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PII: S0014-4894(10)00235-3
DOI: 10.1016/j.exppara.2010.06.029
Reference: YEXPR 6039

To appear in: *Experimental Parasitology*

Received Date: 11 February 2010
Revised Date: 23 May 2010
Accepted Date: 1 June 2010

Please cite this article as: Hong, Y., Han, H., Peng, J., Li, Y., Shi, Y., Fu, Z., Liu, J., Lin, J., Li, X., Schistosoma japonicum: Cloning, expression and characterization of a gene encoding the α5-subunit of the proteasome, *Experimental Parasitology* (2010), doi: 10.1016/j.exppara.2010.06.029

Schistosoma japonicum: Cloning, expression and characterization of a gene encoding the <alpha>5-subunit of the proteasome

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Abstract The development of an effective vaccine against the schistosome is thought to be the most desirable means to control schistosomiasis, even though there is an effective means of chemotherapy with praziquantel. A full-length cDNA encoding the *Schistosoma japonicum* proteasome subunit alpha type 5 protein (SjPSMA5) was first isolated from 18-day-schistosomulum cDNAs. The cDNA had an open reading frame (ORF) of 747 bp and encoded 248 amino acids. Real-time quantitative RT-PCR analysis revealed that SjPSMA5 is up-regulated in 18-day and 32-day schistosomes, and the level of expression in male is around 4-fold higher than that in female worms at 42 days. The SjPSMA5 was subcloned into pET28a(+) and expressed as inclusion bodies in *E. coli* BL21 (DE3) cells. Western blotting showed that the recombinant SjPSMA5 (rSjPSMA5) was immunogenic. After immunization of BALB/c mice with rSjPSMA5, reductions of 23.29% and 35.24% were obtained in the numbers of worms and eggs in the liver, respectively. The levels of specific IgG antibodies and CD_4^+ cells were significantly higher ($P < 0.01$) in the group vaccinated with rSjPSMA5 combined with Seppic 206 adjuvant than in the other groups, as detected by enzyme linked immunosorbent assay (ELISA) and flow cytometry. The study suggested that rSjPSMA5 induced partial immunoprotection against *Schistosoma japonicum* in BALB/c mice, and it could be a potential vaccine candidate against schistosomiasis.

Keywords:

Schistosoma japonicum (*S. japonicum*); proteasome subunit alpha type 5 (PSMA5); immunity; vaccine

1. Introduction

Schistosomiasis is the second most prevalent tropical disease caused by parasitic blood flukes. It causes symptomatic infection in approximately 200 million individuals and more than 200 thousand deaths per year in 74 endemic countries, with more than 600 million people at risk of infection (Bencergquist, 2002; Engels et al, 2002). The current strategy for control of schistosomiasis aims at the reduction of morbidity and involves treatment with praziquantel (WHO, 2002). However, chemotherapy does not prevent re-infection, and some isolates of *Schistosoma mansoni* (*S. mansoni*) that are resistant to high doses of praziquantel have been found in Egypt (Ismail et al, 1996). Thus it has been argued that the identification of target proteins for use in the development of vaccines or new drugs would contribute enormously to the control of this disease.

During the last two decades, many laboratories have attempted to identify schistosome antigens that could induce protective immune response (Afzal et al, 2008). A wide array of antigens has been discovered recently via the use of transcriptome and proteome analyses (Verjovski et al, 2003; Curwen et al, 2004; Cheng et al, 2005). Some of the antigens identified through use of these new technologies may help us to find additional potential vaccine candidates in the future.

The schistosomulum stage is critical for the maturation and development of schistosomes from cercaria into adult worms. Successful development of the schistosome in the final host involves profound structural, biochemical and physiological changes that are vital for adaptation to environmental variation (Ram et

al, 1999). Some of these changes depend to a large degree on the synthesis and degradation of proteins. Therefore, proteins that are expressed highly in the schistosomulum stage, and those that are involved in the associated pathway of regulating protein degradation, may be good candidates for vaccines or new drugs targeted against schistosomiasis. Our previous studies on protein differences between stages and genders in *S. japonicum* identified a protein (GenBank accession no. **AAP06025**) that is differentially expressed in schistosomula at 8 days and at 19 days. This corresponded to the mRNA sequence of *S. japonicum* clone ZZD1079 (GenBank accession no. **AY223002**) (Hu et al, 2003). This stage-specific highly expressed gene is similar to the PSMA5*Mus musculus*, and was named SjPSMA5.

In eukaryotic cells, the turnover of intracellular proteins is mediated primarily by the ubiquitin–proteasome system (Goldberg et al, 1997). Following ubiquitination, proteins are unfolded and degraded by the 26S proteasome. The 26S proteasome is made up of two 19S complexes and a proteolytically active 20S proteasome core. The 20S proteasome is composed of four stacked heptameric rings of α- and β-subunits that are organized into a barrel-shaped structure. The outer rings consist of α-subunits and the inner rings of β-subunits. The α-subunits have the capacity to form rings and they are necessary for the formation of the β rings. They constitute a physical barrier that limits access of cytosolic proteins into the inner proteolytic chamber and they are the sites for binding of the 19S and 11S regulatory complexes. (Coux et al, 1996).

Compared with those of yeasts, mammalian cells (Voges et al, 1999) and parasitic protozoa (Paugam et al, 2003), considerably less is known about the

proteasome and the associated regulation of protein degradation in schistosomes. An earlier study of the proteasome in *S. mansoni* suggested that its activity may be modulated by calcium, and that this modulation is mediated via a calcium-binding protein (CaBP) molecule of 8 kDa (Ram et al, 2003). A recent study demonstrated the presence of a functional proteasomal complex in *S. mansoni*, and that its function could be inhibited by proteasome inhibitors (Guerra-Sa et al, 2005). Further studies indicated that components of the *S. mansoni* proteasome were differentially expressed among cercariae, schistosomula and adult worms, and that the subunit SmRPN11/POH1 was an essential gene in schistosomes (Nabhan et al, 2007). All these findings indicate that the proteasome may play an important role in the development and survival of schistosome. Given that the α-subunits have several clear and important functions in the formation of the proteolytically active 20S proteasome core (Coux et al, 1996), it is reasonable to propose that SjPSMA5 may play an important role during the schistosomulum developmental stage. In this study we cloned, expressed and characterized a putative SjPSMA5, which was identified in the aforementioned proteomic studies of *S. japonicum*, and evaluated its potential efficacy as a vaccine candidate against schistosome challenge.

2. Materials and methods

2.1. Parasites, sera and animals

Schistosomula of the Anhui strain of *S. japonicum* at 7, 13, 18 and 23 days were obtained by perfusion of artificially infected rabbits. Adult worms at 32 and 42 days

were obtained as described previously (Verjovski et al, 2003). Serum samples were collected from normal rabbits and from rabbits infected with *S. japonicum*, as well as from rabbits immunized with crude extracts of adult worms. New Zealand rabbits (male) and BALB/c mice (male) were used for all experiments. They were raised in a sterilized room and feed sterilized food and water.

2.2. RNA isolation and reverse transcription

The total RNAs were extracted from the schistosomes mentioned previously using TRIzol reagent (Invitrogen, USA). The mRNAs were purified with an RNeasy mini Kit according to the manufacturer's instructions (Qiagen, Germany). The RNA was quantitated by spectrophotometry using a Biophotometer (Eppendorf, Germany). Ten micrograms of total RNA from the various developmental stages were used for reverse transcription (RT). The RT was performed with random hexamer primers and Superscript III reverse transcriptase (Invitrogen, USA) according to standard protocols. The resulting cDNA was used for quantitative PCR (qPCR) and RT-PCR.

2.3. Real-time RT-PCR analysis

The cDNA was amplified with the SYBR Premix Ex Taq^{TM} (Takara, Japan) in a Rotor-Gene 3000A Dual Channel Multiplexing System (Corbett Research, Australia). Primers for qPCR were designed using the Real-Time PCR primer design tool (Beacon Designer 7.0) and the settings were adjusted to the highest possible stringency to generate amplicons of 100–200 bp, as recommended. The primers

selected for SjPSMA5 (forward: 5′ CGT ACA GAA GCA GCT CAT CAT TGG 3′ and reverse: 5′ CAA ATA ACA AGG CAA CAC CGA ATG G 3′) amplified a product of 154 bp. The primers targeting *S. japonicum* NADH-ubiquinone reductase (forward: 5′CGA GGA CCT AAC AGC AGA GG 3′ and reverse: 5′TCC GAA CGA ACT TTG AAT CC 3′, product size 174bp) were used as the endogenous housekeeping reference for the qPCR (Gobert et al, 2009). The reaction conditions were as described in the SYBR green kit and the cycling protocol was as follows: 95℃ for 10 s and 40 cycles of 95℃ for 5 s, 60℃ for 10 s, and 72℃ for 15 s; fluorescence was acquired at the end of each extension step. The PCR products were detected in real time by the Rotor-Gene 3000A Dual Channel Multiplexing System. Specific PCR products were confirmed by dissociation curve analysis and agarose gel electrophoresis. At the completion of the run, the dynamic tube was turned on and the data were slope-corrected. After preliminary testing, the threshold line was set to 0.01 for all assays. Cycle threshold (Ct) scores, which correspond to the cycle number at which the amplification curve crosses the threshold line, were recorded for each sample. Negative (no template) controls were included in each PCR run. Five positive controls of known concentration were included in every run to confirm consistent amplification. Finally, quantitation of relative differences in expression was performed using Rotor-Gene version 6.0.38 software (Corbett Research, Australia) (Moertel et al, 2006; Gobert et al, 2009). Expression of the gene encoding NADH-ubiquinone reductase was used as a control. The relative mRNA expression was determined as the ratio of SjPSMA5 to NADH-ubiquinone reductase (Hu et al,

2009).

2.4. Cloning and molecular characterization

Primers were designed according to the nucleotide sequences of the mRNA sequence of clone ZZD1079 of *S. japonicum* reported in GenBank. The 5′ and 3′ oligonucleotides GCG CGA ATT CAT GTT TCT CA and GCG CCT CGA GTT AAG AGG AT were used to amplify the complete open reading frame (ORF) of SjPSMA5. The PCR was conducted according to the following amplification parameters: 94℃ for 10 min, 30 cycles of amplification (94℃ for 30 s, 55℃ for 30 s and 72℃ for 1 min), and a post-PCR step at 72℃ for 10 min. The PCR fragment obtained was cloned into the pMD19-T vector (Takara, Japan) and sequenced.

2.5. Phylogenetic and sequence analysis

Blast and PSI-Blast searches against the NCBI non-redundant protein sequence database, using SjPSMA5 as a query, were used to identify orthologues of SjPSMA5. For phylogenetic analysis, alignments of protein sequences were performed using the ClustalX 1.83 software. The tree was constructed using Clustal with the Neighbour Joining method, excluding positions with gaps. The TreeView program (taxonomy.zoology.gla.ac.uk/rod/treeview.html) was used to visualize the tree. CD-Search (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) was used to find conserved domains.

2.6. Expression and purification of recombinant protein

The cDNA fragment encoding SjPSMA5 was amplified by PCR with the forward primer 5′ GCG CGA ATT CAT GTT TCT CA 3′ and reverse primer 5′ GCG CCT CGA GTT AAG AGG AT 3′). *Eco*R I and *Xho* I (Takara, Japan) endonuclease sites (underlined) were included in these two primers to facilitate the subsequent cloning steps. The SjPSMA5 cDNA fragment was purified, then digested with *Eco*R I and *Xho* I to generate inserts with overhang ends that were ligated into the same sites of the expression vector pET28a(+) (Novagen, USA) to produce a protein that contained an N-terminal hexahistidine tag. For protein expression, transformed BL21 (DE3) cells (Invitrogen) were grown in 500 ml LB plus kanamycin (1 mg/ml) until OD_{600} = 0.6. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 1mM, and cells were incubated for another 5–6 h at 37°C.

The cells were harvested by centrifugation at 10,000×g for 10 min and resuspended in 40 ml of 1×Binding Buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9) per 100 ml culture volume. The cell suspension was sonicated briefly to resuspend the pellet thoroughly and shear the DNA. Inclusion bodies were collected by centrifugation at 5,000×g for 15 min. For protein purification, the inclusion bodies of rSjPSMA5 were rinsed three times with 1×Binding Buffer, and finally resuspended in 5 ml 1×Binding Buffer containing 8 M urea. After incubation on ice for 1 h, the insoluble materials were removed by centrifugation at 16,000×g for 30 min. The supernatant, which contained the soluble protein, was filtered through a 0.45-μm membrane. The recombinant protein was then purified by metal affinity chromatography using His•Bind®Resin Chromatography (Novagen, USA) under

denaturing conditions. The sample was loaded onto a Ni^{2+}-NTA column (5 ml bed volume) that was pre-equilibrated with the 1×Binding Buffer containing 8 M urea. The column was washed with 10 bed volumes of the same buffer, then cleaned with 6 bed volumes of 1×Wash Buffer (500 mM NaCl, 20 mM Tris-HCl, 60 mM imidazole, 8 M urea, pH 7.9) and 1×Elute Buffer (500 mM NaCl, 20 mM Tris-HCl, 1 M imidazole, 8 M urea, pH 7.9). The fractions encompassing the main peak were pooled and the purity of the preparation was assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was refolded by slow dialysis in phosphate buffered saline (PBS), pH 7.4, containing decreasing concentrations of 6 M, 4 M, 3 M, 2 M, and 1 M urea, and PBS only.

2.7. Vaccination and challenge infection

The 206 adjuvant (Seppic, France) was used according to the manufacturer's instructions. Six-week-old specific pathogen free (SPF) BALB/c mice were purchased from Shanghai SLAC Laboratory Animal Co. Ltd, and were randomly allotted into three groups of 10 mice per group. The mice were injected subcutaneously (SC) three times at 3-week intervals with rSjPSMA5 in 206 adjuvant (20 μg/100 μl/mouse), 206 adjuvant in PBS (100 μl/mouse) and PBS only (100 μl/mouse) respectively. Sera were collected from the mice by retro-orbital bleeding before the first vaccination and 2 weeks after each vaccination. All sera were preadsorbed with *E.coli* BL21 cell extracts lacking SjPSMA5 protein (Kumamoto, 1989; Kimsey et al, 1995) and stored at -20°C until further assay. Three weeks after the last vaccination, all mice were

infected with 30±1 viable cercaria percutaneously via a wet glass lid as documented previously (Dupre et al, 2001).

2.8. Western blotting

Parasitic proteins of these stages were prepared in 40 mM Tris, pH 7.4, 2% SDS plus protease inhibitors (Sigma, USA). Worms were homogenized and sonicated for 10s × 5 with an interval of 15s and centrifuged at 12000 × g for 40 min at 4°C. The supernatant was recovered and protein concentrations were determined with a DC Protein Assay Kit (Bio-Rad, USA). Then purified rSjPSMA5 (5 μg), total parasite protein extracts of each stage (60 μg) were subjected to 12% SDS-PAGE.

Western blotting assays were performed according to standard procedures (Sambrook et al, 2001). After 12% SDS-PAGE, the gels were soaked in the transfer buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.4) and the resolved proteins were transferred electrophoretically onto a 0.45-μm pore size nitrocellulose membrane (Whatman, Germany) at 100V for 1 h 30 min at 4°C. The nitrocellulose membrane with transferred rSjPSMA5 was incubated with 3% bovine serum albumin (BSA, Amresco, USA) in PBS (blocking buffer) overnight at 4°C to block the nonspecific sites, followed by five successive washes of 5 min each with 0.05% Tween 20 in PBS. Subsequently, the membrane was incubated in a 1:100 dilution of anti-SjPSMA5 primary antibody in blocking buffer or in a 1:1000 dilution of anti-tubulin primary antibody (Beyotime, China) in blocking buffer for 1 h at 37°C. After five washes as above, the membrane was incubated in a 1:2500 dilution of

secondary goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma, USA) for 1 h, which was followed by another five washes using the same buffer. Detection was performed with 3,3'5,5'-tetramethyl benzidine dihydrochloride (TMB, Sigma, USA) according to the manufacturer's instructions and imaged using an ImageQuant 300 Capture Imaging System (GE Healthcare, USA).

2.9. Immune response assays

Specific IgG antibodies against SjPSMA5 were detected by ELISA using serum samples from individual mice. Soluble rSjPSMA5 was diluted to a concentration of 10 µg/ml in carbonate bicarbonate buffer (pH 9.6) and 100 µl were used to coat 96-well microtitre plates (Costar, USA) overnight at 4°C. The plates were blocked with 3% BSA in PBS and incubated for 1 h at 37°C, then washed six times using 0.05% Tween 20 in PBS (PBS-T). All testing sera were diluted in 1:100 with 3% BSA in PBS-T solution (blocking buffer), added at 100 µl/well and incubated for 1 h 30 min at 37°C. After washing, goat anti-mouse IgG conjugated to horseradish peroxidase (Sigma, USA) diluted 1:2000 in dilution buffer was added to the wells (100 µl/well). The plates were incubated for 1 h at 37°C, washed five times with 0.05% PBS-T, and 100 µl of the substrate, 3,3'5,5'-tetramethyl benzidine dihydrochloride, were added to each well. The plates were incubated at 37°C in the dark for 10 min and the reaction was stopped using 2 M sulfuric acid (50 µl/well). Optical densities (ODs) were measured with a microplate reader (BioTek, USA) at 450 nm.

257 To evaluate the specific cellular immune response, mice were killed 2 weeks
258 after the last vaccination and their spleens were harvested aseptically. The splenocytes
259 were enriched by passage through a nylon wool column, then they were washed twice
260 and resuspended in PBS at a concentration of 10^7/ml, with 100 μl of each cell
261 suspension incubated with PE-conjugated rat anti-mouse CD_4 antibody (0.2 μg/μl)
262 and FITC-conjugated rat anti-mouse $CD_{8\alpha}$ antibody (0.5 μg/μl) (BD Pharmingen™)
263 for 40 min at room temperature in the dark. After two washes with PBS, the cell
264 suspensions were resuspended in a fluorescence preservative fluid and the proportions
265 of CD_4^+ and CD_8^+ cells were analyzed by flow cytometry (FCM).

266 *2.10. Evaluation of immune protection against S. japonicum challenge*

267 The efficacy of immunization was evaluated according to the reduction in worm
268 and egg counts. Adult worms were obtained by perfusion (Smithers, et al, 1965) of
269 infected BALB/c mice from hepatic portal system 42 days after challenge, and the
270 worms were also manually removed from mesenteric veins and counted. Samples of
271 0.5 mg liver tissue from each infected mouse were homogenized in 10 ml 5% NaOH,
272 respectively. The mixture was incubated at 56°C for 1 h and then mixed thoroughly.
273 An average of three counts per 20 μl mixture was taken to estimate the number of
274 eggs, and this count was converted to eggs per gram (EPG). The worm and egg
275 reduction rates were calculated as follows:
276 Percentage reduction in worm burden = (mean worm burden of control group – mean
277 worm burden of vaccinated group)/ mean worm burden of control group×100%.

Percentage reduction in liver egg count = (mean EPG from control group – mean EPG from vaccinated group)/ mean EPG from control group×100%.

2.11. Statistical analyses

Statistical analysis was performed by analysis of variance (ANOVA) and Duncan's multiple range tests using SPSS 11.5 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cloning and molecular characterization of SjPSMA5

The full-length sequence of the *S. japonicum* cDNA encoding a putative PSMA5 was obtained by RT-PCR from the mRNA of 18-day-old schistosomula with specific oligonucleotides that were designed using the corresponding EST sequence (GenBank accession no. **AY223002**). The resulting full-length cDNA (GenBank accession no. **FJ595238**) contained an ORF of 747 bp, and encoded a protein of 248 amino acids with a predicted molecular mass of approximately 27.34 kDa and an isoelectric point of 5.21. Six nucleotide sequence differences were found between the cDNA sequence obtained in this study and the EST sequence from GenBank (accession no. **AY223002**). These occurred at nucleotide positions 78 (C instead of T), 126 (A instead of G), 129 (C instead of T), 153 (G instead of A), 367 (T instead of C), 693 (G instead of A). Only oine amino acid sequence difference was found, at amino acid position 123 (phenylalanine instead of leucine). The gene displays no extended

298 signal peptide sequence.

299 BlastP comparisons of the deduced *S. japonicum* protein sequence with GenBank
300 sequences showed that the best match (*E*-value = 4×10^{-111}) was with the PSMA5 of
301 *Opisthorchis viverrini*. The following closest orthologues of SjPSMA5 were the
302 PSMA5 sequences from *Mus musculus* (*E*-value = 1×10^{-99}), *Homo sapiens* (*E*-value
303 = 6×10^{-99}), *Danio rerio* (*E*-value = 8×10^{-99}), *Salmo salar* (*E*-value = 1×10^{-98}),
304 *Gallus gallus* (*E*-value = 3×10^{-98}), *Xenopus laevis* (*E*-value = 1×10^{-97}) and
305 *Drosophila melanogaster* (*E*-value = 3×10^{-89}).

306 On searching the genome (Gene DB) for PSMA5, we found three related protein
307 sequences (accession nos. **Smp_032580.1, Smp_032580.2, Smp_032580.3**). They are
308 all putative PSMA5 proteins, and they have lengths of 221, 246 and 237 amino acids,
309 respectively. Bioinformatics analysis of the 26S proteasome of *S. mansoni* showed
310 that the sequence Smp_032580.2 was a complete proteasome subunit alpha type 5
311 (SmPSMA5) (Nabhan et al, 2007). BlastP comparison revealed that the SmPSMA5
312 sequence had 97% similarity with that of SjPSMA5. Therefore we designated this
313 gene SjPSMA5 (Fig. 1).

314 The phylogenetic analyses of the PSMA5 are shown in Fig. 2. The results indicate
315 that SjPSMA5 is most closely related to SmPSMA5, and the next closest relation is
316 the PSMA5 of *Opisthorchis viverrini*.

317 *3.2. mRNA expression analysis by quantitative RT-PCR*

318 The expression of SjPSMA5 at the mRNA level was evaluated in *S. japonicum* at

7, 13, 18, 23, 32 and 42 days using real-time quantitative RT-PCR analysis with NADH-ubiquinone reductase as the housekeeping gene (Fig. 3). The results showed that the SjPSMA5 mRNA was found in all investigated stages, and that the level was much higher in the schistosomes at 7, 13, 18, 23 and 32 days than in that at 42 days. In addition, the level in the male was almost 4-fold higher than that in the female at 42 days. This revealed that SjPSMA5 is up-regulated at the stage at which the schistosomes develop quickly and their morphology changes greatly.

3.3. Preparation of rSjPSMA5 and western blotting assay

The gene was cloned into the pET28a(+) expression vector and expressed in the *E. coli* BL21 (DE3) strain upon induction with IPTG. The SDS-PAGE analysis showed that the recombinant protein had a molecular weight of 32 kDa (Fig. 4). After the bacteria had been sonicated briefly, the lysate was separated into soluble and insoluble fractions. The inclusion bodies contained the majority of the recombinant protein, which was mostly solubilized by extraction with 8 M urea (Fig. 4). The protein extracted with 8 M urea was purified under denaturing conditions by affinity chromatography on nickel-charged columns. Eluted target fractions were mainly in 1×Elute Buffer (500 mM NaCl, 20 mM Tris-HCl, 1 M imidazole, 8 M urea, pH 7.9) and submitted to refolding by dialysis against PBS. The purity of the preparation was assessed by SDS-PAGE (Fig. 4). The protein yield after dialysis was estimated to be approximately 30 mg per litre of culture. Then the rSjPSMA5 and SjPSMA5 in native were analyzed by western blotting and results were shown in Fig. 5.

3.4. Protective immune efficacy induced by rSjPSMA5

The percentage reductions in the worm burden and in the liver egg count are listed in Table 1. Mice immunized with rSjPSMA5 showed a 23.29% decrease in the number of worms ($P < 0.05$) and a 35.23% reduction in the egg count ($P < 0.05$) compared with the blank control.

The worm burden and the liver EPG were not significantly different ($P > 0.05$) between the blank control and the adjuvant control groups. Our results showed that immunization of mice with rSjPSMA5 induced partial protection against challenge with *S. japonicum*.

3.5. Detection of SjPSMA5-specific IgG antibody

The level of IgG antibody specific to rSjPSMA5 in the sera from both immunized and control mice as detected by ELISA is shown in Fig. 6. A small amount of specific IgG antibody was detected after the first vaccination with rSjPSMA5, and the amount was significantly increased after the second vaccination. The level of antibody was much higher in the vaccinated mice than in those that received 206 adjuvant or PBS only, and the latter were showed no significant differences in specific antibody levels. This result was consistent with the result of western blotting (Fig. 5). The fact that rSjPSMA5 could stimulate a strong antibody response suggested that humoral immunity may play an important role in the induction of protection against schistosome challenge.

3.6. Evaluation of cell-mediated immunity

The results of the assay of the cell-mediated immune responses in all mice in each group are shown in Table 2. The proportions of different subsets of splenocytes in immunized mice were detected by FCM 2 weeks after the boosting immunization. Compared with the results from the PBS and 206 adjuvant groups, the number of CD_4^+ cells was significantly increased in the group vaccinated with rSjPSMA5 ($P < 0.05$), but no significant changes in CD_8^+ cells were observed among the three groups of mice.

4. Discussion

In the present study, the SjPSMA5 gene from the Chinese strain of *S. japonicum* was cloned and expressed successfully in *E. coli*. It is known that recombinant proteins that are overexpressed in bacteria often form insoluble proteins (Marston, 1986). In our study the recombinant SjPSMA5 was produced as insoluble inclusion bodies in the normal induced condition. This result was the same as for the human PSMA5, which was expressed as an insoluble protein (Han et al, 2004).

The results of the real-time quantitative RT-PCR analysis showed that the SjPSMA5 transcript was expressed at a low level in adult worms at 42 days, and was significantly up-regulated in schistosomes at 7, 13, 18, 23 and 32 days. Comparison of the level of expression by gender in adult worms at 42 days revealed that expression of SjPSMA5 mRNA in male worms is around 4-fold higher than that in female worms. The results revealed that the up-regulation of SjPSMA5 mRNA occurs during a period of rapid growth and significant change in morphology in the schistosomes. It is

possible that the process involves profound structural, biochemical and physiological change.

The turnover of intracellular proteins is mediated primarily by the ubiquitin–proteasome system. A large amount of 20S proteasome is needed to degrade unfolded proteins. Expression of *S. mansoni* proteasome subunit alpha type 1 (SmPSMA1) was evaluated at the mRNA level in the developmental stages of *S. mansoni* using real-time quantitative RT-PCR (Nabhan et al, 2007) and the result was similar to our findings (unpublished).

The western blotting result from the present study revealed that rSjPSMA5 had good immunogenicity. The rSjPSMA5 was probed with sera from BALB/c mice immunized with purified rSjPSMA5, and the rSjPSMA5 could be recognized by western blotting. However, the rSjPSMA5 could not be recognized by western blotting using sera from BALB/c mice received 206 adjuvant or PBS. In addition, crude extracts obtained from worms at 7, 13, 18, 23, 32 and 42 days were recognized by the murine sera against rSjPSMA5; a band with a molecular weight of around 28 kDa was identified. This result revealed that the level of expression of native SjPSMA5 was lower at 42 days than other investigated stages, and this is consistent with the result obtained using real-time RT-PCR.

The rSjPSMA5 protein was evaluated as a vaccine candidate against *S. japonicum*. In the present study, the mice were immunized subcutaneously (SC) with rSjPSMA5, and the number of adult worms and liver eggs in vaccinated mice was significantly lower than those in the other groups ($P < 0.05$). A significant level of

specific IgG was observed in mice vaccinated with rSjPSMA5 mixed with the 206 adjuvant, compared with mice that received the 206 adjuvant or PBS alone. The specific IgG antibodies were at a high level after the second immunization, and the peak was obtained 3 weeks after the last vaccination. The BALB/c mice that had been challenged with cercaria maintained a high level of IgG antibodies until they were killed. These results suggest that the purified rSjPSMA5 was able to elicit a strong antibody response and that it may be an effective immunogen. At 2 weeks after the last vaccination, changes in the numbers of T lymphocytes were evaluated in the three groups of BALB/c mice. The percentage of CD_4^+ cells in mice vaccinated with SjPSMA5 increased significantly ($P < 0.05$) when compared with the mice in the adjuvant or blank control groups. It seems that the purified rSjPSMA5 can activate T helper cells, which may stimulate B lymphocytes to differentiate, secrete specific IgG antibodies against rSjPSMA5 and play an active role in protection against schistosome challenge. Our results showed that both humoral immunity and cellular immunity were important for the induction of protection against schistosome infection in BALB/c mice.

Half a century ago, by analogy with successful microbial and viral vaccines, some scientists tried to vaccinate mice with crude worm extracts or purified components, followed by a cercarial challenge (Sadun et al, 1959; Murrell et al, 1975). The experimental data showed that the results lacked consistency even in the same laboratory, and it seemed apparent that crude extracts were inadequate vaccines (Wilson et al, 2006). Vaccination with irradiated cercariae could elicit almost the

highest immune protection against schistosomes by now (Afzal et al, 2008). However, the limitation of an irradiation attenuated vaccine is related to the shortage of sufficient parasites (Abath et al, 1998). Furthermore, the multiplicity of antigens contained in these irradiated preparations may not all be protective and may lead to unexpected immunopathological or immunosuppressive consequences (Mahmoud, 1989). Although no recombinant vaccine could reach the level of immunoprotection elicited by attenuated cercarial vaccine, it is still considered constitute a defined and safe vaccine.

The proteasome, which is composed of a multi-subunit complex, is the site of degradation of most cellular proteins and is necessary for cell viability (Coux et al, 1996). In recent years, there have been many studies on the structure and function of the proteasome (Tanaka, 1998; Bochtler et al, 1999; Pickart, 2001; Glickman et al, 2002; Verma et al, 2004), and the mechanisms of its action are being elucidated. The importance of controlled protein degradation is evident in schistosomes. The body of the schistosome undergoes extensive remodeling, including the emptying of the secretory glands, the shedding of the glycocalyx, the disappearance of the gland cells, the reconfiguring of the musculature and so on. Proteasome-mediated degradation could play an important role in this process (Stirewalt, 1974; Crabtree et al, 1986; Guerra-Sa et al, 2005). If the pathway is disrupted by some means, for example by inhibitors or RNAi, the development of the schistosome is blocked (Guerra-Sa et al, 2005; Nabhan et al, 2007). Proteomic analysis has been conducted with the 20S proteasome of *S. mansoni* (Castro-Borges et al, 2007) and this has provided useful

information about the 20S proteasome and its composition in schistosomes. Previous research has shown that three of the seven β subunits possess chymotrypsin-like, trypsin-like and caspase-like activities, respectively (Coux et al, 1996) and that they play important roles in the process of degradation of proteins. In summary, a better understanding of the involvement of the proteasome and its subunits in the development of schistosomes may lead us to discover novel target proteins for development of vaccines or new drugs to control schistosomiasis.

In conclusion, our results show that the rSjPSMA5 protein has good immunogenicity and can induce partial protective immunity against schistosome infection in BALB/c mice. It shows potential as a potential vaccine candidate or new drug target.

Acknowledgements

We thank Dr. Zhanzhong Zhao for revising the manuscript and for valuable suggestions. We would like to thank Hao Li, and Ke Lu from the Shanghai Institute of Veterinary Research of the Chinese Academy of Agricultural Sciences for technical assistance with parasite collection. This work was supported by National Basic Research Program of China (No. 2007CB513108), National Natural Science Foundation of China (No. 30671581), National High Technology Research and Development Program of China (No. 2006AA10A207-1) and National Key Technology R&D Program of China (No. 2006BAD06A09).

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Table captions:

Table 1

Comparison of protective effectiveness against *S. japonicum* challenge in mice receiving SjPSMA5.

Note: Data are expressed as mean ± S.E. Each group contained 10 mice. Values with different superscripts in the same column differ significantly ($P < 0.05$). Values with same superscripts in the same column do not differ significantly ($P > 0.05$).

Table 2

Changes of CD_4^+ and CD_8^+ T cells in splenocytes from immunized BALB/c mice.

Note: Data are expressed as mean ±S.E. Each group contained five mice. Values with different superscripts in the same column differ significantly ($P < 0.05$). Values with same superscripts in the same column do not differ significantly ($P > 0.05$).

Figure legends

Figure 1 Comparison of the protein sequence of SjPSMA5 with those of the other species. Clustal X alignment of the derived amino acid sequences of SjPSMA5 (**FJ595238**), SmPSMA5 (**Smp_032580.2**), OvPSMA5 (**ABD64146.1**), DmPSMA5 (**AAB93421.1**), DrPSMA5 (**NP_991271.1**), SsPSMA5 (**NP_001134432.1**), XlPSMA5 (**DAB42871.1**), GgPSMA5 (**NP_001026578.1**), MmPSMA5 (**NP_036097.1**) and HsPSMA5 (**AAV38522.1**). The regions with high identity and similarity between PSMA5 sequences are shown as black and gray columns, according to the Clustal X algorithm. Conserved domain was indicated by continuous box.

646 **Figure 2** The phylogenetic tree analysis of SjPSMA5 with its homologues (the accession numbers
647 of the other members are cited in the legend of Figure 1).
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668 **Figure 3** Stage and gender differential expression of SjPSMA5 in *S. japonicum* by real-time
669 RT-PCR
670 7 d, 13 d, 18 d, 23 d, and 32 d represent worms at 7 days, 13 days, 18 days, 23 days, and 32 days
671 respectively. 42 d (m): male adult worms at 42 days; 42 d (f): female adult worms at 42 days.
672 Expression of the gene encoding NADH-ubiquinone reductase was used as a control.
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Figure 4 SDS-PAGE (12%) analysis of the expression of recombinant protein SjPSMA5. Lanes 1 and 2, total extract from a clone after and before induction with 1mM IPTG. Lanes 3 and 4, total extract of pET28a(+) after and before induction with 1mM IPTG. Lanes 5 and 6, inclusion bodies and supernatant of pET28a(+)-SjPSMA5 after lysis, respectively. Lane 7, rSjPSMA5 purified through Ni^{2+}-charged column chromatography and after dialysis.

712 **Figure 5** Western blotting analysis of rSjPSMA5 and protein extracts from *S. japonicum*

713 (A) M: marker; 1, 2 and 3: Purified rSjPSMA5 was probed with the serum from BALB/c mice

714 immunized with rSjPSMA5, 206 adjuvant and PBS, respectively.

715 (B) protein extracts from *S. japonicum* using anti-rSjPSMA5 or anti-tubulin antibodies

716 M: marker; 7 d, 13 d, 18 d, 23 d and 32 d: protein extracts from worms at 7 days, 13 days, 18 days,

717 23 days, and 32 days respectively. 42 d (m) and 42 d (f): protein extracts from male and female

718 adult worms at 42 days respectively. Tubulin was used as a control.

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734 **Figure 6** Antibody responses specific to rSjPSMA5. Mice were injected SC with rSjPSMA5, 206
735 adjuvant and PBS. Sera were collected and analyzed with ELISA. Each bar represents the mean
736 OD (±S.E., n = 10), the asterisks (*) indicate significantly increased serum antibody titers
737 compared with the PBS control ($P < 0.01$).
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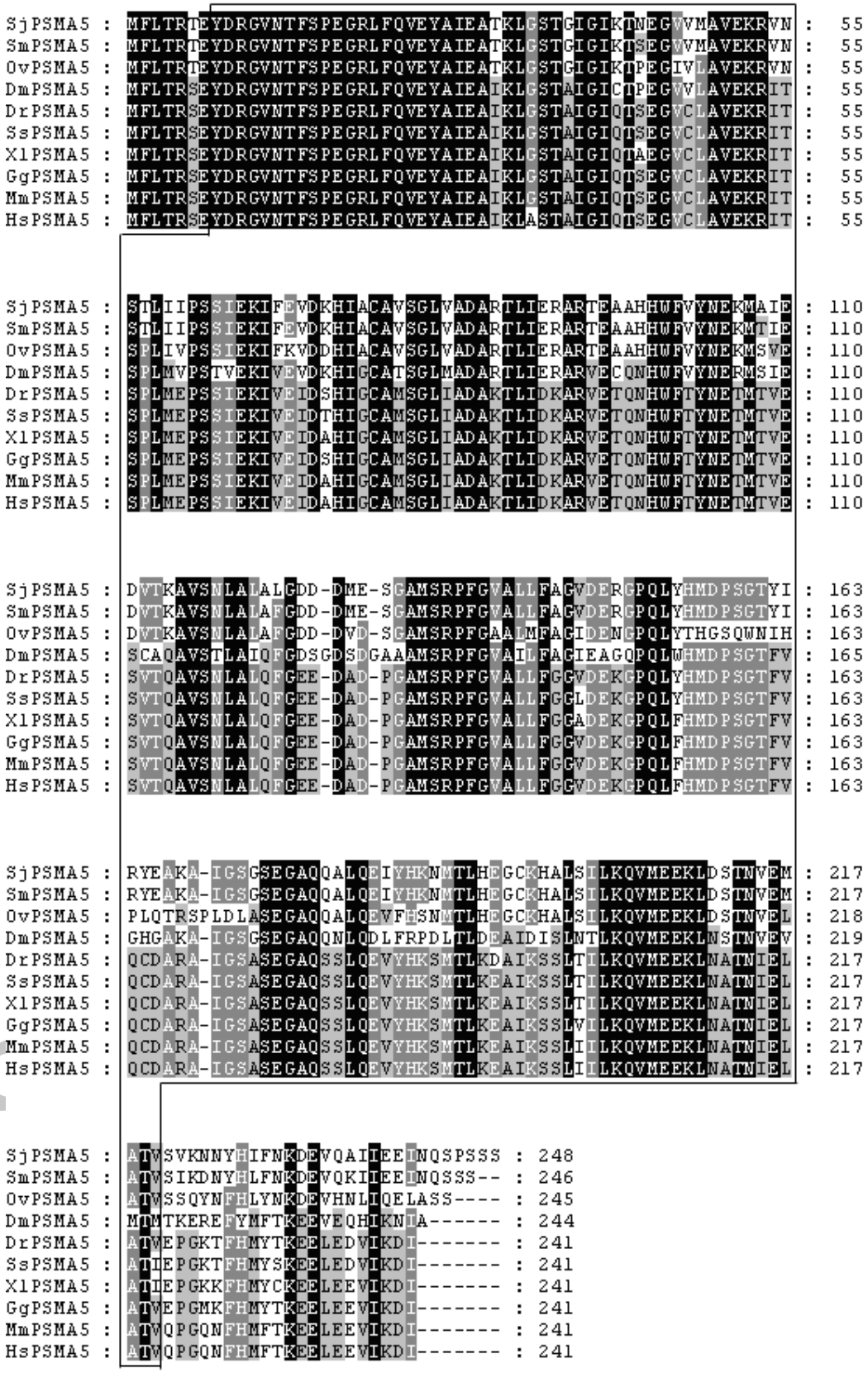

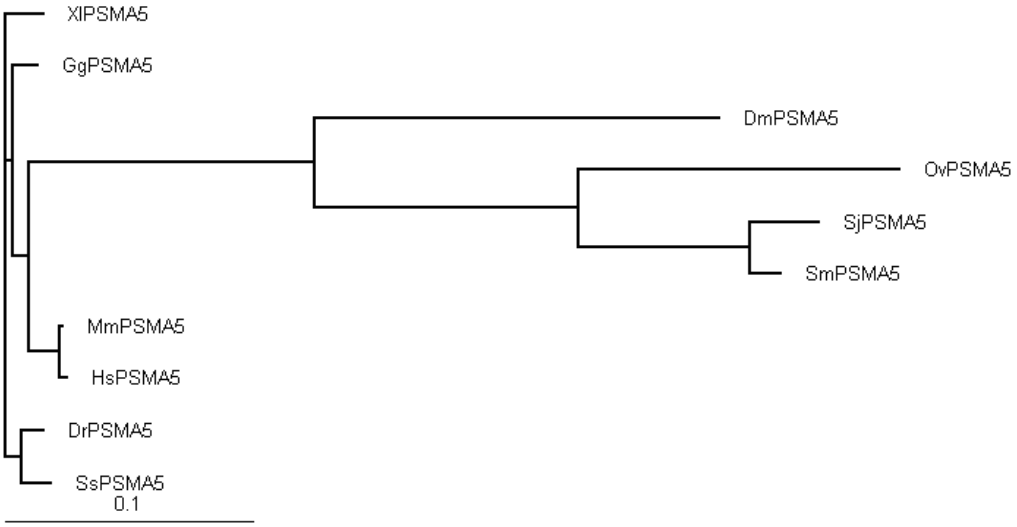

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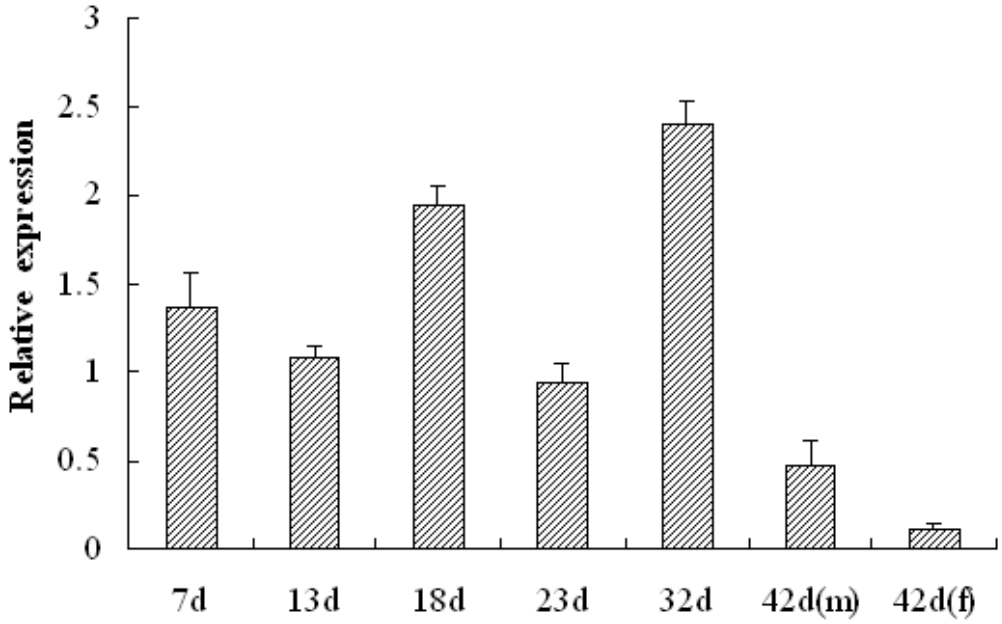

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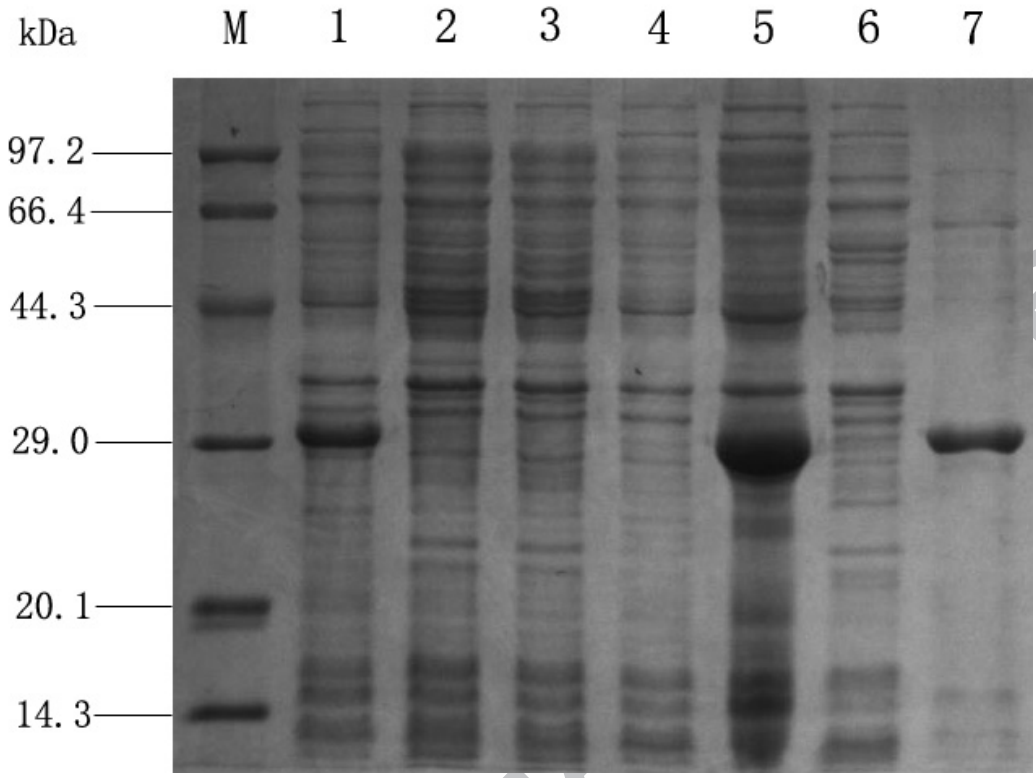

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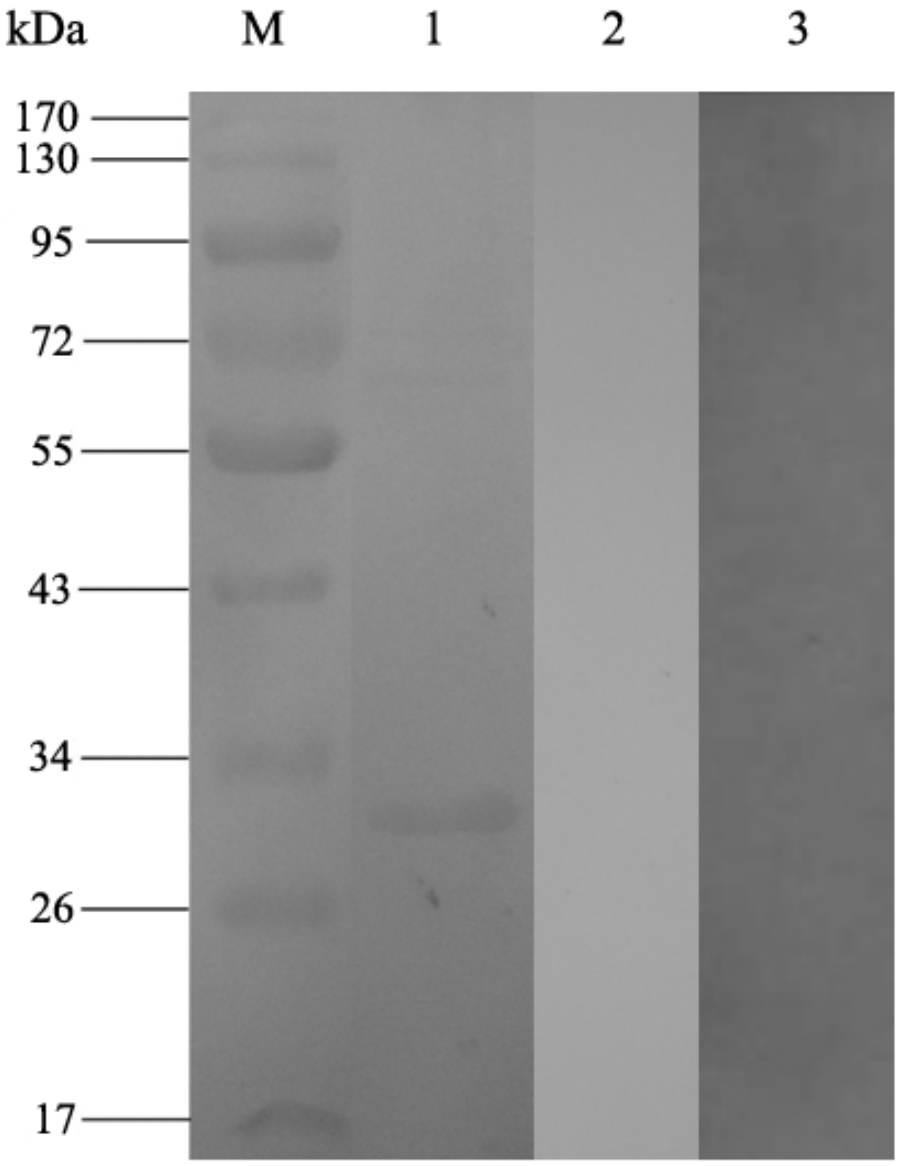
751 Fig4



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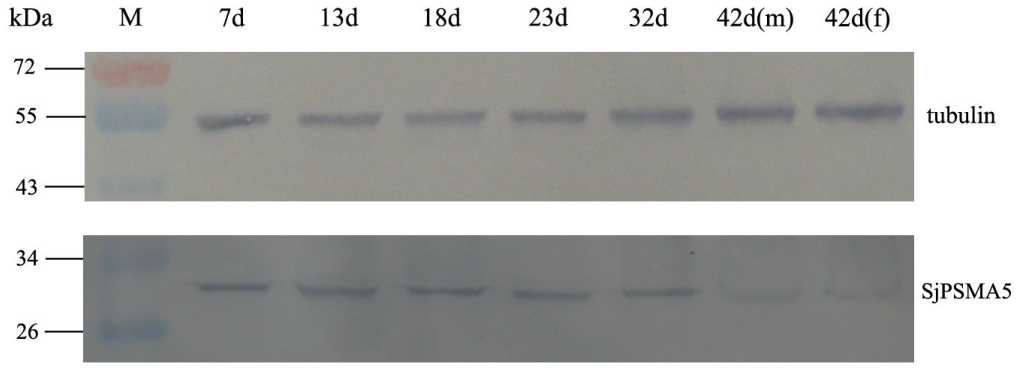
753 Fig5a



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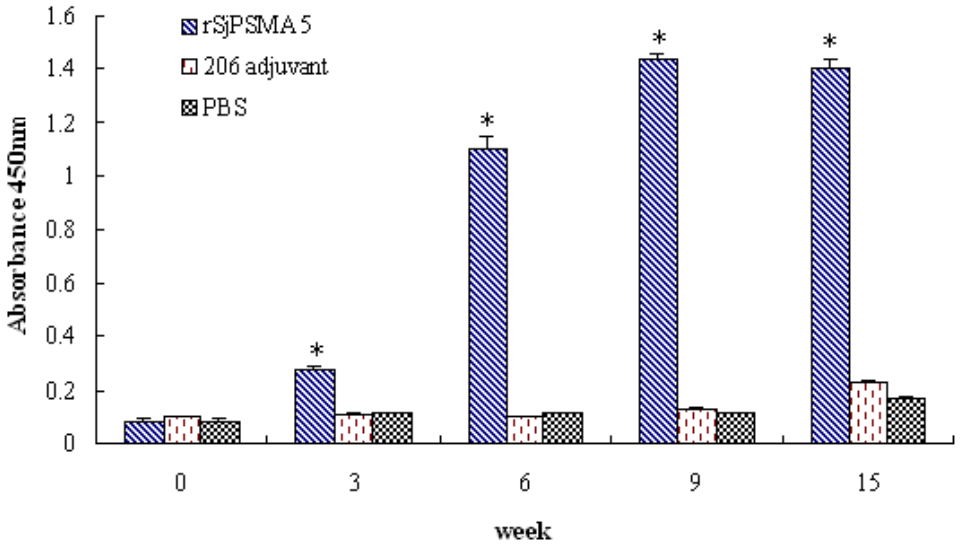
755 Fig5b



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Group	Worm burden	Percent reduction in worm burden (%)	EPG	Percent reduction in liver egg count (%)
SjPSMA5	16.8±6.39[A]	23.29	45833.3±22987.0[A]	35.23
206 adjuvant	21.5±6.36[B]	1.37	74370.4±33876.6[B]	-----
PBS	21.9±4.63[B]	-----	70766.7±31005.8[B]	-----

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Group	CD_4^+ (%)	CD_8^+ (%)
SjPSMA5	21.9 ± 1.10^{A}	8.8 ± 0.72^{A}
206 adjuvant	17.7 ± 0.86^{B}	7.9 ± 0.57^{A}
PBS	17.9 ± 0.47^{B}	7.2 ± 0.53^{A}

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