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Molecular characterization and tissue localization of glutathione *S*-transferase from adult *Ancylostoma ceylanicum*

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Research Paper

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Abstract

Glutathione *S*-transferases (GSTs) are a detoxifying enzyme family that is essential for parasite blood-feeding and survival, and represent potential targets for hookworm vaccine development. Multiple GST-encoding complementary DNAs (cDNAs) have been cloned from *Ancylostoma caninum* and *Necator americanus*, but there are no reports about the cloning of this enzyme from *Ancylostoma ceylanicum*, the animal-derived zoonotic hookworm. To study the molecular nature and tissue localization of GST of *A. ceylanicum* (*Ace*-GST), we designed primers based on the GST gene sequence of *A. ceylanicum* in GenBank, amplified the *Ace*-GST cDNA by reverse transcription polymerase chain reaction, and analysed its homology and genetic evolution relationship. The amplified product was cloned into the pET-32a vector and transformed into *Escherichia coli* BL21 (DE3) for expression. To prepare anti-GST polyclonal antibodies, the recombinant protein was purified and used to immunize Kunming mice. The level of immunoglobulin G (IgG) antibody in the serum of immunized mice was detected by indirect enzyme-linked immunosorbent assay, and the *Ace*-GST localization in adult worm was determined using the immunofluorescence method. The results showed that the full-length cDNA encoding *Ace*-GST was 468 bp, which had the highest homology with *Ac*-GST-1 (60.1%) and clustered into one branch (*v*-class) with *Ac*-GST-1 and *Na*-GST-1 in a phylogenetic tree. Mice immunized with recombinant *Ace*-GST showed specific IgG antibody response. Immunolocalization revealed that natural *Ace*-GST is mainly located in the epidermis, muscle and intestine of the adult. These results may lay a foundation for further studies on the biological function of *Ace*-GST.

Introduction

Hookworms are soil-transmitted nematodes that inhabit the small intestines of humans, dogs and cats, and can cause iron deficiency anaemia, protein malnutrition, diarrhoea in their hosts, as well as growth retardation and mental retardation in children (Crompton, 2000; Traub *et al*., 2008; Bowman *et al*., 2010). The disability-adjusted life year (DALY) caused by hookworm has surpassed the three tropical diseases of the Special Program for Research and Training in Tropical Diseases (WHO-TDR) project – namely, leprosy, trypanosomiasis and Chagas disease (Murphy, 2013). Human hookworm infection causes more DALYs than dengue fever and is the second-highest DALY-causing disease only after malaria. The main hookworms infecting humans are *Ancylostoma duodenale* and *Necator americanus* (Hotez *et al*., 2005). Hookworms infecting dogs and cats include *Ancylostoma ceylanicum*, *A. caninum*, *A. tubaeforme*, *A. brasiliense* and *Uncinaria stenocephala*. Among them, *A. ceylanicum* is the only animal-derived hookworm that can develop into an adult in the human intestine, causing diarrhoea, anaemia and occult blood in stool (Ngui *et al*., 2012). Currently, regular use of anthelmintic drugs is the main method for controlling hookworm disease. However, because of hookworm re-infection and resistance to drugs after long-term use, anthelmintic drugs fail to eliminate this disease (Albonico *et al*., 2003). Therefore, it is urgent to explore new methods to control hookworm infection.

Recently, some scholars began to explore the prevention and treatment of hookworm disease by targeting the molecules involved in the pathogenic mechanism of hookworm, such as the detoxifying enzyme glutathione *S*-transferase (GST). The parasite GST not only plays an important role in detoxifying endogenous and host-derived reactive oxygen species (ROS), but also can be used as a vaccine or drug target against parasitic infection (Brophy & Pritchard, 1994; Torres-Rivera & Landa, 2008). GSTs detoxify endogenous and xenobiotic electrophilic toxins by catalysing their conjugation with glutathione (GSH). Hence, many parasitic helminth GSTs are involved in the protection of parasites against membrane damage induced by cytotoxic products of immune-initiated lipid peroxidation (Brophy & Barrett, 1990; Cervi *et al*.,

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1999; KampköTter et al., 2003). GSTs from the blood-feeding nematodes *Haemonchus contortus* (*Hc*-GST), *N. americanus* (*Na*-GST-1) and *A. caninum* (*Ac*-GST-1) showed a limited conjugating activity on products of lipid peroxidation, but these enzymes can form a binding site with high affinity to free haem through self-dimerization, and thus can detoxify and/or transport haem and haem-related compounds produced from haemoglobin digestion (van Rossum et al., 2004; Zhan et al., 2005, 2010). Animals immunized with recombinant hookworm GSTs can produce protective immunity against hookworm infection, among which *Na*-GST-1 has been selected as a candidate vaccine against human hookworm infection (Zhan et al., 2005, 2010).

At present, a total of 13 GSTs has been identified in the intestinal transcriptome of *A. ceylanicum*. Among which, one GST (GenBank ANCCEY-00737) had the highest similarity with *Na*-GST-1 (Wei et al., 2016), but no studies have investigated the molecular cloning of these enzymes from *A. ceylanicum*. This study aims to clone and express the complementary DNA (cDNA) encoding the GST enzyme from *A. ceylanicum*, and to investigate its molecular characterization and tissue localization, which may provide a basis for further studies on the biological function of this protein.

Materials and methods

Parasites

Adult hookworms were collected from the small intestine of a dead naturally infected dog obtained from the Surgical Laboratory of South China Agricultural University. *Ancylostoma ceylanicum* hookworms were identified by polymerase chain reaction (PCR) amplification of internal transcribed spacer (ITS) sequences as described previously in our laboratory (Liu et al., 2013) using the parasite genomic DNA as a template, followed by sequencing. Positive *A. ceylanicum* samples were stored in ribonucleic acid (RNA) sample preservation solution at −20°C.

Cloning of Ace-GST cDNA

Total RNA was extracted from adult *A. ceylanicum* using a MicroElute Total RNA Kit (Omega, Georgia, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Massachusetts, USA). The *Ace*-GST gene was amplified using a pair of specific primers, GST-AF (5′-GGATCCATGAGTAAGCGACATCAAGC-3′) and GST-AR (5′-GAATTCTCAGAAGGTGGAGCTCGGT-3′) designed based on the *A. ceylanicum* GST gene sequence (ANCCEY_00737). The underlined portions represent the restriction site of *Bam*H I and *Eco*R I, respectively. PCR products were connected to the pMD-18T cloning vector and transferred into *Escherichia coli* DH5α (Sangon, Shanghai, China). Positive clones were screened by bacterial PCR and sent to the Sangon Biotech (Shanghai, China) company for sequencing.

Sequence analysis

The MegAlign module of DNASTAR software version 5.0 (DNASTAR Inc., Madison, USA) was used to compare the *Ace*-GST sequence with other GST amino acid sequences. The phylogenetic tree of *Ace*-GST and the other 18 GST amino acid sequences downloaded from the National Center for Biotechnology Information was constructed with the neighbour-joining method by MEGA 5.0 software (Institute of Genomics and Evolutionary Medicine, Temple University, USA). Phylograms were drawn using FigTree v.1.4.2 software (Institute of Evolutionary Biology, University of Edinburgh, UK).

Expression and purification of Ace-GST

The purified recombinant plasmid and the pET-32a expression vector (TaKaRa, Dalian, China) were digested with the same restriction enzymes (*Bam*H I and *Eco*R I) and then ligated by T4 DNA ligase. The resulted recombinant plasmid (pET-32a-*Ace*-GST) was transformed into *E. coli* BL21 (DE3)-competent cells (Sangon, Shanghai, China) for expression. After cultivation at 25°C and induction for 8 h with 1 mm Isopropyl-β-d-thiogalactoside, the bacteria were collected by centrifugation and suspended in 10 mL phosphate buffer saline (PBS). The bacterial liquid was ultrasonicated on ice, and the obtained cell lysates were centrifuged at 12,000 g for 10 min. The supernatant and the precipitate were separately analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to interpret the solubility of the recombinant protein. The recombinant *Ace*-GST was purified using a His-Tag protein purification kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Briefly, the BeyoGold™ His-Tag purification resin-packed column was equilibrated twice by non-denaturing lysate (provided by the kit). The clear suspension of bacterial lysate was loaded to the purification column, and the lid at the bottom of the column was opened to allow the liquid to pass out of the column by gravity. The column was washed with eight bed volumes of washing buffer (10 mM imidazole). The polyhistidine-tagged protein was eluted with five bed volumes of different elution buffers (20, 40 and 250 mM imidazole) and then analysed by SDS-PAGE to test its purity.

Polyclonal antibody preparation

A total of 30 female Kunming mice aged six weeks were purchased from the Laboratory Animal Center of Southern Medical University and randomly divided into vaccination group (group A) and control group (group B). Group A ($n = 15$) was immunized with recombinant *Ace*-GST protein emulsified with Freund's adjuvant (Sangon, Shanghai, China). These mice were immunized by intraperitoneal injection at the antigen dose of 100 μg/mouse three times at two-week intervals. Freund's complete adjuvant was used for the first immunization and Freund's incomplete adjuvant was used for the last two immunizations. Group B ($n = 15$) was injected with PBS with adjuvant only. Blood samples were collected by the eyeball method 14 days after each immunization. To separate serum, the blood was placed at 37° for 1 h, precipitated overnight at 4°C in a refrigerator, and then centrifuged at 2400 g for 10 min. The collected sera were stored at −70°C until use.

Determination of antibodies to rAce-GST

The serum anti-r*Ace*-GST immunoglobulin G (IgG) level in immunized mice was assessed by an indirect enzyme-linked immunosorbent assay (ELISA) according to standard protocols. Briefly, a 96-well ELISA plate was coated overnight with sodium carbonate buffer solution (pH 9.6) and the purified recombinant *Ace*-GST (0.4 μg) at 4°C. The plate was later washed three times

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with PBS-Tween and blocked overnight with the blocking solution at 4°C. After washing, diluted mice sera were added to the plate (100 µL in each well), and incubated at 37°C for 2 h. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG was used as the secondary antibodies (1:400, 100 µL in each well) and incubated at 37°C. Freshly prepared 3,3′,5,5′-Tetramethylbenzidine (100 µL in each well) was used as a substrate, left for 10 min and then the reaction terminated by adding 25 µL 2 M sulfuric acid. The absorbance of coloured reaction products was measured at 450 nm (OD_{450} value) using a microplate reader. The serum samples were judged as positive when the ratio of its OD_{450} value/OD_{450} of non-immunized mice serum was ≥2.1, and the highest dilution ratio of serum was the titre of the serum; otherwise, it was judged as negative.

Recombinant protein Western blotting (WB) analysis

The recombinant proteins (10 µg) were separated by SDS-PAGE gel electrophoresis, and then analysed by WB using His-Tag mouse monoclonal antibody (1:5000), mouse anti-GST serum (1:1000) and infected dog serum (1:2000) as the primary antibodies. The corresponding secondary antibodies were HRP-conjugated sheep anti-mouse IgG (1:20000, Beyotime, Shanghai, China) in the first two reactions, and HRP-conjugated rabbit anti-dog IgG (1:10000, Beyotime, Shanghai, China) for the third reaction. The immunoreactive bands were visualized by chromogenic staining with Diaminobenzidine as the HRP-substrate.

Immunolocalization of Ace-GST

Ace-GST was immunolocalized in adult worms according to the method previously described by Don *et al.* (2007). Briefly, *A. ceylanicum* adult worms embedded in paraffin were cut longitudinally or transversely and continuous sagittal sections were fixed to glass slides. Each section was probed with anti-*Ace*-GST mouse sera diluted at 1:250 (experimental group) and pre-immune mouse serum (control group) and incubated overnight at 4°C. Then, sections were incubated for 2 h at room temperature with fluorescein isothiocyanate-conjugated sheep anti-mouse IgG. Sections were observed using a Nikon C2 fluorescence microscope with 488 nm excitation and 525 nm emission filters.

Results

Cloning, expression and analysis of Ace-GST

Ace-GST cDNA was cloned from adult *A. ceylanicum* total RNA using reverse transcription PCR (RT-PCR) and sequenced. The full-length cDNA sequence was 468 bp, encoding an open reading frame of 155 amino acids. The nucleotide and translated amino acid sequences of the *Ace*-GST gene were deposited in GenBank (MN103336). After that, the *Ace*-GST recombinant protein was expressed and purified. SDS-PAGE results showed that significant bands were observed at about 36 kDa (including about 20 kDa of pET32a vector protein) (fig. 1a), which was consistent with the expected molecular weight of the protein. Solubility analysis showed that the protein mainly existed in the precipitation of lysates. When eluted by 250 mM of imidazole solution, a large amount of recombinant protein was obtained at about 36 kDa with a protein concentration of 0.521 mg/mL. The WB analysis showed that the recombinant protein antigen could be recognized by His-Tag mouse monoclonal antibody and anti-*Ace*-GST immune serum (fig. 1b).

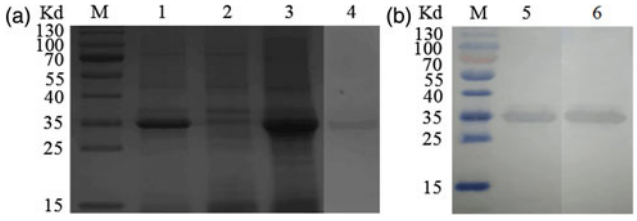

Fig. 1. Expression of cDNA coding *Ace*-GST (a) and WB analysis of recombinant *Ace*-GST (b). 1. product from induced recombinant plasmid pET32a-Ace-GST; 2. supernatant of bacteria lysates; 3. precipitate of bacteria lysates; 4. purified *Ace*-GST proteins; 5. recombinant proteins detected by His-Tag murine monoclonal antibody; 6. recombinant proteins detected by immune serum. M, protein molecular weight marker.

Homology and phylogenetic analysis of Ace-GST amino acid sequence

Homology analysis showed that the *Ace*-GST protein was composed of 155 amino acids with a typical GST C-terminal structure (IPR004046), which had the highest similarity (60.1%) with *Ac*-GST-1 (fig. 2). Phylogenetic analysis showed that the *Ace*-GST was closely related to the nu (ν) class of the GST family (fig. 3) from other nematodes. Within this group, our *Ace*-GST was most closely related to *Ace*-GST (EPB80182.1), a part of an unpublished draft genome of *A. ceylanicum* obtained by conceptual translation, followed by GST from *A. caninum* and *N. americanus*. However, each of the other GST classes, such as kappa (κ), theta (θ), alpha (α), mu (μ) and omega (Ω) GST, clustered into a separate branch.

Antibody response to Ace-GST immunization

The results showed that mice immunized with recombinant *Ace*-GST formulated with Freund's adjuvant produced a high IgG antibody response. The titre of IgG antibody in the immunized group began to increase two weeks after the first immunization (1:25600-1:102400), and reached the peak at two weeks after the third immunization (1:204800). The antibody titre at four and six weeks after the third immunization was still high (1:133120-1:163840).

Immunolocalization of Ace-GST in adult A. ceylanicum

The localization of native *Ace*-GST protein in tissues of adult *A. ceylanicum* was detected by probing of adult worm sections with anti-*Ace*-GST mice serum. The immunofluorescence analysis demonstrated that the *Ace*-GST protein was mainly localized to the cuticle, muscle and intestine of the adult, while no significant staining was detected with negative serum (fig. 4).

Discussion

GSTs are a group of isoenzymes with multiple functions that are widely expressed in various organisms (Torres-Rivera & Landa, 2008). GSTs not only act as detoxifying enzymes to catalyse the reaction of a series of endogenous and exogenous toxic molecules with the tripeptide GSH, but also as ligand-binding proteins to capture toxic substances (Hayes *et al.*, 2005; Oakey, 2011).

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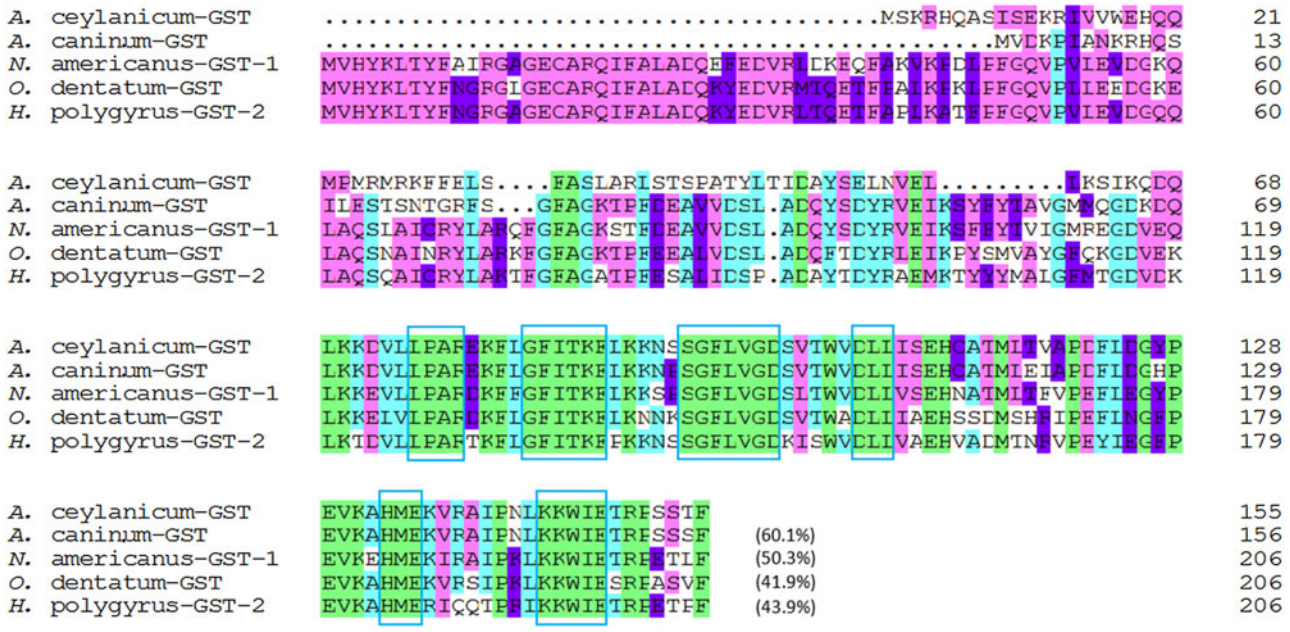

Fig. 2. Multiple alignments of *Ace*-GST with GST amino acid sequences of the other four nematodes.

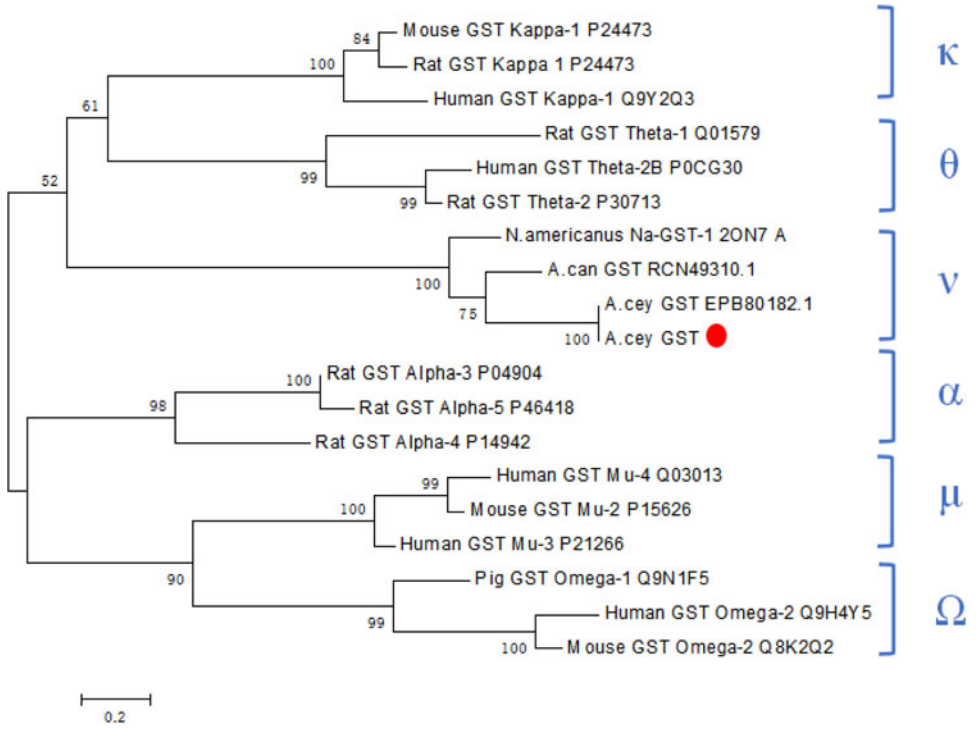

Fig. 3. Phylogenetic tree of *Ace*-GST and the other 18 GST amino acid sequences constructed with the neighbour-joining method.

Based on GST-conjugation enzyme activity, many parasite GSTs play an essential role in detoxifying the secondary products of the parasite membrane lipid peroxidation caused by free radical attacks triggered by the host immunity (Brophy & Barrett, 1990; Cervi *et al.*, 1999; KampköTter *et al.*, 2003). Parasite GSTs also have immunomodulatory functions. Recombinant *Ac*-GST was highly immunogenic in vaccinated animals and induced not only Th2-associated antibody (IgG1) and cytokine (IL-4) responses but also a strong Th1-like response (Zhan *et al.*, 2005). In addition, the GSH transferase fraction from *Taenia solium* activated macrophages and induced the differentiation of $CD4^+$ T cells toward a Th1-type response (Vega-Angeles *et al.*, 2019). Free haem is the end product of haemoglobin digestion in the blood-feeding parasites, which produce oxygen free radicals potentially toxic to the parasite (Klonis *et al.*, 2011). The blood-feeding parasite *Plasmodium* responds to this threat by combining haem ferrous and methaemoglobin into an inert pigment called the malaria parasite pigment (hemozoin) (Klonis *et al.*, 2011).

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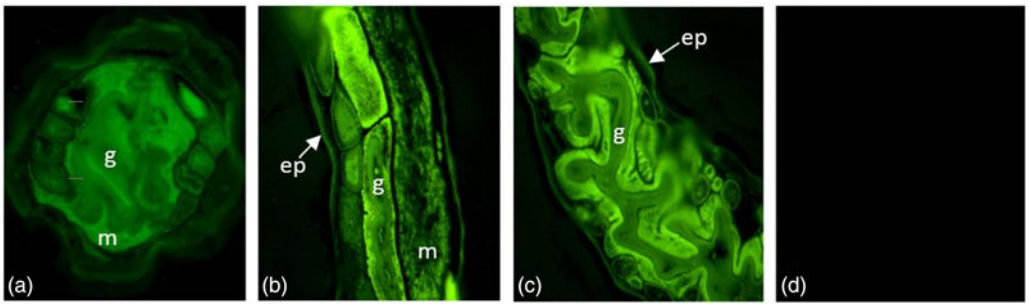

Fig. 4. Immunolocalization of *Ace*-GST. The cross section (a) and longitudinal sections (b, c) of adult *A. ceylanicum*, showing *Ace*-GST mainly located in epidermis (ep), muscle (m) and intestine (g). No significant staining was observed with pre-immune mouse serum (d).

Hookworms, like other blood-feeding parasites (*Plasmodium* and *Schistosoma*), have also evolved a detoxification mechanism for free haem by expressing nematode-specific *v*-class GSTs (Deponte & Becker, 2005; Jani *et al*., 2008; Skelly *et al*., 2014). This GST class differs in structure and function from other GST classes and is marked with a high-affinity binding site for haem and its related molecules and low activity with other substrates (Zhan *et al*., 2005).

Previous studies on *v*-class GSTs mainly focused on the cloning, expression and biological functions of GSTs from *Helicoverpa armigera*, *Haemonchus contortus*, *Ancylostoma caninum* and *N. americanus* (Deponte & Becker, 2005; Zhan *et al*., 2005, 2010; Jani *et al*., 2008; Skelly *et al*., 2014). Although 13 GSTs were identified in the intestinal transcriptome of *A. ceylanicum* (Wei *et al*., 2016), there are no reports on the cloning and molecular characterization of the GST from *A. ceylanicum*. In this study, the full-length *Ace*-GST cDNA was isolated for the first time from adult *A. ceylanicum* by RT-PCR. *Ace*-GST cDNA was 468 bp in length and encoded 155 amino acids without signal peptide sequence. *Ace*-GST homology to *Ac*-GST-1 and *Na*-GST-1 amino acid sequence was 60.1% and 50.3%, respectively. The absence of both signal peptide and transmembrane domain from *Ace*-GST sequence and the insolubility of recombinant protein indicate that this molecule is mainly a cytosolic enzyme. This finding differed from that found in *Ac*-GST-1 and *Na*-GST-1, where both enzymes contained a signal peptide sequence and were detected in adult worm excretory/secretory products (Zhan *et al*., 2005, 2010). In the phylogenetic tree, *Ace*-GST clustered with *Ac*-GST-1 and *Na*-GST-1 in one branch, indicating that this enzyme also belongs to *v*-class GSTs, which is the class of GSTs unique to nematodes. *Ace*-GST share the *v*-class GST unique H-site, which is characterized with a high affinity to haem, which may be related to the fact that nematodes cannot synthesize haem by themselves and must depend on external haem captured from blood (Zhan *et al*., 2010). Although iron-containing haem is a potent generator of toxic ROS, haem is also essential for several biological processes, such as cellular differentiation (Nakajima *et al*., 1999), protein expression regulation (Sun *et al*., 2002), mitochondrial protein transport (Lathrop & Timko, 1993) and protein degradation (Qi *et al*., 1999). Therefore, the presence of a haem -binding site in *Ace*-GST implies its possible dual function as a detoxifying enzyme for the excess haem by conjugating it to reduced GSH and as a transporter of reduced haem to biological sites inside the cells (Zhan *et al*., 2010).

To date, there are many reports on the GST localization in *A. caninum* and *N. americanus*. Zhan *et al*. (2005) confirmed that *Ac*-GST-1 was mainly located in the epidermis and muscular tissue of adults, and it could be weakly observed in the intestine. Moreover, *Na*-GST-1 was widely distributed in the parasite tissues including cuticle, epidermis, muscle, intestine and oesophagus (Zhan *et al*., 2010). To study the immunolocalization of *Ace*-GST, we firstly prepared polyclonal antibodies against this protein by immunizing Kunming mice with *Ace*-GST emulsified with Freund's adjuvant three times. The indirect ELISA was used to measure the fluctuation in IgG levels in immunized mice sera. The results showed that mice immunized with *Ace*-GST produced a high persistent IgG antibody response. Immunofluorescence results showed that *Ace*-GST was mainly localized to the epidermis, muscle and intestine of the adult, which was similar to the localization results of *Ac*-GST-1, suggesting that it may have similar functions to that of *Ac*-GST-1. *Ace*-GST localization to the gut suggests its potential role in the detoxification and/or transport of haem-related substances released as products of haemoglobin digestion during adult hookworm blood-feeding (Zhan *et al*., 2010). Additionally, the presence of *Ace*-GST in the epidermis implies that this nematode may obtain exogenous haem from the worm surface in addition to the intestine, possibly through the cuticular connection channels (Zhan *et al*., 2010).

In conclusion, we successfully cloned and expressed the *Ace*-GST recombinant protein in *E. coli*, which was closely related to *Ac*-GST-1, belonging to *v*-class GSTs. The protein was immunolocalized in the epidermis, muscle and intestine of adult *A. ceylanicum*. This study may promote further studies on the biological function of this protein. However, more studies are required to evaluate this protein as a vaccine candidate against hookworm infection.

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Conflicts of interest. None.

Ethical standards. Animal experiments involved in this study were performed in accordance with the guidelines of the Animal Welfare Law and Regulations of the Department of Health and Human Services, China, and were reviewed and approved by the South China Agricultural University Animal Care and Use Committee (approval number SYXK(Yue)2019-0136).

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