Molecular characterization of cathepsin B from *Clonorchis sinensis* excretory/secretory products and assessment of its potential for serodiagnosis of clonorchiasis

Wenjun Chen1,2, Xiaoyun Wang1,2, Xuerong Li1,2, Xiaoli Lv1,2, Chenhui Zhou1,2,3, Chuanhuan Deng1,2, Huali Lei1,2, Jingtao Men1,2, Yongxiu Fan1,2, Chi Liang1,2 and Xinbing Yu1,2*

**Abstract**

**Background:** Cathepsin cysteine proteases play multiple roles in the life cycle of parasites such as food uptake, immune invasion and pathogenesis, making them valuable targets for diagnostic assays, vaccines and drugs. The purpose of this study was to identify a cathepsin B of *Clonorchis sinensis* (*Cs*CB) and to investigate its diagnostic value for human helminthiases.

**Results:** The predicted amino acid sequence of the cathepsin B of *C. sinensis* shared 63%, 52%, 50% identity with that of *Schistosoma japonicum*, *Homo sapiens* and *Fasciola hepatica*, respectively. Sequence encoding proenzyme of *Cs*CB was overexpressed in *Escherichia coli*. Reverse transcription PCR experiments revealed that *Cs*CB transcribed in both adult worm and metacercaria of *C. sinensis*. *Cs*CB was identified as a *C. sinensis* excretory/secretory product by immunoblot assay, which was consistent with immunohistochemical localization showing that *Cs*CB was especially expressed in the intestine of *C. sinensis* adults. Both ELISA and western blotting analysis showed recombinant *Cs*CB could react with human sera from clonorchiasis and other helminthiases.

**Conclusions:** Our findings revealed that secreted *Cs*CB may play an important role in the biology of *C. sinensis* and could be a diagnostic candidate for helminthiases.

**Background**

*Clonorchis sinensis* is the causative agent of clonorchiasis, a chronic liver infection of human acquired through consumption of raw or undercooked fish and shrimps with infectious metacercariae. Clonorchiasis is endemic in Asian countries and over 35 million people globally are infected *C. sinensis*, including an estimated 15 million in People’s Republic of China [1]. Recently, this infection has emerged in non-endemic regions and developed countries following growing international markets, improved transportation systems and demographic changes such as population movements [2]. *C. sinensis* adults reside chronically in the biliary tract and cause periductal inflammation, fibrosis, pyogenic cholangitis, biliary calculi, cholecystitis, liver cirrhosis and pancreatitis [3]. Like *Opisthorchis viverrini*, *C. sinensis* is one of the direct causes of cholangiocarcinoma announced by the International Agency for Research on Cancer (IARC) in 2009 [4]. It is important to take some measures to control clonorchiasis due to its public health threat. Until now, the main prevention and control strategies for this parasite are treatment of individual patients with praziquantel, and interrupting transmission at the intermediate host level [5]. However, there have been little effective measures to prevent this neglected tropical disease [6].

Cysteine proteinase is ubiquitous in all species [7-9]. In parasites, cysteine proteases have attracted much attention for their essential roles in parasite physiology as well as in host-parasite interactions through their modulation of various pathobiological events, including host tissue invasion, nutrient uptake, host immune...
evasion and molting [10-13]. Research has been conducted to characterize the biochemical properties and pathophysiological roles of cysteine proteases from trematode parasites. The essential roles of cysteine proteases in parasite survival or growth make them attractive targets for vaccines or chemotherapeutic agents [14-16]. Several genes encoding *C. sinensis* cysteine proteases have been identified and partially characterized [17-19]. Lee et al. [20] reported that cathepsin F-like cysteine protease of *C. sinensis* is a good vaccine candidate against clonorchiasis. Li et al. [21] found that endogenous cysteine proteases of *C. sinensis* metacercariae are probably involved in the excystment process. Kang et al. [22] indicated that partially purified cysteine protease from excretory/secretory products (ESP) of *C. sinensis* adults exhibited significant cytotoxic effects against cultured cells. ESP of parasites have attracted more attention for their significant roles in the diagnosis, vaccine, drug target and host-parasite interactions etc. *In vitro* biochemical studies have predicted that ESP from liver flukes have definitive roles in feeding behavior, detoxification of bile components and immune evasion [23]. Ju et al. [18] have identified legumain from ESP as a serodiagnostic antigen of clonorchiasis. In addition, several genes encoding *C. sinensis* cysteine proteases have also been identified and their value as diagnostic antigens for clonorchiasis was investigated [24,25]. However, little is known about cathepsin B (CB) in *C. sinensis* except five distinct sequences deposited in Genbank.

As members of the cysteine protease family, cathepsins have been assayed in the serodiagnosis of both human and animal in parasite infections. Cornelissen et al. [26] reported a specificity of 75.3% in naturally infected cattle using *Fasciola hepatica* cathepsin L as coating antigen. Carnevale et al. [27] found that recombinant pro-cathepsin-L was 100% specific in the diagnosis of serum samples determined by checker board technique. Sripa et al. [28] indicated that partially purified cysteine protease from *E. coli* adults exhibited significant cytolytic triad (Cys116, His285 and Asn305) as well as the Gin could be searched in this sequence. The putative protein contained an occluding loop that is the signature of cathepsin B8 and a haemoglobinase motif which is shared by helminth blood-feeders (Figure 1).

**Cloning, expression and purification of recombinant CsCB (rCsCB) in E. coli**

The recombinant pET-28a(+) plasmid containing the CB gene coding region (signal peptide removed) was confirmed by digestion with restriction enzyme. DNA sequencing revealed that the construct was correct with 6 x His tag at the N terminus of the recombinant protein. The recombinant CsCB was expressed in inclusion bodies in *E. coli*. SDS-PAGE showed that the molecular mass of fusion protein was about 40 kDa, which was in correspondence with the predicted 39.2 kDa (including 34 amino acids of vectors). After purification and renaturation, the concentration of the recombinant protein was about 0.25 mg/ml (Figure 2A).

**Identification of CsCB as ESP by western blotting**

Rat anti-rCsCB antibody titers were higher than 1:102,400 as determined by ELISA. In western blotting assay, both *C. sinensis*-infected rat serum and anti-CsCB rat serum could react with the CsCB while the normal rat serum could not. Comparing with the control group, sera from rats immunized with total ESP could recognize CsCB. In addition, ESP has been shown to react with anti-CsCB rat serum (data not shown). The results above indicated that CsCB was a component of ESP (Figure 2B).

**RT-PCR analysis of CsCB at life-stage of C.sinensis**

CsCB transcripts were detected both in adults and metacercaria of *C. sinensis* (Figure 3A), and the expression level in adults was higher than that of metacercaria when normalized by β-actin (Figure 3B, P < 0.05).

**Immunohistochemical localization of CsCB in C. sinensis adults**

Using the antisera against CsCB as the primary antibody and fluorescence labeling IgG as the secondary antibody, immunolocalization showed that CsCB distributed in the intestine of adults, while no specific fluorescence except autofluorescence was detected in adults treated with normal serum (Figure 4).

**Serodiagnosis of human helminthiases**

The optimal concentration of coating antigen and dilution of serum samples determined by checker board
titration were 3 μg/ml and 1:400, respectively. The cut-off value for positive infection status in ELISA tests evaluated by receiver operating characteristic (ROC) curve was 0.47 (Figure 5). Sera from humans infected with *C. sinensis* showed a sensitivity of 79% by ELSA based on rCsCB and healthy sera and sera from those infected with other helminths showed a specificity of 81% (Table 1). Both ELISA and western blotting assays showed that rCsCB could react with human sera from clonorchiasis, fascioliasis, schistosomiasis, paragonimiasis, cysticercosis and echinococciosis (Figure 6).

**Discussion**

Cathepsins in general are of interest to parasitologists, as there is considerable evidence that they play a key role in the biology of parasites [29]. In this study, a CB of *C. sinensis* was cloned and overexpressed in *E. coli*. It was classified as CB due to its sequence homology to cathepsin B protein and structure. The putative amino acid sequence shared 63%, 52%, 50% and 53% identities with CB of *S. japonicum*, *H. sapiens*, *F. hepatica* and *E. multilocularis*, respectively. Sequence analysis showed that CsCB could react with human sera from clonorchiasis, fascioliasis, schistosomiasis, paragonimiasis, cysticercosis and echinococciosis (Figure 6).

**Figure 1** Alignment of cathepsin B (CsCB) deduced amino acid sequence from *C. sinensis* and other species. The deduced amino acid sequence shares 63%, 52%, 50% and 53% identity with CB of *S. japonicum*, *H. sapiens*, *F. hepatica* and *E. multilocularis*, respectively. Highly conserved residues are shaded in gray. Residues of the catalytic triad (Cys116, His285 and Asn305) are indicated with black triangles. The oxyanion Gln is marked with white triangle; arrow shows cleavage point of mature enzyme, the occluding residues and hemoglobinase motif are boxed.
key molecule in *F. hepatica* biology and drug therapy [34]. In *S. mansoni*, SmCB1 and SmCB2 have been identified and the function of SmCB1 was examined using RNAi silencing, which illustrated that SmCB1 played a role in nutrient acquisition [35,36]. Based on the key role of CB in the biology of parasites, we expect that CsCB may play the same role in nutrition intake and immune invasion of host. In multicellular parasites such as trematodes, the intestine is a major source of secreted proteases and also a place for nutrition digestion and absorption. According to results of immunolocalization, CsCB was distributed in the intestine of adult worm, indicating that CsCB might be involved in digestion of host protein and nutrient uptake for this parasite itself. In our RT-PCR experiments, CsCB could be detected in both adult worm and metacercaria of *C. sinensis*. As a
secreted protease, these observations suggested that CsCB may play an important role in the biology of this parasite.

Proteases contained in ESP of parasites released to the environment, play key roles in bile duct malignancy and the subsequent development of cholangiocarcinoma [37,38]. Our previous studies revealed that ESP of adult C. sinensis lysophospholipase and phospholipase A2 might be pathogenic factors of human hepatic fibrosis caused by infection of C. sinensis [39,40], which could deepen our understanding of the pathogenesis of C. sinensis. CB has already been found in ESP of C. sinensis and some other helminths [41]. Moreover, western blotting revealed that CsCB was a component of ESP, and CsCB could react with C. sinensis-infected rat serum. In ELISA results, rats injected with recombinant CsCB developed high antibody titers. Our results showed that CsCB might be involved in the pathogenesis of C. sinensis-related hepatobiliary diseases as a component of ESP with antigenicity and immunogenicity. Indeed,

Figure 3 Transcriptional level of C. sinensis CB at metacercaria and adult worm. A. 1% agarose gel. DNA marker (M), CsCB PCR products amplified from adult worm cDNA (lane 1), metacercaria cDNA (lane 2), β-actin amplified from adult worm cDNA (lane 3), metacercaria cDNA (lane 4) and CsCB PCR product amplified from recombinant CB plasmid (lane 5). B. PCR products were quantified and analyzed. PCR products were quantified by Tanon Gis software, compared normalized by C. sinensis β-actin, analyzed by Student’s t test (Software package SPSS16.0). P-value of < 0.05 indicated statistical significance.

Figure 4 Immunolocalization of CsCB in adult worm of C. sinensis. Rat anti-CsCB serum was used as primary antibody and goat anti-rat IgG as the secondary antibody. Panel A and D show tissues of adult worm under fluorescence microscope. Panel A and C show the same part under white light. Panel A and B were the negative control treated with preimmune rat serum. i, intestine s, sucker. The images were magnified at 100x for photograph.
C. sinensis adults dwell in the bile duct of host, persistently released ESP resulting in mechanical damage and chemical stimulus which must have brought on infiltration of inflammatory cells surrounding bile ducts and adenomatous hyperplasia of biliary epithelia [42-44]. However, there remains much to do to elucidate the mechanism of C. sinensis causing hepatobiliary diseases. We have also approached the antigenicity and diagnostic value of CsCB in human helminthiases. Both the ELISA and western blotting showed that CsCB could react with sera from most of human helminthiases, but not with sera from healthy people. High identity in the predicted amino acid sequences of C. sinensis and other helminths could be responsible for the observed cross-reactivity. Though cross-reactivity existed, CsCB could also be applied as a diagnostic candidate of clonorchiasis since treatment with praziquantel has the same efficacy on helminths.

**Conclusion**
A gene encoding cathepsin B protein of adult C. sinensis was cloned and expressed for the first time. Our findings revealed that CsCB, which is expressed in the intestine and released outside the worm as a component of ESP, may play an important role in the biology of C. sinensis. Moreover, CB may play conserved roles in helminths for the high homology analyzed by bioinformatics. Further investigations are required to characterize the conserved functions of such important protease and the role as a potential vaccine candidate against C. sinensis infection.

**Materials and methods**

**Sequence analysis of CsCB gene sequence**
A complete coding sequence encoding CB was isolated from GenBank (Accession no. EF102086.1) in NCBI http://www.ncbi.nlm.nih.gov/, the physicochemical properties and the functional domains in deduced amino acids were predicted by proteomics tools in ExPaSy web site http://www.expasy.org/. Based on the similarity, the homologous sequences of different species, including E. multilocularis (BAJ83491.1), F. hepatica(ABU62925.1), S. japonicum (CAX71086.1), H. sapiens (NP_001899.1), Paragonimus westermani (AAB93494.1) and Taenia solium (AAS00027.1) were identified using the basic local alignment search tool (BLAST) server. Multiple sequences alignments were performed using bioinformatics analysis software Vector NTI suite 8.0.

**Cloning, expression, purification and refolding of CsCB in E. coli**
Gene sequence encoding CsCB (signal peptide excluded) was amplified from cDNA of C. sinensis by polymerase...
chain reaction (PCR) using forward primer: 5'-CGC GGATCCGAGTATATTCCATCTTTCCA-3' and the reverse primer: 5'-GTC GAGTACAGTTTTGGATGACC-3' with BamHI/XhoI restriction enzyme sites (underlined). PCR was carried out for 30 cycles at 94°C for 45 s, 57°C for 45 s, and 72°C for 60 s, and the reaction continued for 10 min at 72°C after the last cycle. Purified PCR products were cloned into the His6 tag expression vector pET-28a(+) (Novagen; USA) with corresponding incision enzymes. The recombinant plasmid was transformed into E. coli for expression and insertion confirmed by digestion with restriction enzyme and DNA sequencing. Expression of recombinant CsCB protein was induced by isopropyl-β-D-thiogalactoside (IPTG) at a final concentration of 0.2 mM for 3 h at 30°C. The bacterial cells were collected by centrifugation at 4°C, and the inclusion bodies containing the recombinant fusion protein were solubilized completely with 6 M urea in 20 mM Tris-HCl buffer (pH 8.0), followed by purification with His Bind Purification kit (Novagen; USA) and elution with 150 mM imidazole. Renaturation was carried out by stepwise diluting urea in dialysate buffer (20 mM Tris-HCl, 5 mM EDTA buffer, pH 8.0). Purified protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue, final recombinant protein concentration was estimated by Bradford assay using BSA method used as a standard.

Semi-quantitative reverse transcription-PCR (RT-PCR) analysis of CsCB

In order to determine the mRNA transcriptional level of CsCB in various stages of the parasite, total RNA of adult worms and metacercariae were extracted and quantitated by nucleic acid/protein analyzer (Beckman Coulter; USA). Reverse transcription reactions were carried out by transforming equal amounts of total RNA (1 μg each) to cDNA using RT-PCR Kit (TaKaRa; PR China). RT-PCR experiments were employed to amplify the transcripts of CsCB from cDNA of adult worms and metacercariae, respectively, and C. sinensis β-actin (GenBank accession no. EU109284) was used as a positive control. The forward and reverse primer for CsCB were 5’-GGATTCGGCCTGGAAAAAC-3’, 5’-CAGTTTTGGATGACCAGCAT-3’ and for β-actin were 5’-GGTGACGCTGAAGTATCC-TATTTGA-3’, 5’-CCAAAAGCATAGCCCTCGTAGAT-3’, respectively. The programs for PCR were the same as described above. PCR products from two life stages were quantified by Tanon Gis software (Tanon 4100; PR China) normalized by C. sinensis β-actin and analyzed by
Student’s t test (Software package SPSS16.0), P-value of < 0.05 indicated statistical significance.

Preparation for the antisera of recombinant protein and C. sinensis excretory/secretory product (ESP)

Living adults of C. sinensis were collected and cultured in RPMI-1640 (Gibco; USA) at 37°C under 5% CO2 for 6 h. The culture was centrifuged at 12,000× g at 4°C for 30 min to remove insoluble debris. The supernatant was dialyzed in PBS for 12 h and concentrated with sucrose. Six-week-old male Sprague-Dawley rats were purchased for animal experiments under the Guide for the Care and Use of Laboratory Animals. Both the recombinant CB and ESP were emulsified with complete Freund’s adjuvant and immunized subcutaneously to SD rats. Each rat was given 200 μg rCsCB or ESP at the first injection, and 100 μg protein (emulsified with incomplete Freund’s adjuvant) was given for 2 booster injections at 2-week intervals; antisera were collected before each injection and serum antibody responses to rCsCB and ESP were tested by ELISA.

SDS-PAGE and western blots

The recombinant protein (1 μg per lane) was subjected to SDS-PAGE and electrotransferred onto polyvinylidene difluoride membrane (PVDF, Whatman; USA) at 100 V for 1 h. The membrane was blocked with 5% skim milk in phosphate buffered saline tween-20 (PBST, pH 7.4) at 37°C for 2 h, washed 5 times with PBST, then incubated with different antisera (antisera against the recombinant CsCB protein, antisera against the ESP, sera from C. sinensis-infected rats and normal rats, respectively, 1:200 dilutions for all sera) at 37°C for 2 h. After washing, the membrane was incubated with rabbit anti-rat IgG HRP-conjugated horse radish peroxidase (1:2,000 dilution; Boster; PR China) at 37°C for 1 h. Diaminobenzidine (DAB) substrate solution (Invitrogen, USA) was employed as the secondary antibody. Subsequent reactions were developed with 3,3′,5′,5′-tetramethyl benzidine (TMB; BD Biosciences; USA) and stopped with 2 M H2SO4. All assays were tested in triplicate and repeated twice. The absorbance value was measured at 450 nm.

In western blotting assay, rCsCB was subjected to 12% SDS-PAGE and electrotransferred onto PVDF membrane (Whatman; USA). The membrane was incubated with different human helminthiases sera (clonorchiasis, echinococcosis, fascioliasis, schistosomiasis, paragonimiasis, and cysticercosis, respectively) which showed high OD450 values by ELISA test at 37°C for 2 h, human healthy serum was used as negative control, all sera were 1:400 diluted in PBST containing 0.1% BSA. Goat anti-human IgG HRP-conjugated horse radish peroxidase (1:2,000 dilution; Boster; PR China) was the secondary antibody to react with the membrane and DAB substrate solution was used to visualize the reaction.

Serodiagnosis of human helminthiases by ELISA and western blotting

The optimal antigen concentration and serum dilution required for ELISA were determined by checker board titration. Briefly, 96-well microtiter plates (Costar, USA) were coated with 100 μl 1 μg/ml, 3 μg/ml, 5 μg/ml, 7 μg/ml rCsCB (in 0.05 mol/l NaHCO3 buffer, pH 9.6) and incubated at 4°C overnight. After three washings with PBST, the microplates were blocked with 5% skimmed milk (w/v, in PBST containing 0.1% BSA) for 2 h at 37°C. Following another washing procedure, the plates were incubated with human helminthiases sera (1:50, 1:100, 1:200, 1:400 dilutions for each coating concentration in PBST containing 0.1% BSA) for 2 h at 37°C. Goat anti-human IgG (1:10,000 dilutions in 0.1% BSA-PBST recommended by producer; Invitrogen, USA) were employed as the secondary antibody. Subsequent reactions were developed with 3′, 3′, 5′, 5′-tetramethyl benzidine (TMB; BD Biosciences; USA) and stopped with 2 M H2SO4. All assays were tested in triplicate and repeated twice. The absorbance value was measured at 450 nm.
Statistical analysis
Software package SPSS16.0 was used in the present study for all statistical analysis. Briefly, Student’s t test was used to analyze the measurement data among groups. P value of < 0.05 was considered statistically significant. The optimal cut-off value was calculated based on ROC curve analysis which correlated with sensitivity and 1-specificity [45]. ROC curve, area under the curve (AUC) and scatter plot were carried out using SPSS16.0. The sensitivity and specificity were calculated using microscopic examination of stool samples as gold standard method.

Ethical approval
Centers for Disease Control and Prevention of Nanning, Jiangsu, Gansu and Fujian provinces granted ethical approval for human sera used in the present study. All animal experiments were approved by institute’s ethical committee of Sun Yat-sen University.

Acknowledgements
This work is supported by the National S & T Major Program (Grant No. 2008BZX100004-011) and Development Program of China (973 program; no. 2010CB535000).

Authors' contributions
WXC, XYW and XBY conceived and designed the experiments; WJC, XYW, XRL and XLL analyzed the data. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 23 March 2011 Accepted: 27 July 2011
Published: 27 July 2011

References
1. Lun ZR, Gasser RB, Lai DH, Li AX, Zhou XQ, Yu XB, Fang YY: Clonorchiasis: a key foodborne zoonosis in China. Lancet Infect Dis 2005, 5:31-41.
2. Chai JY, Darwin Murrell K, Lymberry AJ: Fish-borne parasitic zoonoses: status and issues. Int J Parasitol 2005, 35:1239-1254.
3. Srupa B: Pathobiology of opisthorchiosis: an update. Acta Trop 2003, 88:209-220.
4. Shin HR, Oh JK, Masuyer E, Cuadu MP, Bouvard V, Fang YY, Wongnanon S, Srupa B, Hong ST: Epidemiology of cholangiocarcinoma: an update focusing on risk factors. Cancer Sci 2010, 101:579-585.
5. Lee MJ, Chung YB, Lee SK, Chung BS, Li S, Choi MH, Hong ST: The identification of a Clonorchis sinensis gene encoding an antigenic egg protein. Parasitology Res 2005, 95:224-226.
6. Zhou XN, Lv S, Yang GJ, Kristensen TK, Bergquist NR, Utzinger J, Malone JB: Spatial epidemiology in zoonotic parasitic diseases: insights gained at the 1st International Symposium on Geospatial Health in Lijiang, China, 2007. Parasit Vectors 2009, 2:10.
7. Mespleet M, Echaide I, Dominguez M, Mosqueda JJ, Suarez CE, Schnittert L, Florin-Christensen M: Bowipain-2, the falcipain-2 ortholog, is expressed in intraerythrocytic stages of the tick-transmitted hemoparasite Babesia bovis. Parasit Vectors 2010, 3:113.
8. Cruz CE, Fogaca AC, Nakayasu ES, Angeli CB, Belmonte R, Almeda IC, Miranda A, Miranda MT, Tanaka AS, Braz GR, et al: Characterization of proteinases from the midgut of Rhizophalus (Boophilus) microplus involved in the generation of antimicrobial peptides. Parasit Vectors 2010, 3:63.
9. Sojka D, Franta Z, Horn M, Hajdusek O, Caffrey CR, Mares M, Kopacek P: Profiling of proteolytic enzymes in the gut of the tick ixodes ricinus reveals an evolutionarily conserved network of aspartic and cysteine peptidases. Parasit Vectors 2008, 1:7.
10. Sajid M, McKerrow JH: Cysteine proteinases of parasitic organisms. Mol Biochem Parasitol 2002, 120:1-21.
11. Dalton JP, Neill SO, Stack C, Collins P, Walshie A, Sekiya M, Doyle S, Mulcahy G, Hoyle D, Khazadji E, et al: Fasciola hepatica cathepsin L-like proteinases: biology, function, and potential in the development of first generation liver fluke vaccines. Int J Parasitol 2003, 33:1173-1181.
12. Meemon K, Grans R, Vachairat-Grans S, Hoffmann A, Korge G, Vuykant V, Upatham E, Habe S, Sobhon P. Molecular cloning and analysis of stage and tissue-specific expression of cathepsin B encoding genes from Fasciola gigantica. Mol Biochem Parasitol 2004, 136:1-10.
13. Na BK, Kim SH, Lee EG, Kim TS, Bae YA, Kang I, Yu JR, Sohn WM, Cho SY, Kong Y: Critical roles for excretory-secretory cysteine proteinases during tissue invasion of Paragonimus westermani newly excysted metacercariae. Cell Microbiol 2006, 8:1034-1046.
14. Loukas A, Bathony JM, Williamsson AL, Goud GN, Mendez S, Zhan B, Hawdon JM, Elena Bottazzi M, Bridley PJ, Hotz PJ: Vaccination of dogs with a recombinant cysteine proteinase from the intestine of canine hookworms diminishes the fecundity and growth of worms. J Infect Dis 2004, 189:1592-1601.
15. Abdulla MH, Lim KC, Sajid M, McKerrow JH, Caffrey CR: Schistosomiasis mansoni: novel chemotheraphy using a cysteine proteinase inhibitor. PLoS Med 2007, 4:e14.
16. Behrie JK, Buttle DJ, Stepek G, laws D, Duc I: Developing novel anthelmintics from plant cysteine proteinase. Parasit Vectors 2008, 1:29.
17. Kang JG, Bahl YY, Cho PY, Hong SJ, Kim TS, Sohn WM, Na BK: A family of cathepsin F cysteine proteinases of Clonorchis sinensis is the major secreted proteins that are expressed in the intestine of the parasite. Mol Biochem Parasitol 2010, 170:7-16.
18. Ju JW, Joo MH, Lee MR, Cho SH, Cheun HI, Kim JY, Lee YH, Lee KJ, Sohn WM, Kim DM, et al: Identification of a serodiagnostic antigen, legumain, by immunoproteomic analysis of excretory-secretory products of Clonorchis sinensis adult worms. Proteomics 2009, 9:3066-3078.
19. Li Y, Hu X, Liu X, Xu J, Hu F, Ma C, Yu X: Molecular cloning and analysis of stage and tissue-specific expression of cathepsin L-like protease from Clonorchis sinensis. Parasitol Res 2009, 105:447-452.
20. Lee JS, Kim JS, Sohn WM, Lee J, Yong TS: Vaccination with DNA encoding cysteine proteinase confers protective immune response to rats infected with Clonorchis sinensis. Vaccine 2006, 24:2358-2366.
21. Li S, Chung YB, Chung BS, Choi MH, Yu JR, Hong ST: The involvement of the cysteine proteinase of Clonorchis sinensis metacercariae in excystment. Parasitol Res 2004, 93:36-40.
22. Kang TH, Yoon DH, Lee EH, Chung YB, Bae YA, Chung YY, Kang I, Kim J, Cho SY, Kong Y: A cathepsin F of adult Clonorchis sinensis and its phylogenetic conservation in trematodes. Parasitology 2004, 128:195-207.
23. Morpheus RM, Wright HA, LaCourse EJ, Woods DJ, Broyphy PM: Comparative proteomics of excretory-secretory proteins released by the liver fluke Fasciola hepatica in sheep host bile and during in vitro culture ex host. Mol Cell Proteomics 2007, 6:963-972.
24. Nagano I, Pri F, Wu Z, Wu J, Gui H, Boorman T, Takahashi Y: Molecular expression of a cysteine proteinase of Clonorchis sinensis and its application to an enzyme-linked immunosorbent assay for immunodiagnosis of clonorchiasis. Clin Diagn Lab Immunol 2004, 11:411-416.
25. Nak B, Lee HJ, Cho SH, Lee HW, Cho JH, Kho WG, Lee JS, Song KJ, Park PH, Song YK, Kim TS: Expression of cysteine proteinase of Clonorchis sinensis and its use in serodiagnosis of clonorchiasis. J Parasitol 2002, 88:1000-1006.
26. Cornelissen JB, Gassenbeek CP, Borgsteede FH, Holland WG, Harmsen MM, Boersma WJ: Early immunodiagnosis of fasciolosis in ruminants using recombinant Fasciola hepatica cathepsin L-like protease. Int J Parasitol 2001, 31:728-737.
Carnevale S, Rodriguez M, Guarrera EA, Carmona C, Tanos T, Angel SO: Immunodiagnosis of fascioliasis using recombinant procathepsin L cystein proteinase. Diagn Microbiol Infect Dis 2001, 41:43-49.

Sripa J, Brandley PJ, Sripa B, Loukas A, Kaewkes S, Laha T: Evaluation of liver fluke recombinant cathepsin B-1 protease as a serodiagnostic antigen for human oesopohochiases. Parasitol Int 2011.

McKerrow JH, Caffrey C, Kelly B, Loke P, Sajid M: Proteases in parasitic diseases. Annu Rev Pathol 2006, 1:497-536.

Ilic C, Quraishi O, Wang J, Pursima E, Vernet T, Mort JS: Role of the occluding loop in cathepsin B activity. J Biol Chem 1997, 272:1197-1202.

Baq S, Darman RT, Peterson DS: A novel cathepsin B active site motif is shared by helminth bloodfeeders. Exp Parasitol 2002, 101:83-89.

Ranjit N, Zhan B, Stenzel DJ, Mulvenna J, Fujiwara R, Hotez PJ, Loukas A: A family of cathepsin B cysteine proteases expressed in the gut of the human hookworm, Nector americanus. Mol Biochem Parasitol 2008, 160:90-99.

Beckham SA, Law RH, Smooker PM, Quinsey NS, Caffrey CR, McKerrow JH, Pike RN, Spithill TW: Production and processing of a recombinant Fasciola hepatica cathepsin B-like enzyme (FhcatB1) reveals potential processing mechanisms in the parasite. Biol Chem 2006, 387:1053-1061.

McGonigle L, Mousley A, Marks NJ, Brennan GP, Dalton JP, Spithill TW, Day TA, Maul GD: The silencing of cysteine proteases in Fasciola hepatica newly excysted juveniles using RNA interference reduces gut penetration. Int J Parasitol 2008, 38:149-155.

Correnti JM, Brindley PJ, Pearce EJ: Long-term suppression of cathepsin B levels by RNA interference retards schistosome growth. Mol Biochem Parasitol 2005, 143:209-215.

Smooker PM, Jayaraj R, Pike RN, Spithill TW: Cathepsin B proteases of flukes: the key to facilitating parasite control? Trends Parasitol 2010, 26:506-514.

Wang X, Liang C, Chen W, Fan Y, Hu X, Wu Z, Yu X: Experimental model in rats for study on transmission dynamics and evaluation of Clonorchis sinensis infection immunologically, morphologically, and pathologically. Parasitol Res 2009, 106:15-21.

Ma C, Hu X, Hu F, Li Y, Chen X, Zhou Z, Lu F, Xu J, Wu Z, Yu X: Molecular characterization and serodiagnosis analysis of a novel lysophospholipase from Clonorchis sinensis. Parasitol Res 2007, 101:419-425.

Smooker PM, Jayaraj R, Pike RN, Spithill TW: Cathepsin B proteases of flukes: the key to facilitating parasite control? Trends Parasitol 2010, 26:506-514.

Wang X, Liang C, Chen W, Fan Y, Hu X, Wu Z, Yu X: Experimental model in rats for study on transmission dynamics and evaluation of Clonorchis sinensis infection immunologically, morphologically, and pathologically. Parasitol Res 2009, 106:15-21.

Sripa B, Kaewkes S, Sithithaworn P, Maingum E, Lahal T, Smout M, Pairojkul C, Bhudhisawasdi V, Tesana S, Thinkamrop B, et al: Liver fluke induces cholangiocarcinoma. PLoS Med 2007, 4.e201.

Olins MJ, Erlich R: A review and update on cholangiocarcinoma. Oncology 2004, 66:167-179.

Bon B, Houze S, Talabani H, Magne D, Belgadi G, Develoux M, Senghor Y, Chandrier J, Ancelle T, Henequin C: Evaluation of a rapid enzyme-linked immunosorbent assay for diagnosis of strongyloidiasis. J Clin Microbiol 2010, 48:1716-1719.