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A novel antigenic cathepsin B protease induces protective immunity in *Trichinella*-infected mice

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ABSTRACT

Trichinellosis is a foodborne disease that remains a public health hazard and an economic problem in food safety. Vaccines against the parasite can be an effective way to control this disease; however, commercial vaccines against *Trichinella* infection are not yet available. *Trichinella* cathepsin B proteins appear to be promising targets for vaccine development. Here, we reported for the first time the characterization of a novel cDNA that encodes *Trichinella spiralis* (*T. spiralis*) cathepsin B-like protease 2 gene (*TsCPB2*). The recombinant mature TsCPB2 protein was successfully expressed in *E. coli* system and purified with Ni-affinity chromatography. TsCPB2 expression was detected at all the developmental stages of *T. spiralis* and it was expressed as an excretory–secretory protein of *T. spiralis* muscle larvae. Immunization with TsCPB2 antigen induced a combination of humoral and cellular immune responses, which manifested as a mixed Th1/Th2 response, as well as remarkably elevated IgE level. Moreover, vaccination of mice with TsCPB2 that were subsequently challenged with *T. spiralis* larvae resulted in a 52.3% ($P < .001$) reduction in worm burden and a 51.2% ($P < .001$) reduction in muscle larval burden. Our results suggest that TsCPB2 induces protective immunity in *Trichinella*-infected mice and might be a novel vaccine candidate against trichinellosis.

1. Introduction

Trichinella spiralis (*T. spiralis*) is an intracellular nematode infecting humans and other animals. The entire life cycle of *T. spiralis* takes place in a single host, which involves three different developmental stages, the muscle larvae (ML), adult worm (Ad), and new born larvae (NBL). Trichinellosis, caused by *Trichinella* infection, is a cosmopolitan foodborne disease that is not only a public health hazard but also represents as an important problem in food safety and global trade in various animals and their meat [1,2]. Human trichinellosis outbreaks occur worldwide with an annual global average of 5751 cases and 5 deaths [3]. Currently, the reliable early diagnosis of trichinellosis is still lacking due to its nonspecific clinical manifestations [4]. In this case, invading *Trichinella* muscle larvae are usually established at the time of primary diagnosis, which results in ineffective drug treatment [5]. Therefore, it is of significant interest to develop effective vaccines to prevent *Trichinella* infection.

Excretory-secretory (ES) proteins released by *T. spiralis* muscle larvae have been suggested to play a critical role in modulating the host immune system, thus facilitating the establishment of *T. spiralis* parasitism and survival [6,7]. It has been reported that immunization with ES protein elicits a robust immune response and high protection against *T. spiralis* infection in mice and rats [8,9]. Gamble et al. also showed that inoculation pigs with *T. spiralis* larval ES antigens could significantly reduce the adult worm burden [10]. Thus, *T. spiralis* ES proteins offer promising targets for the development of vaccines.

Secretory cathepsin proteases, a major papain-like cysteine protease, play key roles in parasite survival, host invasion, and host immune response [11,12] and have high potential as vaccine targets [13]. Previously, we reported that treatment with a recombinant *T. spiralis* cathepsin B-like protein, TsCPB, induces a Th2 response in *Trichinella*-infected mice and ameliorates mouse intestinal ischemia/reperfusion injury via promoting a switch from M1 to M2 macrophages [14,15]. These results confirm the important function of the cathepsin protein in host immunity. Recently, a cathepsin F-like protease from *T. spiralis* was cloned and expressed, which might be involved in parasite life-cycle

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regulation [16]. Despite the mentioned progress, few cathepsin proteases from *T. spiralis* have been identified.

In the present study, we cloned and characterized another novel cathepsin protease gene from *T. spiralis* (named *TsCPB2*). We found that TsCPB2 was a muscle larvae ES protein and expressed at all the developmental stages of *T. spiralis*. Furthermore, we showed that vaccination with recombinant TsCPB2 protein elicited a mixed Th1/Th2 response and increased IgE level, and induced immune protection against *T. spiralis* larval challenge. Our data suggest that TsCPB2 might be a novel candidate for the development of vaccines against *Trichinella* infection.

2. Materials and methods

2.1. Parasites and antigen preparation

T. spiralis (ISS 533) were originally isolated from swine and maintained in kunming mice. Adult worms were harvested from intestines of mice at 6 days post-infection (PI). Newborn larvae were collected from female adult worms in culture. Muscle larvae were obtained from the muscle of infected mice as described before [17]. ES products were prepared according to a previous report [18].

2.2. Animals

Female BALB/c mice (6–8 week-old) were obtained from the Experimental Animal Center of Sun Yat-sen University (Guangzhou, China). All experimental procedures were approved by the Animal Care and Use Committee of Sun Yat-Sen University.

2.3. Semi-quantitative reverse transcription PCR (RT-PCR)

Total RNA was isolated from adult worms, newborn larvae, or 35-day-old muscle larvae of *T. spiralis* using TRIZOL reagent (Invitrogen, USA) and reverse-transcribed into the first-strand cDNA with reverse transcriptase (M-MLV RT, Promega). Semi-quantitative RT-PCR was performed using a Ready-To-Go RT-PCR kit (Amersham, USA). *Trichinella 18S rRNA* gene (GenBank No.: U60231) was used as an internal control. The primers were as follows: *TsCPB2*, 5′-ATTGTTGCAGGAATGTCA-3′ and 5′-CGTCAAAGCC TCTTCTTAT-3′; *18S rRNA*, 5′-CGCTACTACCGATTGGATGA-3′ and 5′-TACGGAAACCTTGTTACGACTT-3′. The PCR products were electrophoresed in 1% agarose gel and photographed under UV light. Three independent experiments were performed.

2.4. Cloning of TsCPB2 gene

The full-length *TsCPB2* cDNA was amplified using the 3′-Full RACE Core Set (TaKaRa, Japan) with cDNA of *T. spiralis* muscle larvae at 35-day PI as a template. The gene-specific primers for amplification of the 3′-extremity fragment of *TsCPB2* were designed according to the *TsCPB2* EST sequence (accession No.: EX501780): *TsCPB2*-5′GSP outer primer, 5′-CGGATCTGTTGTCCTGGTTGG-3′; *TsCPB2*-5′GSP inner primer: 5′-CTGTTGTCCTGGTTGGATAAA-3′. The PCR product was ligated into a pMD19-T vector (TaKaRa, Japan) and verified by sequencing.

2.5. Sequence analysis of TsCPB2 gene

The open-reading frame of *TsCPB2* was analyzed by the ORF Finder (https://www.ncbi.nlm.nih.gov/orffinder/). The theoretical protein molecular weight and isoelectric point were analyzed using the Protparam program (http://tw.expasy.org/tools/protparam.html). The signal peptide was predicted by the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Catalytic sites of TsCPB2 protein were determined by ScanProsite (http://www.expasy.ch/tools/scanprosite). Domains of cysteine proteinase were analyzed by the InterProScan EMBL-EBI software (http://www.ebi.ac.uk/Tools/pfa/iprscan/). The B-cell epitope was analyzed using the BiPiPred 1.0 server (http://www.cbs.dtu.dk/services/BepiPred/).

2.6. Expression and purification of recombinant mature TsCPB2

The region containing the predicted mature cysteine proteinase of TsCPB2 (amino acid residues 15–333) was amplified by PCR using following primers: 5′-GGGCCAAGCATATGGAAAATTATGAAG AAAATTATGAAC-3′ (with *NdeI* restriction enzyme site) and 5′-CCCAAGCTTTACTTTTGCACGTCCAGCAC-3′ (with *HindIII* restriction enzyme site) and subcloned into pET-30a(+) expression vector that contains a His-tag at the C-terminus (Novagen, USA). TsCPB2 protein was expressed in *E. coli* BL21 (DE3) after isopropyl-β-D-thioga lactopyranoside (IPTG) induction and purified by Ni-affinity chromatography (Qiagen, USA) as described previously [14].

2.7. Infection sera and anti-TsCPB2 antibody

Sera were obtained from BALB/c mice orally infected with 200 *T. spiralis* larvae at 3, 6, 10, 16, 20, and 44 days PI, respectively. A polyclonal antibody against TsCPB2 recombinant protein was produced in immunized rabbits injected subcutaneously with 200 μg of purified TsCPB2 protein formulated with complete Freund's adjuvant (FA, Sigma, USA), followed by three booster injections of the same dose of the protein formulated with incomplete FA (Sigma, USA) at 2-week intervals. Then the antibody was purified and quantified as described before [14].

2.8. Immunization and challenge infection

Mice were divided into three groups of 21 animals each. The two experimental groups of mice were injected subcutaneously with 40 μg of recombinant TsCPB2 protein or TsCPB protein (another *Trichinella* cathepsin-like protease cloned and expressed in our lab [14], as an unrelated control protein) emulsified with complete FA. The control group was given complete FA formulated with PBS only. Subsequently, the experimental groups of mice were boosted twice with the same dose of the recombinant protein emulsified with incomplete FA at 10 day intervals, while the control group was boosted with incomplete FA formulated with PBS only. Tail blood of immunized mice was collected at 0, 10, 20, and 30 days post-immunization.

One week after the final boost, 5 mice of each group were sacrificed and spleens were collected for cytokine measurement. The remaining 16 immunized mice per group were challenged orally with 400 *T. spiralis* muscle larvae 2 weeks after the final immunization. Eight mice from each group were euthanized at 6 days PI and the numbers of intestinal adult worms were counted. The muscle larvae were examined from the remaining eight mice of each group at 42 days PI and the rate of reduction in muscle larval burden was calculated as reported previously [19,20].

2.9. Western blotting

Protein samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, USA). After blocking with 5% (w/v) milk in TBS-Tween 20, the membranes were incubated with anti-His tag antibody (1:500 dilution, Biorad, USA), *T. spiralis*-infected mouse sera (1:500 dilution), or rabbit anti-TsCPB2 antibody (1:2500 dilution) followed by horseradish peroxidase

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