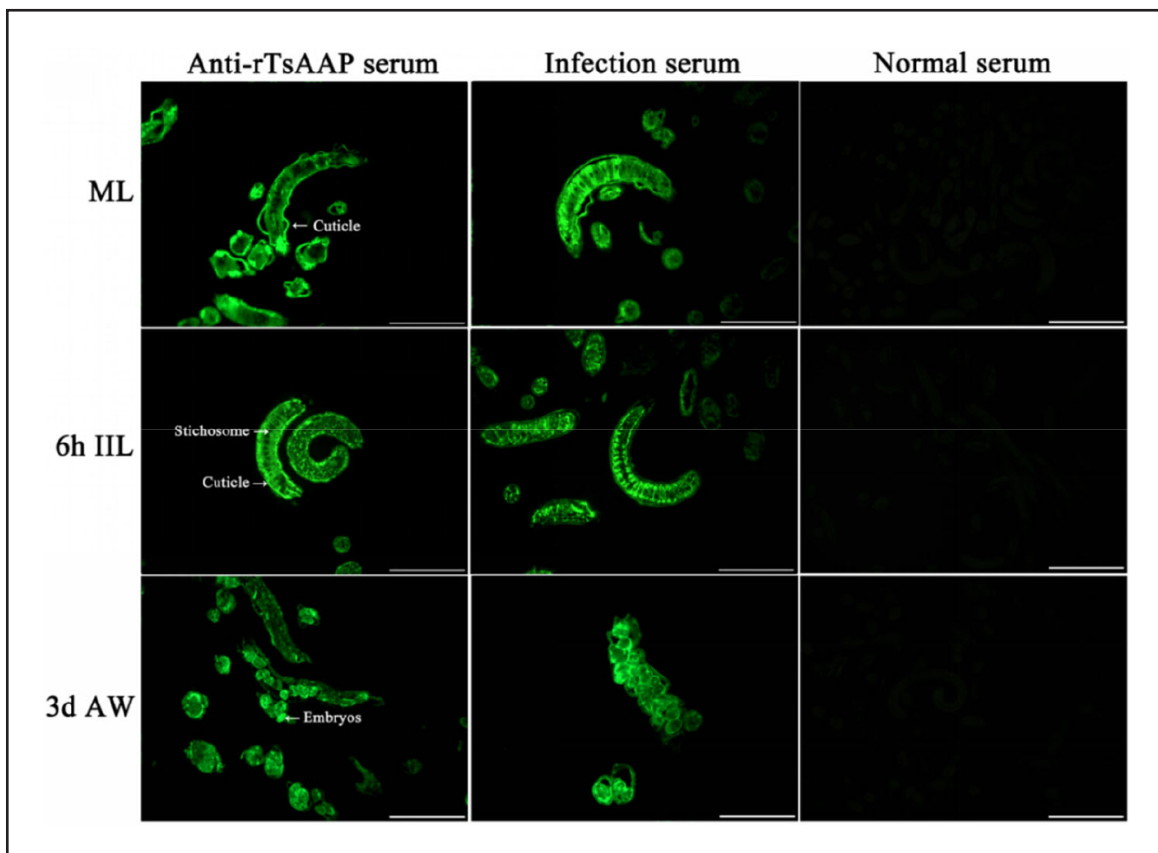
Figure 4. Immunolocalization of TsAAP in cross-sections of diverse *T. spiralis* stages by IIF with anti-rTsAAP serum. Fluorescence staining was observed at cuticle of ML, IIL and intrauterine embryos of adult females. No immunostaining in worm cross-sections was observed by using normal serum as a negative control. Scale bars: 100 μm.
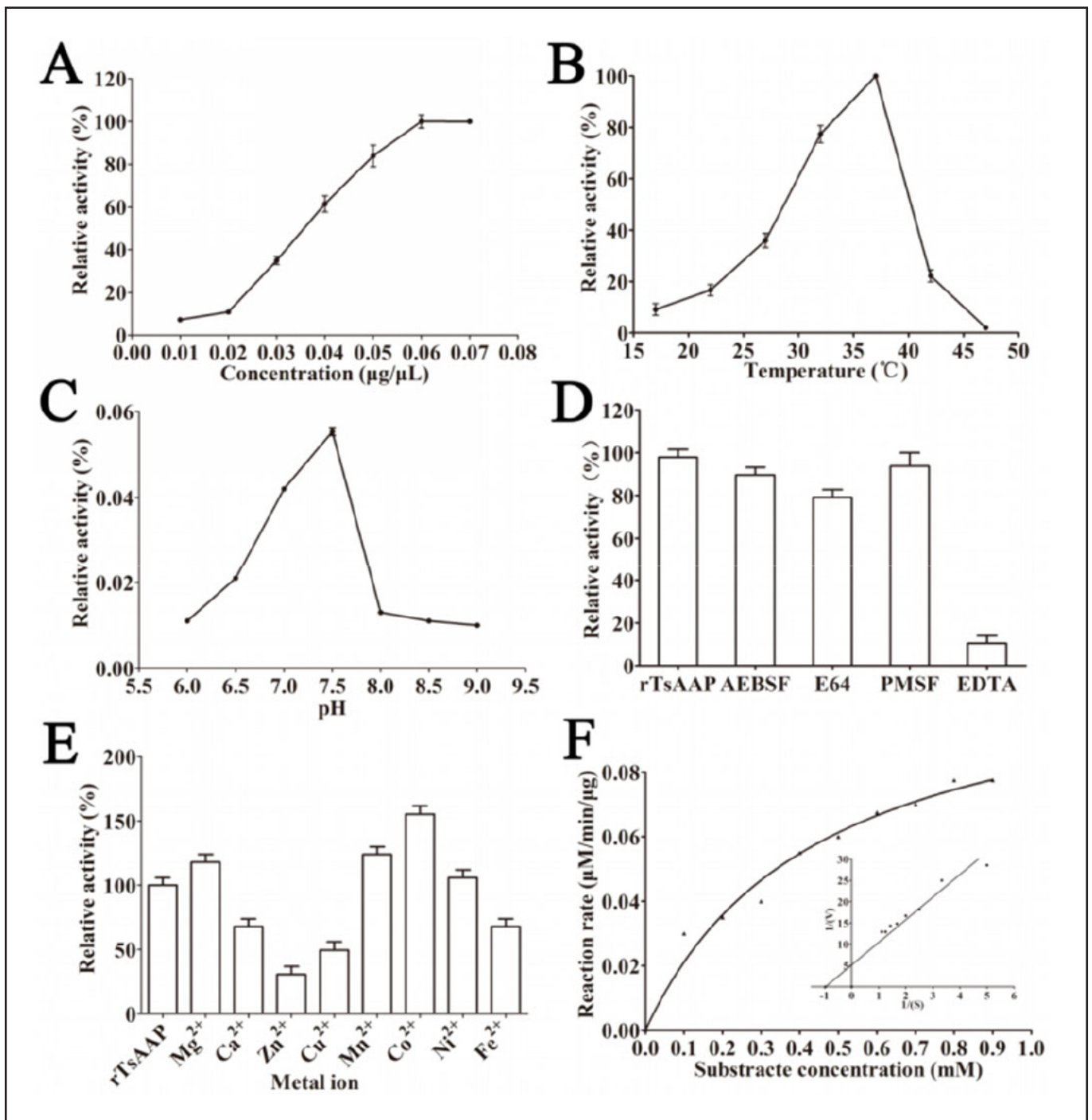
Figure 5. rTsAAP enzyme activity. A: The optimal enzyme amount for rTsAAP to hydrolyze the substrate; B: rTsAAP enzyme activity at different temperatures; C: rTsAAP enzyme activity at different pH values; D: Effects of various inhibitors on rTsAAP enzyme activity; E: Effects of different metal ions on rTsAAP enzyme activity; F: Michaelis-Menten plot and Lineweaver-Burk plot.

by 15.69, 22.14, 29.77, 67.88% ($P<0.05$), and TsAAP protein expression levels were decreased by 17.81, 20.88, 47.02 and 58.56%, respectively ($P<0.05$) (Figure 8A, 8B). On 1, 2 and 3 d after interference with 60 ng/μl dsRNA-TsAAP, TsAAP transcription level was decreased by 29.07, 59.84 and 75.49% ($P<0.05$), and TsAAP protein expression was inhibited by 26.31, 33.11 and 85.47%, respectively ($P<0.05$) (Figure 8C, 8D). There was no significant change of *T. spiralis* cathepsin L (TsCL) protein expression level in ML treated with dsRNA-TsAAP (Figure 8E), indicating that the dsRNA is TsAAP-specific.

Worm crude proteins were prepared after the ML treated with 60 ng/μL dsRNA-TsAAP was cultured for 3 d, and incubated

the TsAAP substrate (H-Glu-PNA). The results showed TsAAP enzymatic activity in soluble proteins of dsRNA-treated ML was decreased by 37.92% compared with in the PBS group ($P <0.05$) (Figure 8F).

Suppression of dsRNA-TsAAP on larval growth, development and fecundity

The mice infected with dsRNA-TsAAP treated ML showed a 21.93% adult reduction at 3 dpi and a 31.46% adult reduction at 6 dpi, compared the PBS group ($F_{3d} = 3.684$, $F_{6d} = 8.942$, $P <0.05$), and the control dsRNA groups did not exhibited any adult burden reduction ($P>0.05$) (Figure 9). Female fecundity
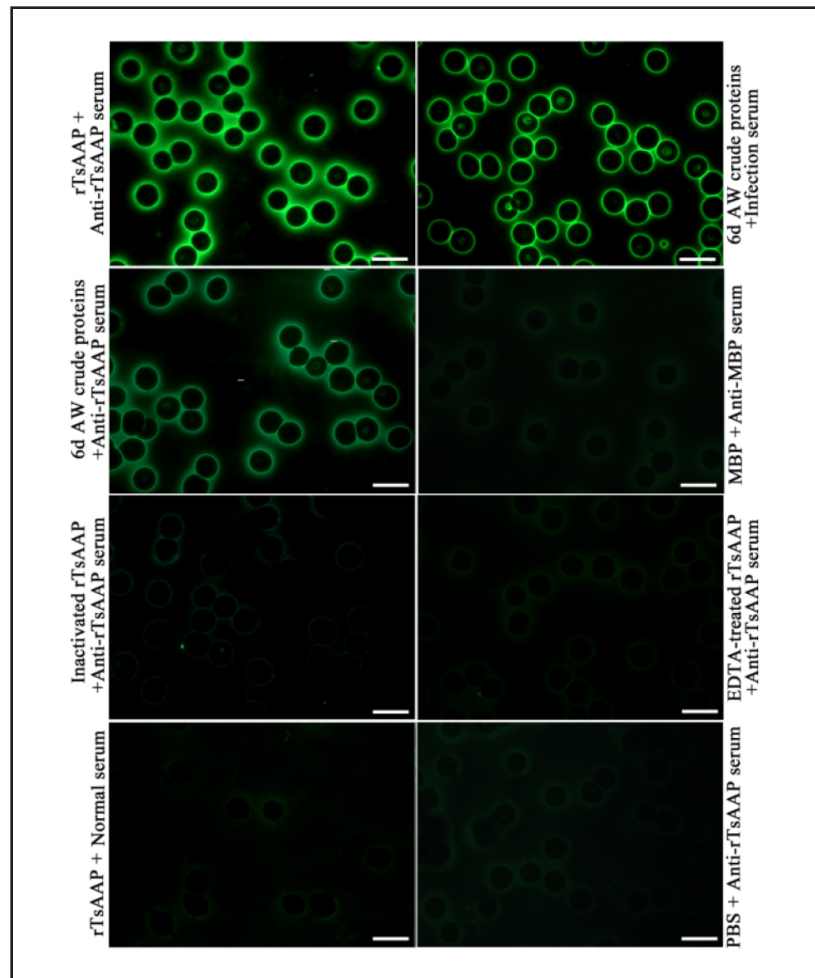
Figure 6. IIF assay of binding of rTsAAP and erythrocytes. IIF results indicated that the binding sites of rTsAAP and erythrocytes were on membrane of red blood cells. The erythrocytes incubating with crude adult protein were probed by anti-rTsAAP serum and infection serum as positive control, and MBP was used the irrelevant protein negative control. Scale bar: 20 μm.
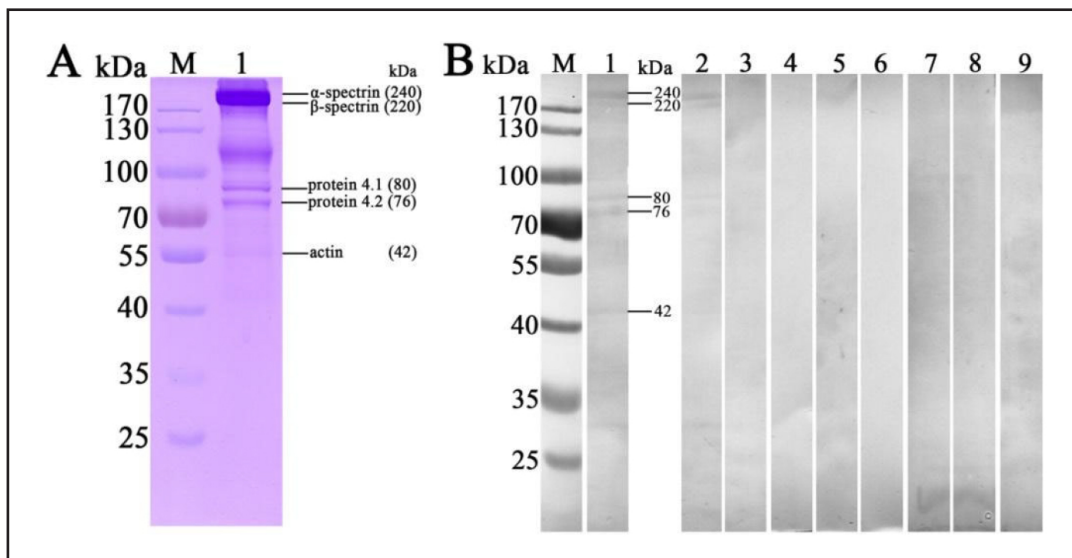
Figure 7. rTsAAP binds to erythrocyte membrane protein. A: SDS-PAGE of erythrocyte membrane proteins. M: protein marker; 1: erythrocyte membrane protein. B: Far-western blot of rTsAAP binding to erythrocyte membrane protein, M: protein marker; 1-3: erythrocyte membrane proteins incubated with rTsAAP; 4-6: erythrocyte membrane protein incubated with MBP; 7-9 erythrocyte membrane protein incubated with PBS. 1 and 7: probed with anti-rTsAAP serum; 2, 5 and 8: probed with infection serum; 3, 6 and 9: probed with pre-immune serum; 4: probed with anti-MBP serum.
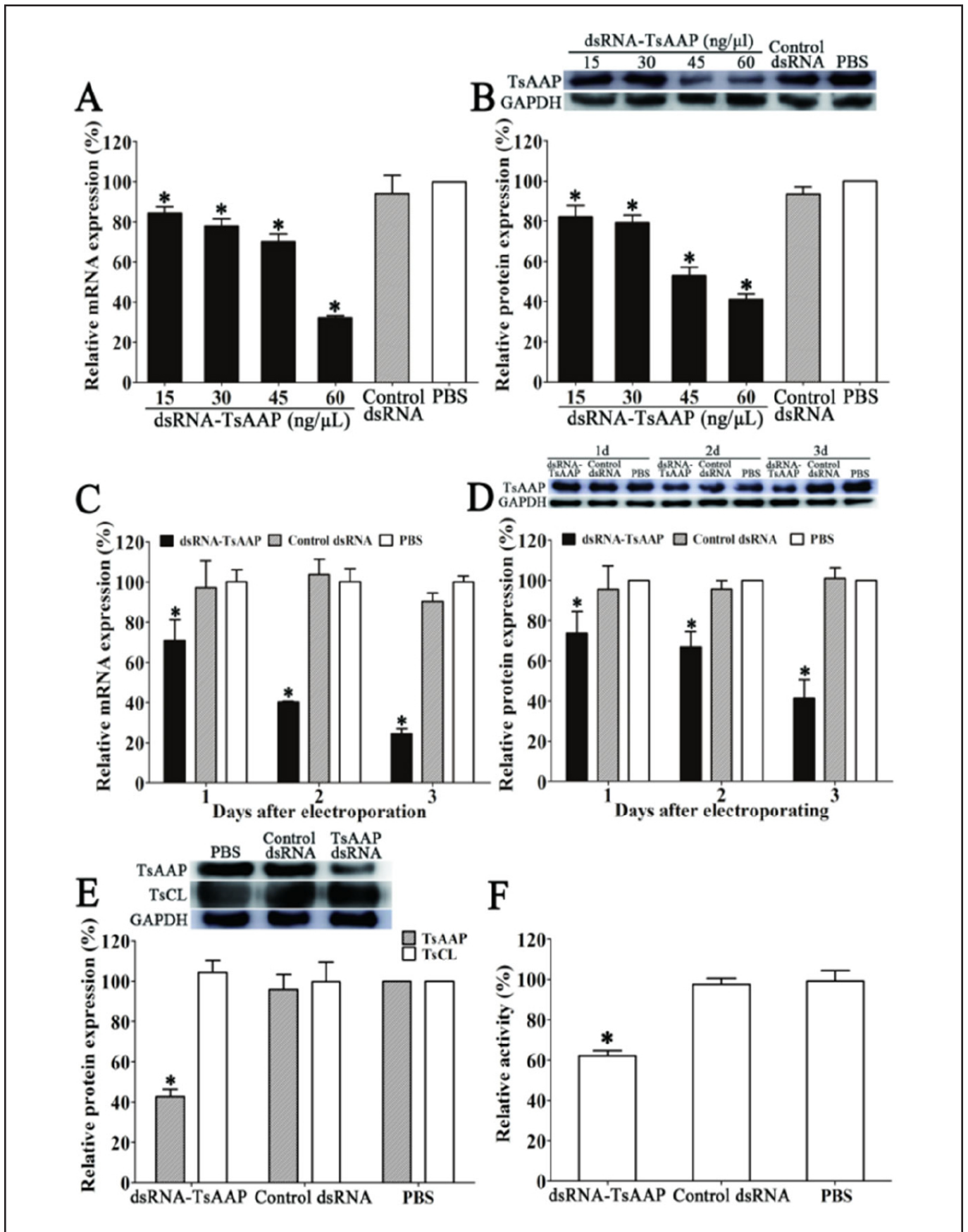
Figure 8. TsAAP transcription expression and enzyme activity after dsRNA interference. A: Effect of different dsRNA-TsAAP doses on TsAAP transcription level. B: Effect of different dsRNA-TsAAP doses on TsAAP expression level. C: TsAAP transcription levels at various days after dsRNA interference. D: TsAAP expression levels at various days after dsRNA interference. E: Expression levels of TsAAP and *T. spiralis* cathepsin L (TsCL) in ML treated using TsAAP-specific dsRNA. F: TsAAP enzymatic activity was significantly reduced in soluble proteins of dsRNA-TsAAP transfected ML. *$P < 0.05$ relative to the control dsRNA and PBS groups as negative controls.
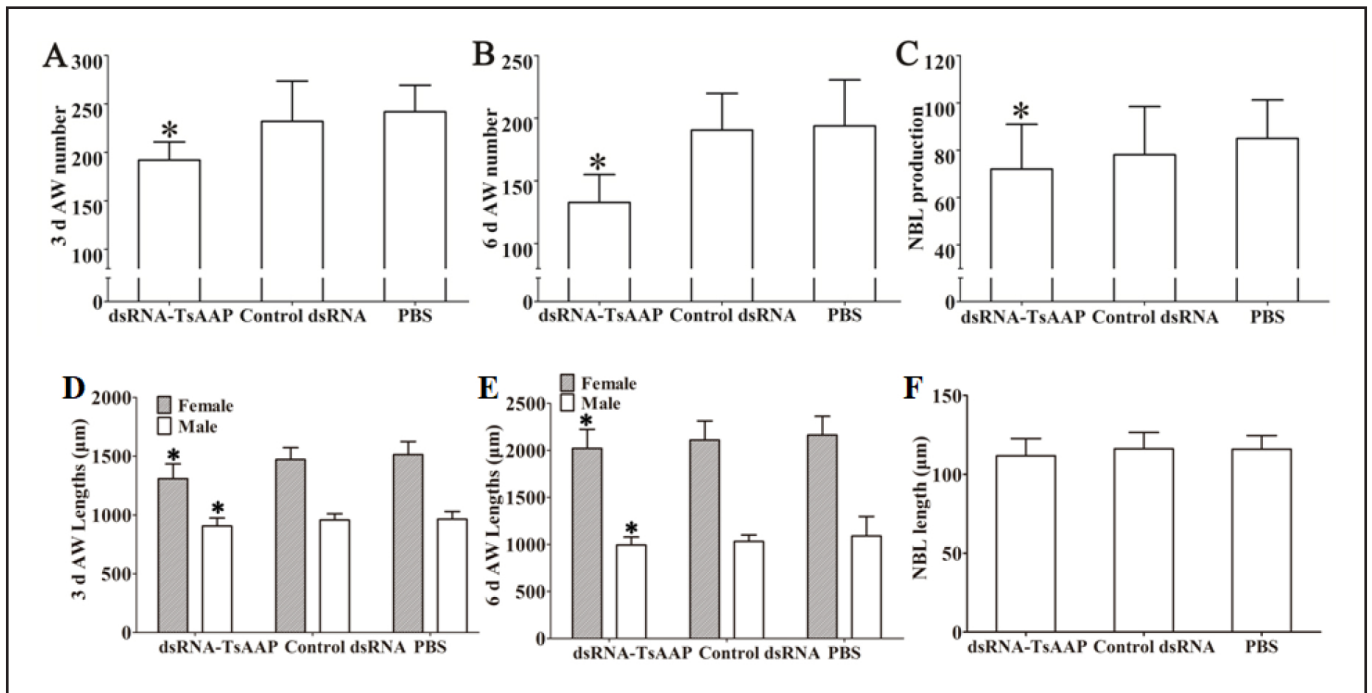
Figure 9. Intestinal adult worm burdens and lengths from mice challenged with muscle larvae transfected with dsRNA-TsAAP, control dsRNA or PBS. A: Intestinal adult worm burden at 3 dpi. B: Intestinal adult worm burden at 6 dpi. C: Newborn larvae (NBL) production of adult female adults in 72 h. D: 3 d adult worm lengths. E: 6 d adult worm lengths. F: NBL lengths. *Represents a significant difference compared with the control dsRNA and PBS groups as negative controls (P <0.05).

in dsRNA-TsAAP treatment group was also evidently lower than the PBS group (F = 3.303, P<0.05), but there were no significant differences of female fecundity between control dsRNA and PBS group (P>0.05), suggested that silencing of TsAAP inhibited intestinal larval development and reduced the female fecundity.

Furthermore, compared with the PBS group, the length of female and male worms at 3 dpi in dsRNA-TsAAP group was decreased by 13.35 and 15.25%, respectively, (P<0.05), the female and male length at 6 dpi was decreased by 6.56 and 8.76% (P<0.05), but the newborn larval length had no significant change (P>0.05), further conformed that silencing of TsAAP impaired intestinal larval growth and development.

DISCUSSION

Various *T. spiralis* enzymes play important roles in penetration, migration, feeding, parasitism and escape from host immune response (Ren *et al*., 2021). These enzyme molecules are usually stage-specific during the growth and development of *T. spiralis,* which may be related to the destruction of host mechanical barrier and humoral barrier (Ros-Moreno *et al*., 2000; Nagano *et al*., 2009). Most of enzymes had good antigenicity and have been proven to induce effective protective immunity against *Trichinella* infection and regarded as candidate vaccine target molecules (Zhang *et al*., 2018). At present, studies on *T. spiralis* enzymes mainly focuses on the serine proteases (Sun *et al*., 2018a), cysteine proteases (Song *et al*., 2018a; Yan *et al*., 2021), metalloprotease, aspartic proteases, and so on (Xu *et al*., 2020b), but there are only a few of studies on the peptidase function in lifecycle of *T. spiralis*. Previous studies showed that *T. spiralis* aminopeptidase is a potential candidate target for anti-*Trichinella* vaccine (Guo *et al*., 2020).

In this study, a TsAAP was cloned into pQE-80L plasmid and expressed in *E. coli* expression system. Bioinformatics analysis showed that TsAAP contained a Peptidase_M18 domain, which has the function of catalysis of the hydrolysis of N-terminal amino acid residues. Sequence analysis showed that TsAAP had 94-99% identity with AAP of the 8 encapsulated species of the genus *Trichinella*, suggesting that AAP of many members of the M18 family have high homology, and their sequences are highly conserved. The phylogenetic tree demonstrated that a monophyletic group of 12 species/genotypes of the genus *Trichinella*. After being purified, the rTsAAP was used to generate anti-rTsAAP immune serum. Immunization of mice with rTsAAP triggered an evidently specific anti-rTsAAP antibody response, and the titre of anti-rTsAAP IgG in immune serum was 1:10^4, demonstrating that rTsAAP had good immunogenicity.

The RT-PCR results showed TsAAP mRNA transcription was observed in various *T. spiralis* stages (ML, IIL, 3- and 6-day-old AWs, and NBL). On Western blotting analysis, rTsAAP was recognized by anti-rTsAAP serum and anti-his McAb, but no by *Trichinella*-infected mouse serum. Western blotting also revealed that natural TsAAP protein band with 51.76 kDa in soluble crude proteins of various stage worms were recognized by anti-rTsAAP serum, demonstrating that TsAAP protein was expressed in various *Trichinella* developmental phases. The results of IIF using whole worms revealed that natural TsAAP was expressed on epicuticle of various *T. spiralis* lifecycle stages other than muscle larvae. The IIF with worm cross sections showed that TsAAP was principally localized at the cuticle, stichosome and female intrauterine embryos of the parasite. Moreover, native TsAAP in ML ES proteins was not recognized by anti-rTsAAP serum (data not shown), suggesting that TsAAP is a surface and somatic protein, but not secretory protein of this nematode. The purified rTsAAP was not recognized by *T. spiralis* infected serum on Western blotting and there was no fluorescence on ML stage by IIF, it is likely because the TsAAP is a worm somatic protein, and neither an ES protein nor a surface protein. The native somatic

TsAAP is not exposed directly to host immune system and could not stimulate the host to produce a high level of anti-TsAAP antibodies during natural *T. spiralis* infection (Li *et al.*, 2015). The results suggested that TsAAP may play a role in intestinal worm and NBL growth and development in *T. spiralis* life cycle.

Enzymatic activity assay indicated that rTsAAP has the enzymatic activity of native AAP to hydrolyze the substrate H-Glu-pNA. The optimal temperature and pH value of rTsAAP was 37°C and pH 7.5, which is similar with that of *P. falciparum* aspartyl aminopeptidase (Lauterbach & Coetzer, 2008). The rTsAAP activity was obviously enhanced by four metal ions (Co^{2+}, Mn^{2+}, Mg^{2+} and Ni^{2+}), and Co^{2+} was the most effective metal ion cofactor of rTsAAP, further verifying that TsAAP is a aminopeptidase which requires divalent metal cations and an optimal basic pH for the hydrolysis (Chen *et al.*, 2012; Zheng *et al.*, 2016). Besides, other four metal ions (Ca^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+}) have obvious inhibitory effect on rTsAAP activity. Excess zinc usually inhibits the enzymatic activity of peptidases, it is likely because only weakly hydrophobic interactions between proteolytic regions can loosely bind together to form tetrahedral pyramids. Excess zinc will lead to the disintegration of the dimer complex and destroy the quaternary structure of peptidases (Barrett, 1994; Park *et al.*, 2017). Similar to other aspartyl aminopeptidase, rTsAAP is sensitive to metalloproteinase inhibitor (EDTA). Cysteine proteinase inhibitor (E64) and serine protease inhibitors (PMSF and AEBSF) have little effect on rTsAAP activity. These results indicated that rTsAAP is a metalloproteinase, and its enzyme activity is dependent on divalent metal ions and can be inhibited by metalloproteinase inhibitors (Guo *et al.*, 2020).

In this study, the protein-protein interaction between rTsAAP and erythrocyte proteins was also evaluated. The IIF results revealed that there is a strong specific binding between rTsAAP and surface of erythrocyte membrane. Far-western analysis showed that rTsAAP can specifically bind to five kinds of erythrocyte membrane proteins (α-spectrin, β-spectrin, protein 4.1, protein 4.2, and actin). The α-spectrin and β-spectrin are macromolecular heterotetramers composed of α and β subunits in the cytoskeleton. The importance of spectrin in maintaining cell shape, mechanical properties and integrity of cell membranes has been clarified by many studies on red blood cells (Machnicka *et al.*, 2019). Previous studies have shown that mutations in the spectrin protein in red blood cells can make the cells more fragile and less flexible, and lead to hereditary hemolytic anemia (Bennett & Healy, 2008). The enhanced saliva acidification of spectrin in erythrocytes in visceral leishmaniasis patients may be related to the rupture of spectrin, leading to severe distortion of erythrocyte development and impaired integrity of erythrocyte membrane (Samanta *et al.*, 2011). Protein 4.1 contains spectrin-actin binding domain (SAB), which serves as a membrane-cytoskeleton crosslinking agent to connect spectrin-actin filament complex and a variety of transmembrane proteins. The formation of the ternary-complex between protein 4.1, spectrin-actin and actin effectively enhances the interaction between spectrin and actin (Baines *et al.*, 2014). Actin is a class of multifunctional globular proteins that form micro-filaments. It has a dynamic structure that can be assembled and disassembled. Erythrocyte actin filaments provide more sites for the spectrin-actin filament complex to connect to the lipid bilayer (Gokhin & Fowler, 2016).

AAP can specifically remove glutamate and aspartic acid at the N-terminal of the peptide chain. It has been found that actin and α-spectrin have three aspartic acids (actin)

and one glutamic acid (α-spectrin) following the N-terminal methionine (Bradshaw *et al.*, 1998). Aspartic acid or glutamic acid near the N-termini of β-spectrin, protein 4.1 and protein 4.2 may be the primary targets of TsAAP (Teuscher *et al.*, 2007). All the above proteins are important components of the cytoskeleton system of cell membranes. Actin and protein 4.1 are located in the junction complex, and protein 4.2 is located in the band 3 complex of the erythrocyte membrane. Actin and protein 4.1 are located in the junction complex, and protein 4.2 is located in the band 3 complex of the erythrocyte membrane (Tse & Lux, 1999). Thus, cleavage of any of these proteins can destabilize the cytoskeleton of the erythrocyte membrane. *P. falciparum* AAP is involved in the digestion of hemoglobin in food vacuoles by cleavage of membrane skeleton proteins (e.g., spectrin) (Lauterbach & Coetzer, 2008). *P. falciparum* digest hemoglobin into peptides, which are converted into single amino acids by the combined action of AAP and other aminopeptidases (Dalal & Klemba, 2007). So hemoglobin is an important nutrient source of amino acids in parasites. *T. spiralis* mainly obtain nutrition from the body fluid and tissue fluid of the host. Although it is not a blood-sucking nematode, there is a large amount of hemoglobin in its pseudocelom of the parasite. Hemoglobin and oxyhemoglobin had been identified in *Trichinella* larvae. Unlike most other parasite hemoglobin, the hemoglobin found in larvae has a very low affinity for oxygen. Hemoglobin appears to accumulate gradually in larvae, and the encapsulated larvae that have resided in skeletal muscles for over 6 weeks have more hemoglobin than younger worms (Campbell *et al.*, 1983). It has been found that *Caenorhabditis elegans* can ingest and digest fluorescently labeled erythrocytes at all stages of parasitic feeding. The nematode uses the suction force of the pharyngeal pump to capture the red blood cells, which rupture and release their contents. The contents of the red blood cells enter the intestine through the pharynx, and the nematode can survive on the red blood cells (Chauhan & Pritchard, 2019). Therefore, it is speculated that TsAAP may have a similar physiological activity to hydrolyze hemoglobin to free amino acids through proteolytic cascade reaction. Aminopeptidase plays a key role in protein turnover and regulation of intracellular amino acid library. They are essential for the metabolism, growth and development of all cells and tissues, (Mucha *et al.*, 2010). As an intracellular peptidase, AAP may play a role in the last step of the cascade reaction, producing free aspartic acid and glutamic acid for *T. spirais* to synthesize the macromolecules. The results suggested that *T. spiralis* might destroy the erythrocyte membrane through interaction between TsAAP and erythrocyte membrane skeleton proteins, hydrolyze hemoglobin into free amino acids with the participation of TsAAP, and consequently get nutrition for maintaining the worm growth and development.

RNA interference (RNAi) technique was extensively used in many organisms to identify genes involved in regeneration and development and to provide information about gene function (Fischer, 2015). In recent years, RNAi has been applied to study the function of *Trichinella* genes (Wang *et al.*, 2015; Xu *et al.*, 2020b; Yang *et al.*, 2020a). In this study, TsAAP-specific dsRNA was designed on the basis of Peptidase_M18 functional domain of TsAAP. dsRNA-TsAAP was delivered into the ML by electroporation, the results showed that 60 ng/µL dsRNA treatments for 3 days had the ideal interference effect, the TsAAP expression and enzymatic activity in dsRNA-transfected ML was decreased by 85.47% and 37.92% respectively. After silencing the TsAAP gene, worm burdens of intestinal adult worms recovered from infected mice at 3 and 6 dpi were significantly decreased, the length of adult

worms became shorter, and the fecundity of 6 d female worms was distinctly reduced. The results suggested that silencing of TsAAP gene might impair *T. spiralis* nourishment uptake, significantly impede worm growth and development, and inhibited female reproduction capacity (Yang *et al.*, 2020b).

In conclusion, TsAAP was transcribed and expressed in all stages of *T. spiralis* lifecycle. It was principally located at cuticle, stichosome and intrauterine embryos of this nematode. rTsAAP had good immunogenicity. rTsAAP has an enzymatic activity of a natural aspartyl aminopeptidase. rTsAAP has the capacity to specifically bind to erythrocyte membrane proteins. Silencing of TsAAP gene by specific dsRNA obviously reduced the levels of TsAAP transcription, expression and enzymatic activity. RNAi also significantly decreased intestinal worm burdens, worm length and female fecundity, suggested that TsAAP might play an important role to provide the parasite with nourishment. The results indicated that TsAAP participates in the growth, development and fecundity of *T. spiralis* and it might be a potential candidate target for anti-*Trichinella* vaccine and therapeutic drug.

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Conflicts of interest
The authors declare no conflicts of interest with regards to this study or the manuscript prepared for publication.

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