 doi:10.20944/preprints201705.0173.v1

Molecular and Biochemical Characterization of Calmodulin from *Echinococcus granulosus*

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 doi:10.20944/preprints201705.0173.v1

Abstract:

Echinococcus granulosus is a harmful cestode parasite which could cause Cystic Echinococcosis in humans, various livestock species and wild animals. Calmodulin (CaM), a Ca^{2+} sensor protein, is widely expressed in eukaryotes and mediates a variety of cellular signaling activities. In our study, the CaM in *Echinococcus granulosus* (rEgCaM) was successfully cloned and the molecular and biochemical characterizations of rEgCaM were identified. The results showed that rEgCaM was a highly conserved calcium-binding protein, consisting of 149 amino acids. Immunoblot analysis revealed that rEgCaM could be identified using *E. granulosus* infected sheep serum. The usage of rEgCaM as antigen was evaluated by indirect ELISA which exhibited a high sensitivity of 90.3% but low specificity (47.1%). rEgCaM was mainly located in the tegument tissues and parenchymal region of protoscoleces, the tegument and inner body of adult worm and predominantly expressed in the germinal layer. The mRNA expression of rEgCaM in PSCs were gradually decreased with the increase death of PSCs. In electrophoretic mobility tests and ANS assays, rEgCaM showed a typical calcium-binding protein characteristics. This is the first report on CaM from *E. granulosus* and rEgCaM is considered to be involved in some important biological function of *E. granulosus* as a calcium-binding protein.

Keywords: *Echinococcus granulosus*; Calmodulin; Ca^{2+}-binding protein; Immunohistochemical localization; Quantitative RT-PCR

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1. Introduction

Cystic echinococcosis (CE), also called hydatid disease, is a serious zoonotic parasitic disease caused by the larval form of *Echinococcus granulosus* and is an important public health issue in both developed and developing counties [1,2]. Larval stage of *E. granulosus* infects livestock and humans, while the adult worm parasitizes the small intestine of canids. The metacestode larva is a unilocular, fluid-filled cyst which includes a germinal layer, laminated layer and external layer derived from dead host cells and fibrosis. CE results in an estimated 1-3 million disability-adjusted life years (DALYs) globally per annum and 20%-90% of CE prevalence is observed in domestic animals, leading to an annual economic loss of approximately US$ 3 billion [3]. The World Health Organization included echinococcosis as one of the 17 neglected tropical diseases in its strategic plan from 2008 to 2015 [4].

Calmodulin (CaM), a small calcium sensor protein, is one of the most evolutionarily ancient proteins in eukaryotes [5]. The function of CaM includes Ca^{2+} binding and conversion of Ca^{2+} signals though downstream proteins to regulate various physiological processes, such as muscle contraction, metabolism and cell motility [6,7]. The structure of CaM is characterized by two globular heads joined by an extended, α-helical linker; each of the globular heads includes two EF-hands which can bind to a calcium ion (Ca^{2+}) [8,9]. After biding to Ca^{2+}, CaM changes its conformation, exposing more hydrophobic residues in order to interact with diverse target proteins [10,11]. In *Caenorhabditis elegans*, 56 Ca^{2+}-bound-calmodulin binding proteins were identified using mRNA-display, including heat shock proteins, myosin family members, CaM-dependent kinases, protein phosphatases and phosphodiesterases [12].

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Although CaM has been widely studied and well characterized in many organisms, it has not been cloned or identified in tapeworms. Only in *Echinococcus multilocularis* (a species that has a close genetic relationship with *E. granulosus*), CaM was predicted as a potential drug target with a high score and available chemical leads (known drugs or approved compounds) [13]. In this report, *E. granulosus* calmodulin (EgCaM) was identified and characterized. Antigenicity and immunoreactivity of rEgCaM had been detected and the preliminary ELISA-based serodiagnostic potential of EgCaM was assessed. The locations of this protein in the adult worm and larval stage and the mRNA expression of different states of PSCs were defined clearly. Moreover, the Ca^{2+}-binding properties of EgCaM was measured.

2. Results

2.1 Sequence analysis of calmodulin

The cDNA sequence of EgCaM with an open reading frame of 450bp (GenBank: KR153481) encoded a protein with 149 amino acids and a theoretical molecular weight of 16.8 kDa (pI = 4.09). The instability index was calculated to be 33.43. No signal peptide or transmembrane regions were found in the deduced amino acid sequence. BLASTp showed that the amino acid sequence of EgCaM shared 84.9%-100% identity with CaMs from *E. multilocularis*, *Hymenolepis microstoma*, *Fasciola hepatica*, *Schistosoma japonicum*, *Caenorhabditis elegans*, *Toxocara canis*, *Plasmodium falciparum*, *Trypanosoma cruzi*, *Homo sapiens* and *Mus musculus* (Figure 1a). Four Ca^{2+}-binding domains were located at residues 21-32, 57-68, 94-106 and 130-142 in EgCaM. A phylogenetic tree showed the relationship of EgCaM with calmodulin from other parasites and hosts; EgCaM clustered with the calmodulins from *E. multilocularis* and *H. microstoma*, but not with other calmodulins (Figure 1b).

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2.2 Expression, purification and Western blotting analysis of EgCaM

That the pET28a(+) plasmid contained the gene of EgCaM was confirmed by double digestion and DNA sequencing. Soluble EgCaM was expressed with a His-tag in *E. coli* BL21 after 4h induction with 1 mM IPTG at 37℃. The purified rEgCaM produced a single band of approximately 20 kDa (including the His-tag), which was agreement with predicted molecular weight (Figure 2). In Western blotting, rEgCaM could react with *E. granulosus* positive sheep sera and anti-rEgCaM rabbit sera. The specific band was visible which was not observed following incubation of the membrane with sera of non-infected sheep or pre-immunized rabbit (Figure 2). In addition, total PSCs extract was blotted with rabbit anti-rEgCaM IgG of approximately 16.0 kDa. The size of this protein was similar with the theoretical molecular weight of EgCaM. It was indicated that EgCaM had a good antigenicity and immunoreactivity.

2.3 Indirect ELISA

Building on the good antigenicity and immunoreactivity of EgCaM, preliminary serodiagnostic potential of EgCaM based on indirect ELISA was assessed. The optimal concentration of the EgCaM antigen was 1.6 μg/well and the best dilution of the sera was 1:160. The cut-off value of the EgCaM-ELISA was 0.472 (mean=0.354, SD=0.040) which was inferred from the *E. granulosus*-negative sera of sheep. Based on the cut-off value, a total of 38 sheep sera (31 for *E. granulosus*-positive and 7 for *T. multiceps*-positive) and 10 goat sera (for *C. tenuicollis*-positive) were tested. 28 sera samples from sheep infected with *E. granulosus* were detected as positive, indicating a sensitivity of 90.3% (28/31). In the cross-reaction assay, the OD values of 5 *T. multiceps*-positive sera isolated from sheep and 3

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C. tenuicollis-positive sera isolated from goat were lower than cut-off value. It was indicated that the specificity of this assay was 47.1% (8/17) .

2.4 Immunohistochemical localization of EgCaM in parasite sections

Anti-rEgCaM rabbit IgG was used to detect the native protein in protoscoleces, cyst walls and adult worms by immunofluorescence analysis (Figure 3). Specific immunofluorescence was detected in the tegument tissues and parenchymal region of protoscoleces, and in the tegument and inner body of adult worms. EgCaM was predominantly localized in the germinal layer. No specific fluorescence was observed in any sections when native rabbit IgG was used.

2.5 mRNA expression of rEgCaM in PSCs treated with H_2O_2

The mRNA expression of rEgCaM in PSCs treated with different concentrations of H_2O_2 (0 mM, 5 mM and 10 mM) was assessed by RT-PCR analysis. The results showed that rEgCaM transcripts in PSCs were gradually decreased with the increase of the concentration of H_2O_2 (Figure 4).

2.6 Biochemical characterization of rEgCaM

rEgCaM in the presence of Ca^{2+} demonstrated increased electrophoretic mobility on SDS-PAGE, compared with protein treated with EDTA (Figure 5a). On native polyacrylamide gels, the electrophoretic mobility of rEgCaM with Ca^{2+} was slower than that of protein exposed to EDTA (Figure 5b). In SDS-PAGE and native gels, the control protein (untreated rEgCaM) showed the same mobility as rEgCaM in the presence of Ca^{2+}, suggesting that the purified protein bound Ca^{2+} during the processes of expression or purification.

The maximum ANS fluorescence emission wavelength from rEgCaM in the presence of

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EDTA was 509 nm. The fluorescence intensity was enhanced when rEgCaM bound Ca^{2+} and the emission maximum moved to 490 nm (Figure 5c), indicating that rEgCaM underwent conformational changes after binding Ca^{2+}.

3. Discussion

Calmodulin as a dynamic Ca^{2+} sensor, is present in all eukaryotic cells, and mediates a variety of cellular signaling activities, such as regulation of gene expression, enzymatic activities and mitochondrial events, modulation of ion channel activities, and specific mechanisms of synaptic transmission [14-16]. Thus, CaM plays an important role in many physiological functions of parasites. Although CaM has been widely studied in trematoda and protozoa , it has not been cloned or identified in tapeworms [17-21]. The characterization and function of CaM in *E. granulosus* is still unreported. In the present study, we have successfully expressed the rEgCaM in *E. coli*, characterized the location in *E. granulosus* sections and mRNA expression of PSCs in different states, as well as assessed its serodiagnostic potential. Furthermore, we also have determined the bioinformatic features and the ability of the protein to bind Ca^{2+} of rEgCaM.

Four Ca^{2+}-binding domains in EgCaM at amino acid positions 21-32, 57-68, 94-106 and 130-142 demonstrated that this protein belongs to the EF-hand calcium-binding protein (CaBP) family, which contain two or more EF-hands [22]. Additionally, the amino acid sequence of EgCaM showed high identity with calmodulin from cestodes, trematodes, nematodes, protozoons and mammals (Figure 1). Calmodulin was previously characterized as a highly conserved protein from the parasite *Schistosoma mansoni*; the alignment of both SmCaM1 and SmCaM2 shared 97-98% identity with other CaMs from mammals, flatworms

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and insects [17]. These data indicate that calmodulin has remained highly conserved across species during evolution. Moreover, mutations and composition of the calmodulin intergenic spacer in *Leishmania* species have been studied as a molecular target that might have taxonomic value [23].

Recently, more and more studies were tending to use recombinant protein antigens to detect animals suspected of being infected by serological diagnosis method [24-26]. It was important to evaluate the serodiagnostic potential of these protein in favor of further research. In our study, western blotting analysis of EgCaM showed that this protein had a good antigenicity and immunoreactivity. However, even the sensitivity was high, the use of rEgCaM in the serodiagnostic ELISA lacks utility because of the low specificity. This might be due to the high conservation of CaM. To conclude, the results were revealed rEgCaM was not a suitable potential diagnostic antigen.

Immunohistochemical localizations showed that EgCaM could be detected in the larva, germinal layer and adult worm sections of *E. granulosus*. These data indicate that EgCaM may play a crucial role in the growth and development of the parasite. In detail, EgCaM was highly expressed in the germinal layer, which is one of the most physiologically active regions of cysts (Figure 3). As a result of this wide distribution of EgCaM in the germinal layer, it is possible to say that various Ca^{2+}-dependent mechanisms occur in this region. Interestingly, EgCaM was mainly distributed in the tegument of the adult stage, which indicates the possibility of Ca^{2+} signaling pathways between *E. granulosus* and its host. A similar result has been reported in *Clonorchis sinensis* [20]. More importantly, EgCaM was mainly expressed under the tegument in the protoscolex stage. Calcium homeostasis can

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induce dose-dependent toxicity of *E. granulosus* protoscoleces [27]. In addition, calcineurin, a Ca^{2+}-calmodulin activated serine-threonine protein phosphatase, has been studied in protoscoleces and it was suggested that calcineurin is specifically involved in exocytic activity [28]. This phenomenon further implies that calmodulin is related to exocytic activity in protoscoleces. By studying other target proteins of calmodulin, such as myosin and GASP180, some important physiological activities which require calmodulin have been discovered in parasites [19,29]. In a previous study, calmodulin depletion resulted in total and catastrophic failure in paraflagellar rod assembly of *Trypanosoma brucei* [21].

In order to further research the function of CaM in *E. granulosus*, PSCs were treated with different concentrations of H_2O_2 (5 mM and 10 mM) for 6 h at 37° C to induce the death. As the activity and metabolic rate of PSCs were declined, the mRNA expression of rEgCaM was significantly decreased (Figure 4). It was demonstrated that CaM was related to the growth and metabolism of PSCs. To sum up, calmodulin plays an indispensable role in many physiological functions in *E. granulosus*.

rEgCaM is an EF-hand CaBP with four Ca^{2+}-binding domains, displaying changes in its electrophoretic mobility in the absence and presence of Ca^{2+}. rEgCaM, presented a typical Ca^{2+}-induced electrophoretic mobility shift in this study (Figure 5). These results were consistent with previous studies of CaBPs in *Clonorchis sinensis* and *Fasciola hepatica* [18,20,30]. Moreover, some calmodulins with mutations in their serine and tyrosine residues retain these characteristics [31]. One of biochemical features of calmodulin is conformational change, exposing more hydrophobic residues on the protein surface after Ca^{2+} binding [32]. rEgCaM had this characteristic, which was verified by ANS fluorescence assay. When Ca^{2+}

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was bound to rEgCaM, ANS fluorescence was enhanced, and the ANS fluorescence emission wavelength shifted from 509 nm to 490 nm.

4. Materials and methods

4.1 Animals and parasites

E. granulosus protoscoleces (PSCs) and cyst walls were aseptically isolated as reported before from liver hydatid cysts of naturally infected cattle presented for routine slaughter in an abattoir in Qinghai Province, China [33]. Every 2000 PSCs were cultured in 1 ml RPMI 1640 medium with 10% BSA (Hyclone), 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma, USA). Adult worms were obtained from a 5-month-old dog after 35 days artificial infection with PSCs. Four New Zealand white rabbits were prepared for production of polyclonal antibody.

4.2 Ethical Statements

All animals involved in this paper were purchased from the Laboratory Animal Center of Sichuan Agricultural University. All procedures were carried out strictly according to the Guide for the Care and Use of Laboratory Animals by the Animal Ethics Committee of Sichuan Agricultural University (Ya'an, China) (Approval No. 2013-028).

4.3 Sera

31 sera were isolated from sheep naturally infected with *E. granulosus*, while 24 negative sera isolated from healthy sheep with no cysts by autopsy. All samples were collected from slaughterhouse in Xinjiang Province. Sera (7 samples) from sheep naturally infected with *Taenia multiceps* and sera (10 samples) from goats naturally infected with *Cysticercus tenuicollis* were collected from slaughterhouse in Sichuan Province.

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4.4 Bioinformatic analysis

The complementary DNA sequence encoding *E. granulosus* calmodulin was uploaded to GenBank (accession code: KR153481) (http://www.ncbi.nlm.nih.gov/). The open reading frame (ORF) was found with an ORF finder tool and analyzed using BLASTp at NCBI. Proteomics tools on the ExPaSy website (http://www.expasy.org/) were used to analyze the physicochemical parameters, signal peptide and transmembrane regions of the amino acid sequence. A phylogenetic tree (neighbor-joining tree) of calmodulin was made using MEGA, version 5.

4.5 Cloning, expression and purification of recombinant EgCaM (rEgCaM)

Total parasite RNA from PSCs was extracted using a commercial kit (Cowin Biotech, China) following the manufacturer's instructions. cDNA was synthesized by using 1 μg of total RNA as the template for the Reverse Transcription System (Thermo, USA). Sequence coding for calmodulin was amplified by polymerase chain reaction (PCR) using the following specific primers: sense primer 5′-CGGGATCCATGGCTGACCAACTTACA-3′ and antisense primer 5′-CCCTCGAGCTACTTCGACTGCATCATC-3′, introducing *BamHI* and *XhoI* restriction enzyme sites, respectively. The PCR protocol included 30 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min. After purification, the PCR product was ligated into the T &A cloning vector and transformed into *Escherichia.coli (E. coli)* DH5α. Digested plasmid DNA using *BamHI* and *XhoI* restriction enzymes was cloned into expression vector pET-28a (Novagen, Germany) and transformed into *E. coli* BL21 (DE3) (Cowin Biotech, China). An *E. coli* clone with the correct DNA sequence was cultured in Luria Bertani broth, and expression of rEgCaM was induced with 1 mM isopropyl-1-thio-β-D -galactopyranoside (IPTG) at 37 °C

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for 6 h. The rEgCaM with a poly-histidine tag was affinity purified from bacterial lysate using Ni-NTA His-tag resin (Qiagen, Germany). The purified rEgCaM was analyzed by 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The final concentration of purified rEgCaM was determined by BCA protein assay kit (Beyotime, China).

4.6 Preparation of polyclonal antibody against rEgCaM

Four rabbits were used to produce the polyclonal antibody. Rabbit sera was collected before immunization to provide a reagent for negative controls. For the first immunization, 200 μg rEgCaM emulsified with an equal volume of Freund's complete adjuvant (Sigma, USA) was injected subcutaneously. The second and third injections for boosting immunization were given by mixing 100 μg protein with an equal volume of Freund's incomplete adjuvant at 2 week intervals. Two weeks after the final injection, rabbit antisera were collected. The antibody titer was determined by enzyme-linked immuno-sorbent assay (ELISA). Immunoglobulin G (IgG) was further isolated from the antisera using a Protein G-Sepharose column (Bio-Rad, USA).

4.7 Western blotting

Purified rEgCaM was firstly separated by SDS-PAGE and subsequently transferred onto polyvinylidene difluoride membrane (Millipore, Germany). The membranes were blocked with 5% (w/v) skimmed milk at room temperature for 2 h, and then incubated with *E. granulosus* positive sheep sera or anti-rEgCaM rabbit sera (1:150 v/v dilutions with 0.01 M PBS) overnight at 4°C. After washing with TBST (40mM Tris-HCl, 0.5M NaCl, 0.5% Tween-20, pH 7.4) the membranes were incubated with horseradish peroxidase

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(HRP)-conjugated goat anti-rat antibody for 1 h. Bands were detected using diaminobenzidine (DAB) reagent (Tiangen, China) according to the manufacturer's instructions. Total PSCs extract were used to detected the specificity and sensitivity of rabbit anti-rEgCaM IgG, the method was same as described above. Non-infected sheep and pre-immunized rabbit sera were used as negative controls.

4.8 Development of Indirect ELISA

96-well microtiter platesplates were coated with 100 μL two-fold diluted rEgCaM antigen (ranging from 1:20-1:2560) diluted in 0.1M carbonate buffer (pH 9.6) at 4°C overnight. After washing three times with PBST to remove non-adsorbed antigen, plates were blocked with 5% skim milk (w/v) in PBS at 37°C for 1 h. Positive and negative sera isolated from sheep were two-fold diluted in PBS ranging from 1:20 to 1:640 (3.2μg/well to 0.1μg/well) and incubated at 37°C for 1 h. After washing, 100 μL rabbit anti-sheep HRP-conjugated antibody (diluted to 1:3,000 with PBS) was added to each well at 37°C for 1 h. Thereafter, followed by a final wash, the enzyme reaction was visualized by the addition of TMB at room temperature for 15 minutes and stopped with Stop Solution (0.5 M phosphoric acid). The OD value (optical density) was measured at 450 nm using a microplate reader.

4.9 Evaluation and statistical analysis

The best dilutions of rEgCaM antigen and sera were determined, and then 62 sheep sera (31 for *E. granulosus*-positive, 7 for *T. multiceps*-positive and 24 for negative control) and 10 goat sera (for C. tenuicollis-positive) were serodiagnosed with the indirect ELISA described as above. *E. granulosus*-positive and negative sera were used in all plates, working as the intra-plate control. The sensitivity of this method was assessed by the percentage value of

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ELISA positive and true positive, while the specificity was evaluated by the cross-reaction with *T. multiceps* and *C. tenuicollis*-positive sera.

The negative cut-off was defined as the mean value + 3×standard deviations (SD) from the OD values obtained from 24 negative sera. The significance of comparisons between test sera groups was estimated by ANOVA (SPSS Inc., Chicago, IL).

4.10 Immunohistochemical localization of EgCaM

Fresh adult worms, PSCs and cyst walls of *E. granulosus* were fixed with 4% paraformaldehyde, embedded in paraffin wax, and then sliced into 5-μm-thick sections. The sections were dewaxed, rehydrated, treated to inactivate endogenous peroxidase activity and incubated in 5% BSA in phosphate buffered saline (PBS) for 1 h at room temperature. Then, the sections were incubated with anti-rEgCaM rabbit IgG or native rabbit IgG (1:500 *v/v* dilutions in PBS) overnight at 4 °C. After washing three times with PBS, the sections were then incubated with FITC-conjugated goat anti-rabbit IgG (1:200 *v/v* dilution in 0.1% Evan's Blue) for 1 h at 37 °C in the dark. After washing three times with PBS, glycerine was added to the sections and images were observed under a fluorescence microscope.

4.11 rEgCaM mRNA expression of PSCs in different states

PSCs were cultured in 12-well microplates and incubated with different concentrations of H_2O_2 (5 mM and 10 mM) for 6 h at 37° C to induce the death of PSCs. PSCs treated without H_2O_2 were used as the control.

Total RNA was extracted and the corresponding cDNA was obtained as described above. To quantify the transcript level of rEgCaM in different states of PSCs, quantitative RT-PCR were conducted [34]. The primers of rEgCaM were 5'-GAAGGATACCGATAGTGAGGAAGA-3' and

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5'-ATCATTTCGTCAACCTCCTCGTC-3'. The primers of the housekeeping gene β-actin were 5'-ATGGTTGGTATGGGACAAAAGG-3' and 5'-TTCGTCACAATACCGTGCTC-3'. The data were calculated using the $2^{-\Delta\Delta CT}$ method.

4.12 Ca^{2+}-binding properties of rEgCaM

To investigate the Ca^{2+}-binding properties of rEgCaM, denaturing and native gel electrophoresis were used. rEgCaM (0.5 mM) was incubated with $CaCl_2$ (50 mM) and EDTA (50 mM), respectively, on ice for 1 h. The control was untreated rEgCaM protein. Subsequently, equal volumes of SDS gel loading buffer were added and the samples were heated in boiling water for 5 min, followed by separation on 15% denaturing polyacrylamide gels. Native gel electrophoresis followed a similar procedure but without SDS, and the mixture was directly loaded onto the gels (not heated). Proteins were visualized by staining with Coomassie Blue.

4.13 ANS fluorescence

1-anilinonaphthalene-8-sulfonic acid (ANS) is a widely used fluorescent probe. rEgCaM (0.5 mM) and ANS (3 mM; Sigma, USA) in PBS were incubated with 50 mM $CaCl_2$ and 50 mM EDTA, respectively, on ice for 1 h. rEgCaM alone (0.5 mM) was the control. The results were measured in a Spectra Max M5 microplate fluorometer at 25°C with 380nm as the excitation wavelength for ANS. The emission wavelength scans were collected between 400 and 700nm. The experiments were repeated three times.

5. Conclusions

In this study, *E. granulosus* calmodulin was cloned, expressed and then characterized. Antigenicity and immunoreactivity of rEgCaM had been detected using western blotting

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assays and usage of CaM as antigen was evaluated in an indirect ELISA. This work indicated that EgCaM was not a suitable potential diagnostic antigen. The locations of this protein in the adult worm and larval stage and the mRNA expression of different states of PSCs were defined. The Ca^{2+}-binding properties of EgCaM was measured. It was demonstrated that EgCaM was considered to be played indispensable role in many physiological functions in *E. granulosus*.

Acknowledgements

The authors thank Junyang Zhu, Min Yan and Xuefeng Yan for their helpful assistance in some experimental steps. We are also extremely grateful to teachers and classmates from the Laboratory of Animal Infectious Disease and Microarray, Laboratory of Animal Diseases and Environmental Hazards of Sichuan Province in Sichuan Agricultural University for having allowing us to conduct the protein purification experiment and use fluorescence microscope in their laboratories, respectively.

Author Contributions

NW and GYY conceived the study. NW and XQZ designed the study and wrote the first version of the manuscript. XQZ, XJS participated in its design, coordination and performed the statistical analysis. XBG and WML conceived of the study and collected the experimental material. YX and XRP collected and analyzed the raw data. GYY* (Corresponding author) is responsible for this study, participated in its design and coordination and help to draft the manuscript. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure Legends

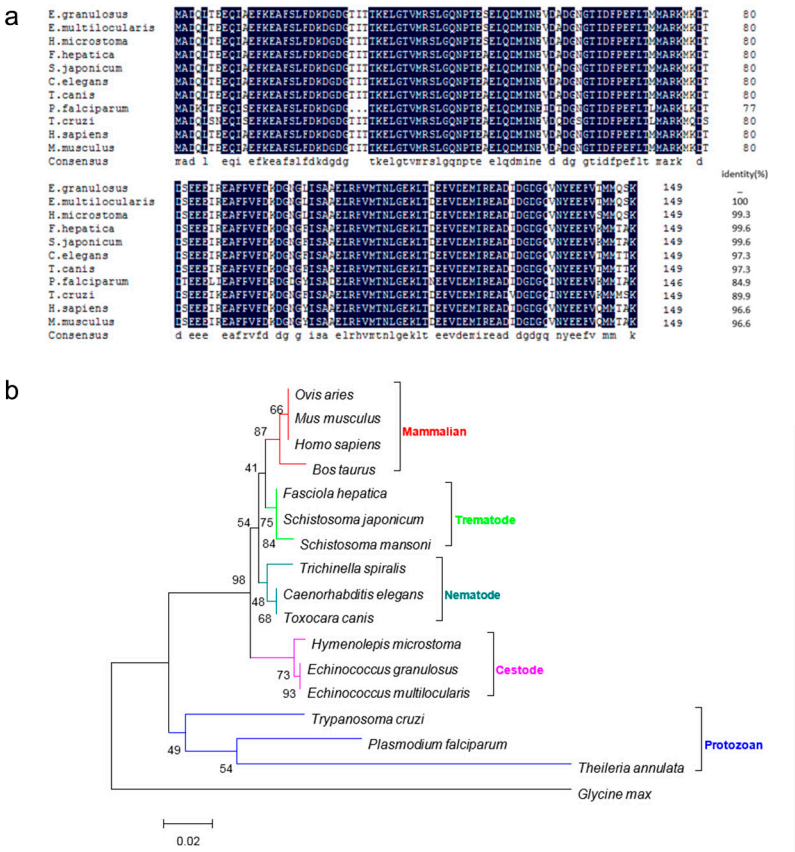

Figure 1. Multiple sequence alignment and phylogenetic analysis. (a) Alignment of the deduced amino acid sequence of *Echinococcus granulosus* calmodulin with sequences of related proteins from other parasites and hosts. *Echinococcus multilocularis* (CDJ00366.1), *Hymenolepis microstoma* (CDS28106.1), *Fasciola hepatica* (CAL91032.1), *Schistosoma japonicum* (AAW27335.1), *Caenorhabditis elegans* (NP_503386.1), *Toxocara canis* (KHN73369.1), *Plasmodium falciparum* (AAA29509.1), *Trypanosoma cruzi* (XP_808093.1), *Homo sapiens* (NP_001734.1), and *Mus musculus* (NP_033920.1). (b) A phylogenetic tree of calmodulin. The tree was built by the neighbor-joining method in MEGA version 5. The bootstrap values are shown at the branching point (1,000 replications).

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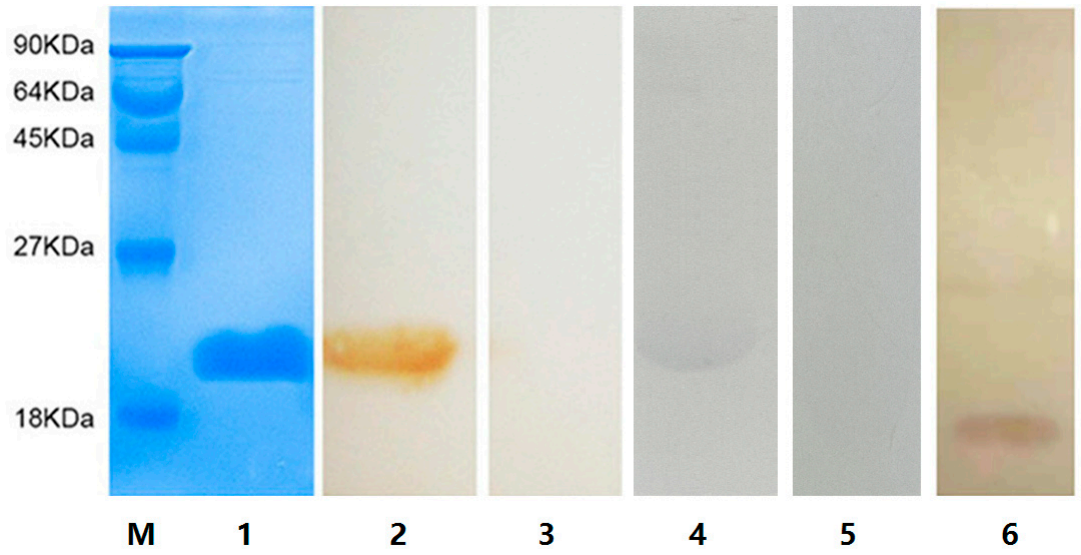

Figure 2. Purification and Western blotting analysis of rEgCaM. M: protein molecular weight markers; Lane 1: purified rEgCaM; Lane 2: rEgCaM was probed with *E. granulosus* positive sheep sera; Lane 3: rEgCaM was probed with sera of non-infected sheep; Lane 4: rEgCaM was probed with anti-rEgCaM rabbit sera; Lane 5: rEgCaM was probed with pre-immunized rabbit sera; Lane 6: Total PSCs extract was probed with rabbit anti-rEgCaM IgG.

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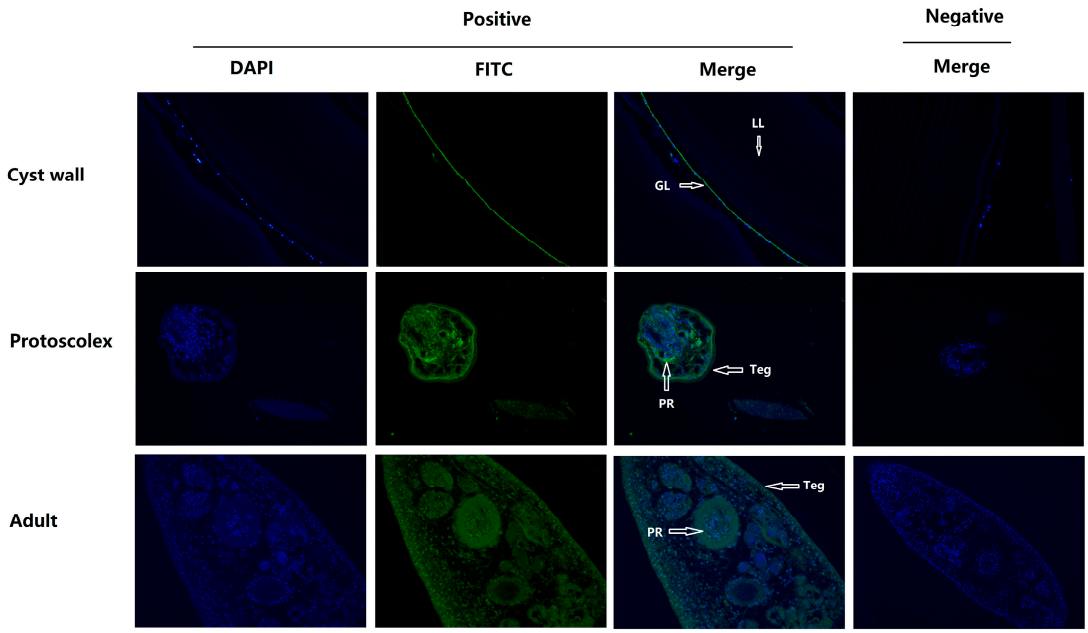

Figure 3. Immunohistochemical localization of EgCaM in sections of *E. granulosus*. Anti-rEgCaM rabbit IgG was used as primary antibody and FITC-conjugated goat anti-rabbit IgG was used as secondary antibody to detect the native protein in protoscoleces, germinal layer and adult worms. The nucleus DNA was stained with DAPI. Pre-immune rabbit sera was used as negative control. Images of protoscoleces are magnified at ×400, germinal layer and adult worm are ×200. The following structures are indicated: LL, laminated layer; GL, germinal layer; Teg, tegument; PR, parenchymal region.

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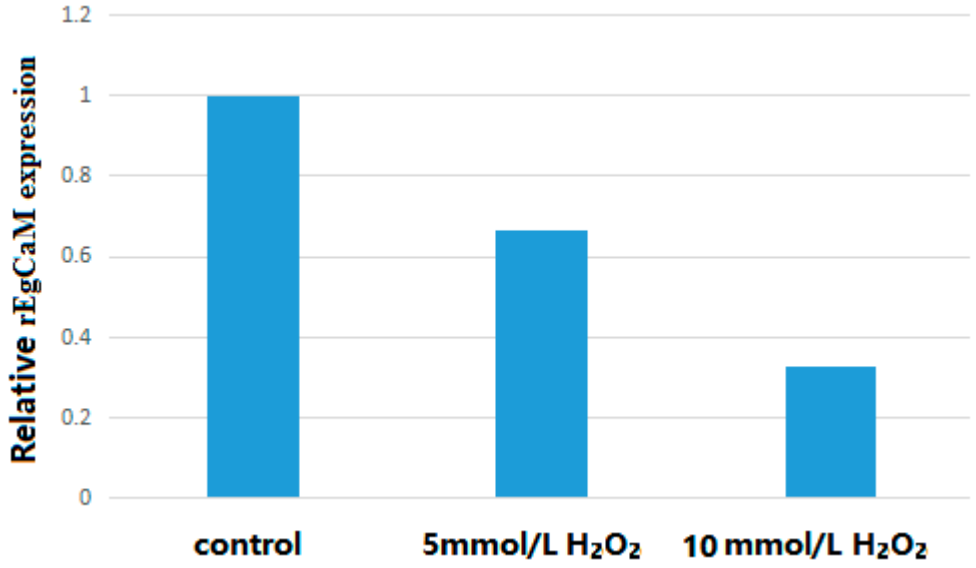

Figure 4. Relative expression levels of rEgCaM in PSCs treated with H_2O_2. The mRNA expression of rEgCaM in PSCs treated with different concentrations of H_2O_2 (0 mM, 5 mM and 10 mM) was assessed by RT-PCR analysis.

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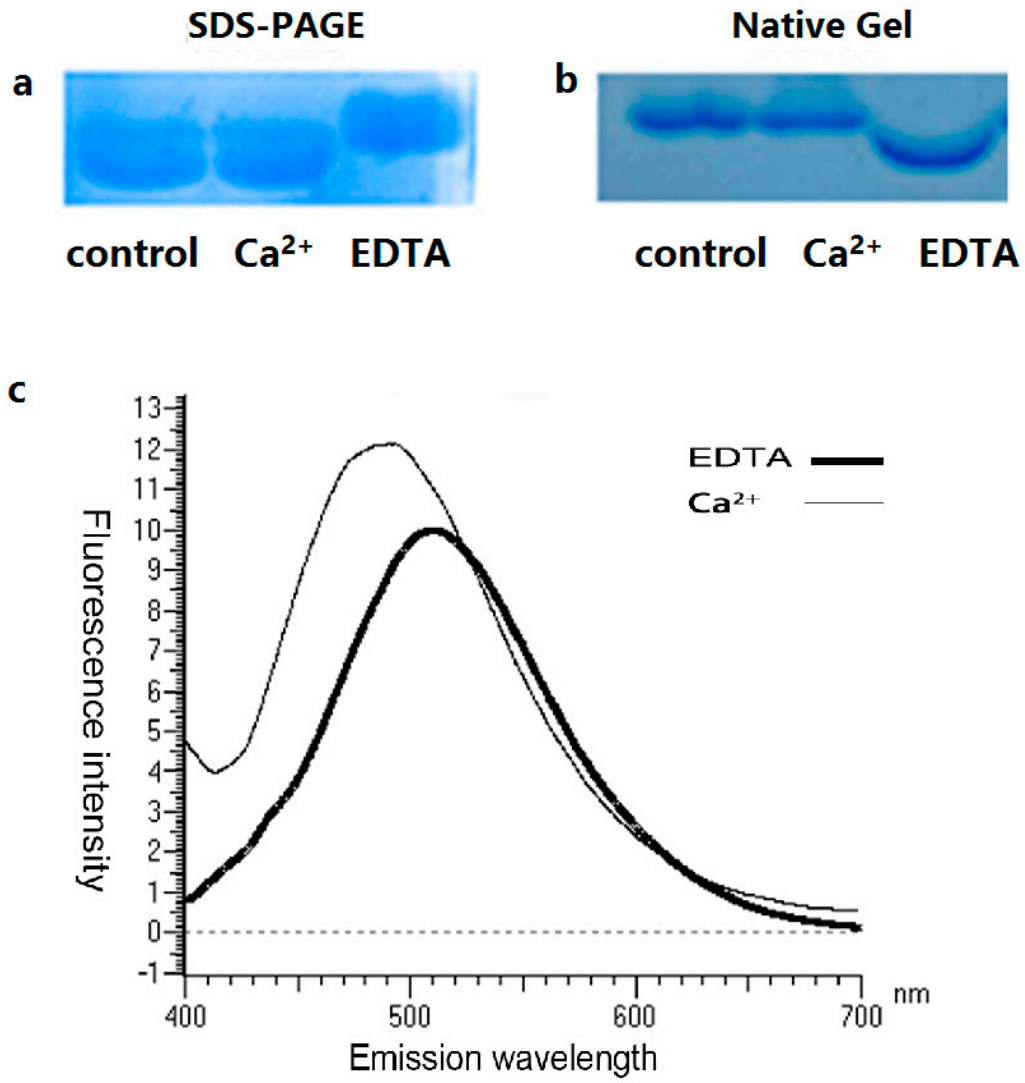

Figure 5. Analysis of Ca^{2+}-binding properties of rEgCaM. The mobility of recombinant protein in the presence and absence of Ca^{2+} was compared on both SDS-PAGE (a) and native gel (b) electrophoresis. (c) ANS fluorescence emission spectra of rEgCaM.