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ORIGINAL PAPER

# A novel tegumental protein 31.8 kDa of *Clonorchis sinensis*: sequence analysis, expression, and immunolocalization

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**Abstract** The complementary DNA (cDNA) plasmid libraries of adult worm, metacercariae and egg of *Clonorchis sinensis* (*C. sinensis*) were constructed for researches on genomics and proteomics of *C. sinensis*. The full-length cDNA sequence encoding tegumental protein 31.8 kDa (*Cs*TP31.8) was identified from the adult cDNA library. The cDNA sequence has been submitted to the GeneBank Database with accession number ABK60086. This novel cDNA sequence contains 828 bp with a putative open reading frame of 275 amino acids. The deduced amino acid sequence shows identity to membrane-associate antigens or tegumental antigens of other species. There were conserved calcium-binding EF hand and dynein light chain type 1 in the sequence. *Cs*TP31.8 transcripts were detected in cDNA libraries of adult worm and metacercariae but not in that of egg. Recombinant *Cs*TP31.8 was expressed and purified from *Escherichia coli* BL21 (DE3). *Cs*TP31.8 was immunolocalized at the tegument of adult *C. sinensis* by using antirecombinant *Cs*TP31.8 sera.

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## Introduction

Human clonorchiasis, caused by the infection of *Clonorchis sinensis* (*C. sinensis*), is endemic in Southern China, Korea, Japan, and other Southeast Asian countries. Nearly 35 million people have been infected with *C. sinensis* globally, of whom 15 million are in China (Lun et al. 2005). People are infected with *C. sinensis* in endemic regions by consuming raw or undercooked freshwater fish and shrimp with metacercariae of *C. sinensis*. The infection rate is increasing in some provinces of China because of people's unhealthy eating habit (Pan et al. 2000; Fang et al. 2000). Despite its threat to human health, little is known about the genomics and proteomics of *C. sinensis*. Many biological and biochemical characteristics of this worm have not yet been defined.

The outermost surface of intramammalian stages of platyhelminth parasite, the tegument, is generally viewed as the most susceptible target for vaccines and drugs because it is importance for host response and parasite survival (Van Hellemond et al. 2006). In the present study, we described sequence analysis, expression, and characterization of one tegumental protein of *C. sinensis*, tegumental protein 31.8 kDa (*Cs*TP31.8). This basic study might be the cornerstone for further application of this protein as a molecule for diagnosis and vaccine of clonorchiasis.

## Materials and methods

### Preparation of C. *sinensis* adult worms sections

*C. sinensis* adult worms were harvested from biliary tracts of infected cats. The worms were fixed with formalin after twice washing gently with sodium chloride. The worms

were embedded with paraffin wax and sliced into 5-μm sections for immunohistochemical localization.

### Identification of complementary DNA sequence encoding CsTP31.8

The complementary DNA (cDNA) library of adult *C. sinensis* was constructed by the method of Switching Mechanism at 5′ end of the RNA Transcript (SMART). The sequences of 8,900 expressed sequence tags and 1,775 Unigenes were obtained as described previously (Song et al. 2004; Yang et al. 2005; Zheng et al. 2005). All Unigenes were screened, and full-length cDNA sequences were determined by BLASTx program at the National Center for Biotechnology Information (NCBI) web server http://www.ncbi.nlm.nih.gov/BLAST). Meanwhile, the identities of the deduced amino acid sequences of these cDNAs with proteins of other species were analyzed.

### Bioinformatics analysis of CsTP31.8

The complete encoding sequence (CDS) of *Cs*TP31.8 was determined by Open Reading Frame (ORF) Finder program at NCBI web server http://www.ncbi.nlm.nih.gov/projects/gorf/). The cDNA and deduced amino acid sequence of *Cs*TP31.8 were analyzed by Vector NTI suite 8. The conserved domain and physico-chemical properties such as molecular weight (MW), isoionic point (pI), halflife and so on were predicted by tools provided by ExPASy Proteomics Server http://expasy.org/).

### Transcript analysis of CsTP31.8 at life-stage of *C. sinensis*

Polymerase chain reaction (PCR) was employed to amplify the transcripts of *Cs*TP31.8 from cDNA library of adult worm, metacercariae and egg, respectively, by using specific primers (forward primer: 5′-*CGGGAATTCATGGATGCTTTTATCG*-3′ and reverse primer: 5′-*GGCTCGAGTCAATAATATGGTGTCTTG*-3′). PCR products were analyzed on a 1% agarose gel by ethidium bromide staining. The specific PCR products amplified from each library were cloned into pGEM-T vector and sequenced.

### Cloning, expression, and purification of CsTP31.8

There were recognition sequences (underlined in the sequences of primers) of *EcoR*I and *Xho*I in the PCR products amplified from cDNA library of adult worm by using the same primers mentioned above. The specific PCR product was purified and digested with *EcoR*I and *Xho*I and then inserted into pGEX-4T-1 vector digested with the same restriction enzyme. The recombinant plasmid was confirmed by DNA sequencing and transformed into *Escherichia coli* BL21. The expression of *Cs*TP31.8-glutathione-*S*-transferase (GST) fusion protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). After induction, the bacteria cells were collected and sonicated. The fusion protein was purified by using affinity column of GSTrapFF (Amersham Biosciences, USA) according to the user manual. The GST tag was cleaved with thrombin (Pharmacia). The efficiencies of *Cs*TP31.8 expression and purification were identified by 15% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie brilliant blue G-250. The final concentration of purified *Cs*TP31.8 was determined by the method of Bradford on nucleic acid/protein analysator (Beckman, USA).

### Acquirement of rat anti-recombinant *Cs*TP31.8 immune sera and Western blotting analysis

Approximately 100 μg of recombinant *Cs*TP31.8 was mixed with equal volume of complete Freund's adjuvant. Sprague–Dawley rats were immunized with the mixture. After 2 weeks, the rats were immunized again with 100 μg of *Cs*TP31.8 mixed with incomplete Freund's adjuvant. After one more pulse with *Cs*TP31.8 alone at fifth week, the immune sera were collected at sixth week from rats. The titer of the antisera to *Cs*TP31.8 was 1:12,800 determined by enzyme-linked immunosorbent assay (ELISA).

The recombinant *Cs*TP31.8 protein were subjected to 15% SDS-PAGE and then electrotransferred to a polyvinylidene difluoride membrane at 100 V for 1 h in a Trans-Blot transfer cell (BioRad, CA). The membrane was incubated in the immune sera (1:100 dilution) and subsequently in horseradish peroxidase-conjugated goat anti-rat IgG (1:5,000 dilution; Bethyl, USA). At last, the membrane was developed color with diaminobenzidine substrate solution.

### Immunolocalization of *Cs*TP31.8 at adult worm of *C. sinensis*

Sectioned worms in paraffin wax were deparaffinized and incubated in the rat anti-*Cs*TP31.8 sera (1:100 dilution). Preimmune rat sera were employed to make a negative control. The sections were subsequently incubated in fluorenscein isothiscyanate (FITC)-conjugated anti-rat IgG (1:50; Boster). The sections were observed under fluorescence microscope.

## Results

### Sequence analysis of CsTP31.8

The cDNA sequence of *Cs*TP31.8 was identified by BLASTx. The full cDNA sequence (GenBank accession number, ABK60086) contained a complete ORF of 828

base pairs (bp) which encoded a putative protein of 275 amino acids.

The MW of *Cs*TP31.8 was predicted to be 31.8 kDa. Its theoretical isoelectric point (p*I*) was 4.33. The estimated half-life of *Cs*TP31.8 was more than 10 h in *E. coli*. The instability index of *Cs*TP31.8 was computed to be 35.57. This classified the protein as stable.

BLASTx showed that *Cs*TP31.8 had more than 40% identity to tegumental proteins from other platyhelminth parasites. It was predicted that there were conserved domains of calcium-binding EF hand and dynein light chain type 1 in *Cs*TP31.8 by Interpro Scan program. The alignments were 96.8 and 89.9%, respectively.

### Transcript analysis of CsTP31.8 at life-stage of *C. sinensis*

*Cs*TP 31.8 transcripts were detected in cDNA library of adult worm or metacercariae of *C. sinensis* but not in that of egg (Fig. 1).

### Expression and purification of *Cs*TP31.8

The recombinant *Cs*TP31.8 was expressed as a fusion protein with a GST tag in *E. coli* BL21 after induction by IPTG. The fusion protein of GST-*Cs*TP31.8 was about 58 kDa on polyacrylamide gel stained by 0.1% Coomassie brilliant blue R250. The MW was consistent with the predicted MW of 57.8 kDa (MW of GST is 26 kDa). After purification, other proteins of *E. coli* BL21 were removed. GST was completely removed (Fig. 2). The concentration of purified *Cs*TP31.8 was 1.8 mg/ml.

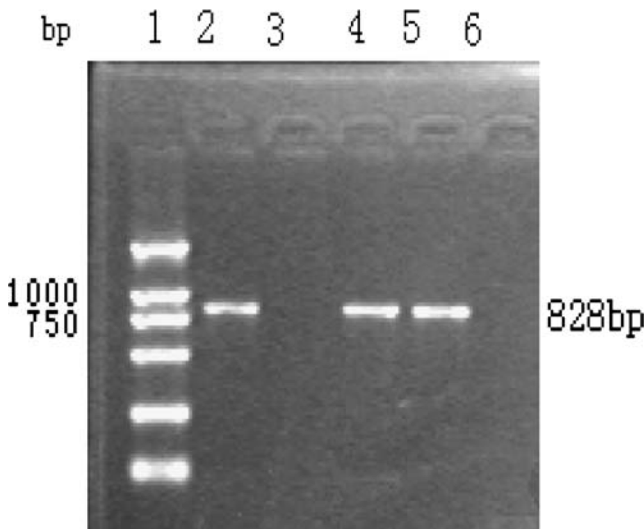


**Fig. 1** Transcript analysis of *Cs*TP31.8 at life-stage of *C. sinensis* by using PCR with specific primers of the complete encoding sequence of *Cs*TP31.8. *Lane 1* DNA marker (2,000, 1,000, 750, 500, 250, and 100 bp); *lane 2* positive control; *lane 3* negative control; *lane 4* adult worm cDNA library; *lane 5* metacercariae cDNA library; *lane 6* egg cDNA library

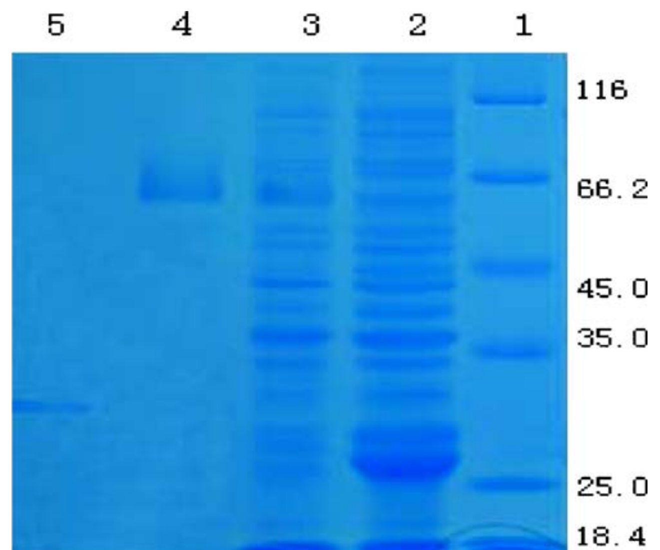


**Fig. 2** Expression and purification of recombinant *Cs*TP31.8 in *Escherichia coli* BL21 identified by 15% SDS-PAGE staining by 0.1% Coomassie brilliant blue R250. *Lane 1* Protein molecular weight markers; *lane 2* lysate of the bacteria with pGEX-4T-1 vector induced with IPTG; *lane 3* lysate of the bacteria with recombinant pGEX-4T-1-*Cs*TP31.8 induced with IPTG; *lane 4* GST-*Cs*TP31.8 fusion protein purified with affinity column of GSTrapFF; *lane 5* recombinant *Cs*TP31.8 without GST tag

### Western blotting analysis of recombinant CsTP31.8

The purified *Cs*TP31.8 could be probed by rat anti-*Cs*TP31.8 sera in the Western blot analysis (Fig. 3).

### Immunolocalization of CsTP31.8 at adult worm of C. *sinensis*

The analysis of immunohistochemical localization by using rat anti-*Cs*TP31.8 sera showed that *Cs*TP31.8 was located at the tegument of adult *C. sinensis*. *Cs*TP31.8 wasn't observed in the internal tissues of the *C. sinensis*. *Cs*TP31.8 could not be detected in sections incubated with preimmune rat sera (Fig. 4).

## Discussion

In this work, we studied a novel tegumental protein *Cs*TP31.8 from *C. sinensis*. We identified its complete encoding sequence from cDNA library. It contains 828 bp which encoded a putative protein of 275 amino acids. Its physico-chemical properties were analyzed by bioinformatics. It showed around 40% identity to tegumental proteins of other species. There might be conserved domains of calcium-binding EF hand and dynein light

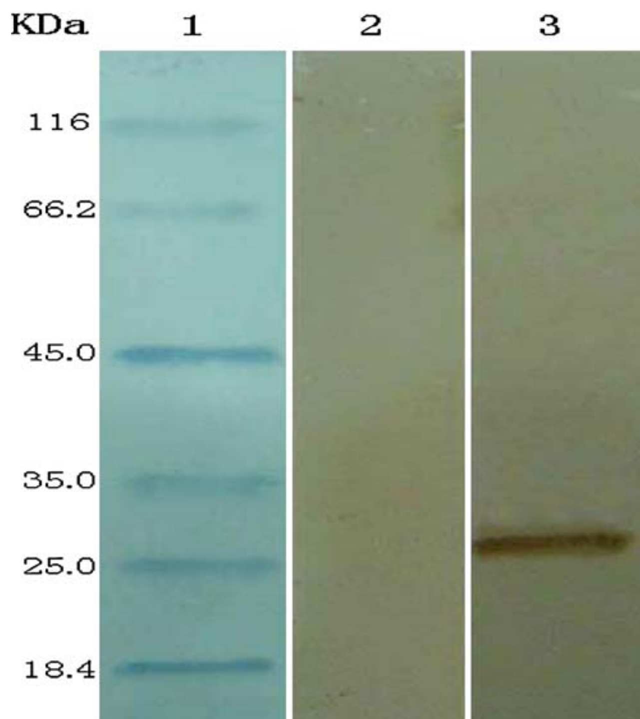


**Fig. 3** Western blotting analysis of recombinant *Cs*TP31.8 by using rat preimmune sera or anti-*Cs*TP31.8 sera as primary antibody. *Lane 1* Protein molecular weight markers; *lane 2* purified recombinant *Cs*TP31.8 reacted with rat preimmune sera; *lane 3* purified recombinant *Cs*TP31.8 reacted with rat anti-*Cs*TP31.8 sera

chain type 1 in it. The alignments were 96.8 and 89.9%. The transcripts of *Cs*TP31.8 could be detected in cDNA libraries of adult worm and metacercariae. *Cs*TP31.8 was immunolocalized at the tegument of adult *C. sinensis* as predicted.

The identity between *Cs*TP31.8 and membrane-associated antigens or tegumental antigens of other species was about 40%. There probably were calcium-binding EF-hand and dynein light chain type 1 domains in *Cs*TP31.8. Such domains were usually involved in the structure of membrane-associated or tegumental antigens from *Schistosoma japonicum*, *Schistosoma mansoni*, and *Fasciola gigantica* (Jeffs et al. 1991; Ruiz de Equino et al. 1999; Fitzsimmons et al. 2004; Hoffmann and Strand 1997). The result of immunolocalization showed that *Cs*TP31.8 was located at the tegument of adult *C. sinensis*. The results of both bioinformatics analysis and actual immunolocalization of *Cs*TP31.8 indicated that the novel protein was one of the tegumental proteins from *C. sinensis*.

The tegument is a dynamic host-interactive layer involved in nutrition, immune evasion and modulation, excretion, and signal transduction. The tegument constitutes the parasite–host interface (Jones et al. 2004; Van Hellemond et al. 2006). Many proteins identified in the tegument of *S. japonicum*, *S. mansoni*, and *F. gigantica* were potentially molecules for vaccine development and for immunological diagnosis (Jeffs et al. 1991; Waine et al. 1994; Xavier et al. 1998). It was documented that the tegument showed the strongest staining in the immunohistochemical staining of *C. sinensis* with *C. sinensis*-infected animal sera (Cho et al. 1986; Choi et al. 1981; Hong et al. 2001). The transcripts of *Cs*TP31.8 were detected in the stage of metacercariae and adult worm, so that if *Cs*TP31.8 might be defined as an important antigen of *C. sinensis*, it could be developed as a vaccine not only for intermediate host but also for definitive host.

In a previous study, another tegumental protein from *C. sinensis* was identified, and its value as a molecule for serologic diagnosis was evaluated in our lab (Zhou et al. 2007). The identification of this novel tegumental protein *Cs*TP31.8 would enhance our understanding of host–parasite interaction as well as provide another potential antigen for diagnosis and vaccine development.

In summary, we identified a novel tegumental protein *Cs*TP31.8 of *C. sinensis*. Basic information of *Cs*TP31.8 was obtained. Further studies will be focused on its ultrastructure location and value as a molecule for diagnosis and vaccine development of clonorchiasis.

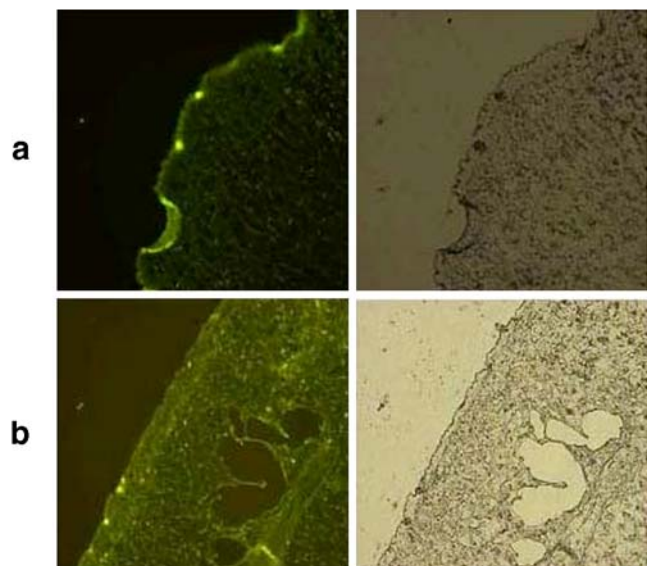


**Fig. 4** Immunohistochemical localization of *Cs*TP31.8 at adult *C. sinensis* by using rat anti-*Cs*TP31.8 sera as primary antibody and FITC-conjugated anti-rat IgG as secondary antibody. The *left images* showed the tegument and underlying tissues of adult worm under fluorescence microscope. The *right images* showed the same part under optical microscope. Sera from nonimmunized rats were used to make negative control. The localization of *Cs*TP31.8 is shown in **a** and the negative control is shown in **b**

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