การวิเคราะห์หายีนของพยาธิ *Opisthorchis viverrini* โดยวิธี Immunoscreening กับซีรั่มผู้ป่วยโรคมะเร็งท่อน้ำดีที่ติดเชื้อพยาธิใบไม้ตับ

Novel Genes of *Opisthorchis viverrini* Identified by Immunoscreening with Cholangiocarcinoma Associated Opisthorchiasis Serum

จิตติยวดี ศรีภา,[1] บรรจบ ศรีภา,[2] ศศิธร แก้วเกษ,[3] เทวราช หล้าหา[4]

Jittiyawadee Sripa,[1] Banchob Sripa,[2] Sasithron Kaewkes,[3] Thewarach Laha[4]

บทคัดย่อ

พยาธิใบไม้ตับ *Opisthorchis viverrini* (OV) เป็นพยาธิที่มีอัตราการระบาดสูงในประชากรของประเทศไทย โดยเฉพาะอย่างยิ่งในภาคตะวันออกเฉียงเหนือ ซึ่งการติดเชื้อพยาธิชนิดนี้มีความสัมพันธ์กับการเกิดมะเร็งท่อน้ำดี โดยมีรายงานวิจัยยืนยันว่าพยาธิใบไม้ตับจัดให้เป็นสารก่อมะเร็งชนิดหนึ่ง และสารที่พยาธิปล่อยออกมามีผลทำให้เกิดการเติบโตที่ผิดปกติของเซลล์ผนังท่อนำดีของผู้ที่ติดเชื้อซึ่งอาจเกี่ยวข้องกับการเกิดมะเร็งท่อน้ำดีได้ การวิเคราะห์คุณลักษณะของยีนของพยาธิชนิดนี้จึงเป็นข้อมูลสำคัญในการค้นหาโมเลกุลที่เกี่ยวข้องหรือก่อให้เกิดพยาธิสภาพที่สำคัญนี้ งานวิจัยนี้ได้ทำการคัดเลือกยีนด้วยวิธี immunoscreening เพื่อตรวจหายีนที่มีความสัมพันธ์กับการติดเชื้อพยาธิชนิดนี้จากห้องสมุดซีดีเอ็นเอของระยะตัวเต็มวัยของพยาธิ OV โดยใช้ซีรั่มของผู้ป่วยโรคมะเร็งท่อน้ำดีที่ติดเชื้อพยาธิใบไม้ตับ จากการทดลองได้คัดเลือกโคลนยีนที่ให้ผลบวกกับซีรั่มของผู้ป่วยจำนวน 6 โคลน ได้แก่ asparaginyl endopeptidases, egg protein, actin, testis enhanced gene transcript และยีนที่ยังไม่ทราบหน้าที่อีกจำนวน 2 ชนิด ซึ่งได้ทำการวิเคราะห์ยีน asparaginyl endopeptidases ของพยาธิ OV มีลำดับ nucleotide 1,302 bp สามารถแปลรหัสเป็นกรดอะมิโนได้ 408 กรดอะมิโน สามารถทำนายน้ำหนักโมเลกุลได้เป็น 47 กิโลดาลตัน สามารถทำนาย cleavage site ได้ระหว่างกรดอะมิโนที่ 17 และ 18 และเมื่อเปรียบเทียบยีนชนิดนี้กับยีนชนิดเดียวกันแต่พบในสิ่งมีชีวิตอื่นพบว่ายีนชนิดนี้มี active sites อยู่ที่ กรดอะมิโนฮิสทิดีน และซิสเตอีน

คำสำคัญ: *Opisthorchis viverrini*, อิมมูโนสกรีนนิ้ง, แอสพาราจีนิล เอ็นโดเปปติเดส

ว วิทย เทคโน มมส 2551;27(4):298-308

Abstract

The liver fluke, *Opisthorchis viverrini* (OV), is an important human pathogen distributed in northeast Thailand. The linkage between the liver fluke and CCA is strongly supported by previous literature. The molecular mechanisms by which OV induces CCA in humans are unknown. Opisthorchis antigens are found in the bile ducts (in regions where parasites reside as well as those in smaller ducts where they do not) as well as damaged liver cells and

[1] *นิสิตปริญญาโท ภาควิชาปรสิตวิทยา, [2] รองศาสตราจารย์, ภาควิชาพยาธิวิทยา, [3] รองศาสตราจารย์, [4] ผู้ช่วยศาสตราจารย์, ภาควิชาปรสิตวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยขอนแก่น อำเภอเมือง จังหวัดขอนแก่น 40002*

[1] *Master degree student, Department of Parasitoloty, [2] Assoc. Prof., Department of Pathology, Assoc. Prof., [4] Assistant Prof., Department of Parasitology Faculty of Medicine, Khon Kaen University, Mueang District, Khon Kaen 40002, Thailand.*

* *Corresponding author: Jittiyawadee Sripa, Department of Parasitology Faculty of Medicine, Khon Kaen University, Mueang District, Khon Kaen 40002, Thailand.* *Received: 30 October 2007; Accepted: 15 March 2008.*

various inflammatory cell infiltrates, further incriminating ES antigens in cholangiocarcinogenesis. Characterization of OV molecules associated with OV infection and cholangiocarcinoma using the immunoscreening method was performed. The adult cDNA library screening was done with cholangiocarcinoma associated opisthorchiasis serum. The results show that 6 protein encoding genes including asparaginyl endopeptidase, egg protein, actin, testis enhanced gene transcript and 2 unknown function genes were positive with strong reaction to human serum. Further investigation on OV asparaginyl endopeptidase show that the full length of the OV asparaginyl endopeptidase gene was 1,302 bp, encoded 408 amino acid residues predicted molecular weight at 47 kDa and predicted cleavage site between amino acid residure 17 and 18.

Keywords: *Opisthorchis viverrini*, immunoscreening, asparaginyl endopeptidases

J Sci Technol MSU 2008;27(4):298-308

Introduction

The liver fluke *Opisthorchis viverrini* (OV) is an important human pathogen distributed in northeast Thailand and Southeast Asia.[1] The infection of this parasite causes opisthorchiasis, which is associated with a number of asymptomatic hepatobiliary abnormalities, including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis.[2] From experimental and epidemiological evidences it is strongly implied that the OV infection is in the etiology of cholangiocarcinoma (CCA).[1,3,4] The molecular mechanisms by which OV induces CCA in humans are unknown. Hamsters are a fully permissive host, and infection results in CCA when accompanied by excessive levels of dietary nitrosamines.[3,5] Opisthorchis antigens are found in the bile ducts as well as damaged liver cells and various inflammatory cells infiltrates further incriminating ES antigens in cholangiocarcinogenesis.[6] This study attempted to isolate and characterize the specific antigen that is associated with OV infection and CCA.

Immunoscreening of adult OV cDNA library was performed with cholangiocarcinoma associated opisthorchiasis serum to target antigenic determinants which react with patient serum. In this study, we showed the result from immunoscreening which obtained six clones that react with patient serum.

Materials and Methods

The adult OV cDNA library. The cDNA library of adult OV was constructed with SMART™ Library Construction Kit (CLONTECH, USA) following the standard protocol of the company. The titer of the library was determined in order to plate the correct number of plaque forming units per plate. Titrated the phage was to determined the plaque forming units per milliliter (pfu/ml).

Serum. Serum from a cholangiocarcinoma associated opisthorchiasis patient was obtained from Srinagarind hospital. Antibody level against ES antigen of OV was measured by ELISA as previous described.[7]

Immunoscreening. We used a picoBlue™ Immunoscreening Kit (Stratagene, USA) for cDNA library screening. Immonoscreening procedures were followed per company instructions. The details of the immunoscreening were described as follows. Briefly, the *Escherichia* coli strain XL1-blue host cells were incubated with the library (1.5x104 pfu) and combined with melted top agar and the mixture was poured onto the agar plates. The culture plates were incubated at 37 °C overnight until appropriated plaque occurred and after that overlaid with 10 mM Isoproply-β-D-thiogalactopyranoside (IPTG) incubated nitrocellulose membranes for 30 min at room temperature. The plates

were incubated at 37 °C overnight after that each membrane was removed and subsequently washed with Tris-Buffered Saline containing Tween 20 (TBST) 4x15 min. The membranes were then blocked for 1 hr at room temperature in blocking solution then incubated the membrane in patient serum (diluted to 1: 400) for 90 min and further washed 4 x 5 min in TBST. The secondary antibody of goat anti-human IgG conjugated alkaline phosphatase was diluted 1:2,000 in blocking solution and incubated with the membrane for 1 hour. After that, membranes were washed 3-5 times for 5 min each with TBST. The membrane was developed using diaminobenzidine (DAB). Fusion protein adsorbed to the membranes that were recognized by the patient serum appeared as brown color spots. Positive plaques were picked corresponding between positive spot on the replica membrane to replica plate and incubated in 500 µl sterile SM buffer and kept at 4 °C.

Preparation of plasmid DNA was by single-clone excision. Plasmid were converted using automatic *in vivo* excision. A single BM25.8 *E. coli* colony was incubated in LB broth at 31 °C overnight. After that, 200 µl of overnight culture were combined with 150 µl of the eluted positive plaque and incubated at 31 °C for 30 min without shaking. Add 400 µl of LB broth and incubate at 31 °C for an additional 1 hr with shaking at 225 rpm. Infected cell suspension was spread on an LB agar/ampicillin plate and incubated at 31 °C overnight. Well-isolated colonies were picked from each clone to LB/ampicillin broth and prepared plasmid DNA separately from each one using plasmid prep (Qiagen, Germany).

Polymerace Chain Reaction (PCR). The size of recombinant plasmid was checked by colony PCR. Briefly, 2 µl of overnight cultured isolated colonies was used as DNA template for PCR. Specific primers, 5'pTriplEx (5'-CTCGGGAAGCGCGCCATTGTGTTGG) and 3'pTriplEx (5'-GCCAATTCGCCCTATAGTGAGTCGTAT) were used. The PCR reactions were 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min for 35 cycles and addition of final extension at 72 °C for 10 min. The PCR samples were resolved by electrophoresis on 0.8% agarose/EtBr gel for checked insert size.

Sequencing and analysis. Nucleotide sequence of recombinant plasmid clones were analyzed using the dye terminator method at BSU, Bangkok. Sequences were edited to remove the vector sequences and poly (A) tails. The edited sequences were translated to protein and analyzed open reading frame that had continuous translation. The sequence was searched for homology with the DNA database using BLAST algorithm at http://www.ncbi.nlm.nih.gov/blast/. Multiple sequences alignment was analyzed using ClustalW in Bioedit program.[8] Prediction analysis of signal peptide from the open reading frames were performed based on the putative identities of the reading frames using SignalP-NN prediction and SignalP-HMM prediction at http://www.cbs.dtu.dk/services/SignalP/.

Phylogenetic analysis. Phylogenetic study of OV legumain was analyzed based on amino acid sequence. OV legumain protein sequence was aligned with other organism using clustalW.[8] Tree was constructed using distance matrix with neighbor-joining method.

Results

The immunoscreening of OV cDNA library can select 6 positive clones, with human serum of opisthorchiasis and cholangiocarcinoma. The cDNA sequences were translated to protein and chosen for correct open reading frame that had continuous translation. From the result of sequence analysis, the genes that isolated from OV library were legumain, egg protein, actin, testis enhanced gene transcript and 2 unknown genes (Table 1).

Table 1 The isolated genes from OV cDNA library by immunoscreening with cholangiocarcinoma associated opisthorchiasis serum.

Names	Type	Length (bp)
1. OVH03	Legumain	1,302
2. OVH04	Egg protein	855
3. OVH06	Actin	1,109
4. OVH08	Testis enhanced gene transcript	~750
5. OVH09	Unknown gene	~700
6. OVH11	Unknown gene	~750

Legumain or asparaginyl endopeptidase gene of OV.

Gene structure analysis showed the OV legumain gene was a full length gene. The OV legumain cDNA clone contained either 5' or 3' ends with overlapping regions. The full-length cDNA was obtained by PCR amplification from OV cDNA library and sequence under the dye terminator method. The nucleotide sequence of the full-length cDNA comprises 1,302 bp, encoding a predicted protein 408 amino acids (Figure 1) with predicted molecular mass of 47 kDa. Analysis of the N-terminus of the deduce amino acid sequence of OV legumain with SignalP-NN prediction and SignalP-HMM prediction can indicated the presence of the signal peptide, predicted to be cleave at cleavage site between residues 17 and 18 (Figure 2). This sequence has been submitted to GenBank with accession number DQ402101. The amino acid sequence of OV legumain was compared to legumain in other organisms. The other legumain amino acid sequences were searched from GenBank for alignment. Multiple alignments of legumain in flat worm, round worm and vertebrate was showed the complete conserve of active sites through these divergent species, including histidine and cysteine that essential for catalysis (Figure 2). The constructed phylogenetic tree was illustrated that OV legumain was closely related to the liver fluke, *Fasciola* spp. and blood fluke, *Schistosoma* spp. respectively (Figure 4). Previous studies show that the legumain can be used for diagnostic tools and vaccine production which is interesting for further study in OV. Finally, the legumain gene is an important gene in the nutrition of parasites by trans-activated other gut-associated proteases to digest host hemoglobin. Functional assay of OV legumain in terms of protease activity is needed to be investigated.

Egg protein or vitelline B precursor encoded gene of OV.

Egg protein is the common antigen found in trematodes such as *C.* sinensis, Paragonimus and Fasciola.[9,10] The full length of egg protein encoded gene of OV is 855 nucleotide encoding 253 amino acids (Figure 4). Recombinant protein of egg protein from Clonorchis has been successfully used as the antigen to develop serodiagnosis method in Clonorchiasis. This molecule needs to be investigated for its potential as an immunodiagnosis tool.

Actin encoded gene of *O. viverrini*

The Actin gene was characterized in many organism and also trematode such as Clonorchis sinensis and Fasciola spp.[11] The full length of the actin encoded gene of OV is 1,020 nucleotide encoding 283 amino acids (Figure 5). The actin protein has been isolated and characterized as the main cytoskeletal in Fasciola.[12]

Discussions

The aim of this study was the isolation of the OV gene from OV cDNA library. Six genes of OV were identified and we choose OV legumain gene for characterized its structure and protein profile.

Legumain or Asparaginyl endopeptidases or propose to call hemoglobinase belongs to the C13 family of cysteine peptidases. Legumain is the member of the clan CD cysteine peptidases. It is a lysosomal cysteine peptidases that hydrolyse peptides and proteins

```
AAACGTGGTTGAGATGCAACGTTCTTGCCTTCTCCTGACGTTCTTGTTGTACGTCAACTA      60
                 M  Q  R  S  C  L  L  L  T  F  L  L  Y  V  N  Y
CGCTGCATGGTTAGGCGCTGTTTGTGTCGGTTCGCGGTTGTTTCACAGTGATCAGGCAAG     120
   A  A  W  L  G  A  V  C  V  G  S  R  L  F  H  S  D  Q  A  R
AAACTGGGTTGTTCTGGTCGCTGGATCCAATGGTTGGGAGAACTACCGACACCAAGCGGA     180
   N  W  V  V  L  V  A  G  S  N  G  W  E  N  Y  R  H  Q  A  D
TGTATATCACGCGTATCAAATCATGAAGCGCAACAACATTTCGACGGAGCAAATAATTAC     240
   V  Y  H  A  Y  Q  I  M  K  R  N  N  I  S  T  E  Q  I  I  T
CTTCGCCTACGATGATATTGCAAACAACCCCGAAAATCCGTTTATGGGCAAGGTGTTCAA     300
   F  A  Y  D  D  I  A  N  N  P  E  N  P  F  M  G  K  V  F  N
TGACTACACTCACAAAGACGTGTACGAAGGTGTGCACATAGATTATCGTGGAGAGGATGT     360
   D  Y  T  H  K  D  V  Y  E  G  V  H  I  D  Y  R  G  E  D  V
GACACCGGACAATTTCCTGCGTGCCATGAGGGGTGATAAAGAACTTGAAGCTAATGGAAA     420
   T  P  D  N  F  L  R  A  M  R  G  D  K  E  L  E  A  N  G  K
GAAGGTACTAAAAAGCGGTCCAGAAGATCACGTCTTTGTCTACTTTTCCGATCATGGTGC     480
   K  V  L  K  S  G  P  E  D  H  V  F  V  Y  F  S  D  H  G  A
AGACGGACTTCTTGCCTTTCCAGAGGATGACCTCCTCGCCTCGGATTTGAACAAAACTTT     540
   D  G  L  L  A  F  P  E  D  D  L  L  A  S  D  L  N  K  T  L
GGGTTACATGCACGAAAACAAAATGTACAAACAAATGGTTCTATACGTGGAAGCATGTGA     600
   G  Y  M  H  E  N  K  M  Y  K  Q  M  V  L  Y  V  E  A  C  E
ATCCGGTTCTATGTTCCAGGATATCCTGCCATCGGATATCGGGATCTATGTGACAACCGC     660
   S  G  S  M  F  Q  D  I  L  P  S  D  I  G  I  Y  V  T  T  A
GGCTAACAGTGAGGAATCCAGCTGGGCTACTTTCTGTCGCGACACAATCATTGGCACTTG     720
   A  N  S  E  E  S  S  W  A  T  F  C  R  D  T  I  I  G  T  C
TCTGGCGGACGAATACTCGTACAACTGGCTCACGGACTCTGAGCATCACGATCTGTCGCA     780
   L  A  D  E  Y  S  Y  N  W  L  T  D  S  E  H  H  D  L  S  H
TCGCACACTGGATGATCAGTTCCAATCGGTGAAACAGAATACCAAGCAAAGTCACGTATC     840
   R  T  L  D  D  Q  F  Q  S  V  K  Q  N  T  K  Q  S  H  V  S
GAGATTCGGGGAACTGCCTCAGGTACTTCATAGCCATCCGTCACGCTGGGCACATTTGGT     900
   R  F  G  E  L  P  Q  V  L  H  S  H  P  S  R  W  A  H  L  V
CACCATGGTCCGACGAATGATGAAAGCCGAAACCGAGGAAGAACATGAATTGGCATCCCG     960
   T  M  V  R  R  M  M  K  A  E  T  E  E  E  H  E  L  A  S  R
AAAACTATATCGTGCACTTCTGCTTGCCCAGATCGTTAAAGAAACATTCGAAGAAATCGT    1020
   K  L  Y  R  A  L  L  L  A  Q  I  V  K  E  T  F  E  E  I  V
CACGGATGTAACAACCTTCCATCAGCCAACCATGCGCATGTTGTCAAAGTCGGAGGAACT    1080
   T  D  V  T  T  F  H  Q  P  T  M  R  M  L  S  K  S  E  E  L
CCAGTGCTATGAAGAAGTATTCCAAGAGTTCAAAAACCGGTGCTTCACCATTCGACAGGT    1140
   Q  C  Y  E  E  V  F  Q  E  F  K  N  R  C  F  T  I  R  Q  V
CCCTGAGGTGGCTCAATACGCAAGACATCTGCGGAAGCTGTGCAAAGAAGGATACGAAAC    1200
   P  E  V  A  Q  Y  A  R  H  L  R  K  L  C  K  E  G  Y  E  T
TGAAGCACTTGTTCAATCTGTTCATGAAGTCTGTTCCTAGTGGACGCTGTCAACATTTGA    1260
   E  A  L  V  Q  S  V  H  E  V  C  S  *
TGGAACAACCTTAATTCGAAAAATGCATAAGTCCTGCAGTGG 1302
```

Figure 1 Nucleotide and deduce amino acids sequence of OV legumain. Upper lines showed the nucleotide sequence. Sequences corresponding to the forward and reverse primers used in PCR are underlined. The predicted cleavage site (↓) utilized to yield the mature enzyme, the active sites histidine and cysteine (in bold) and the termination codon (TAG) was marked with asterisk.

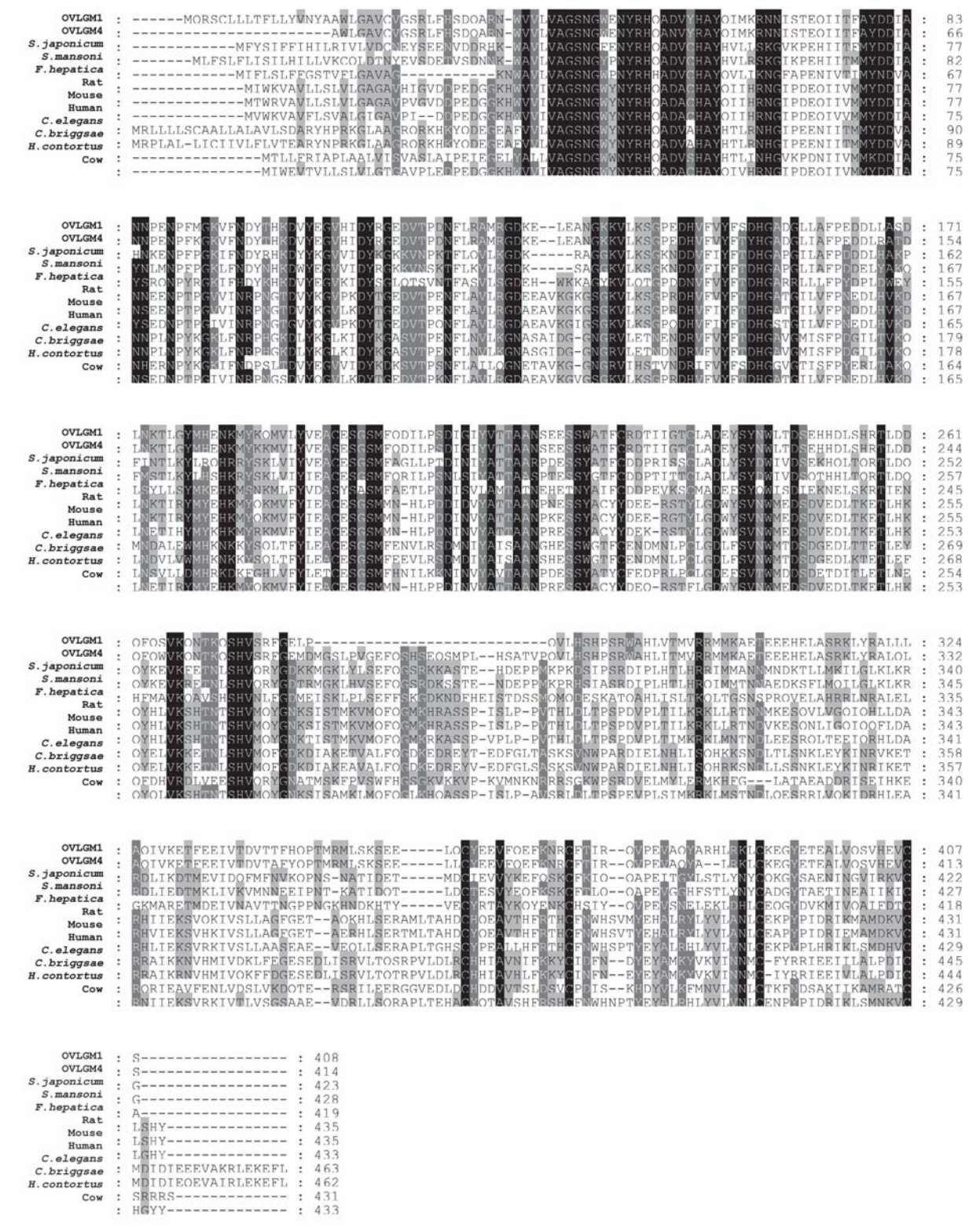

Figure 2 Multiple alignment of amino acids sequences of legumains. The sequence alignment is shown in black and grey. The completely conserved residues are in black, highly conserved residues are in dark–grey and rather highly conserved residues are in light grey. The alphabets, H and C has been used to indicate the conserve active sites histidine and cysteine through these divergent species.

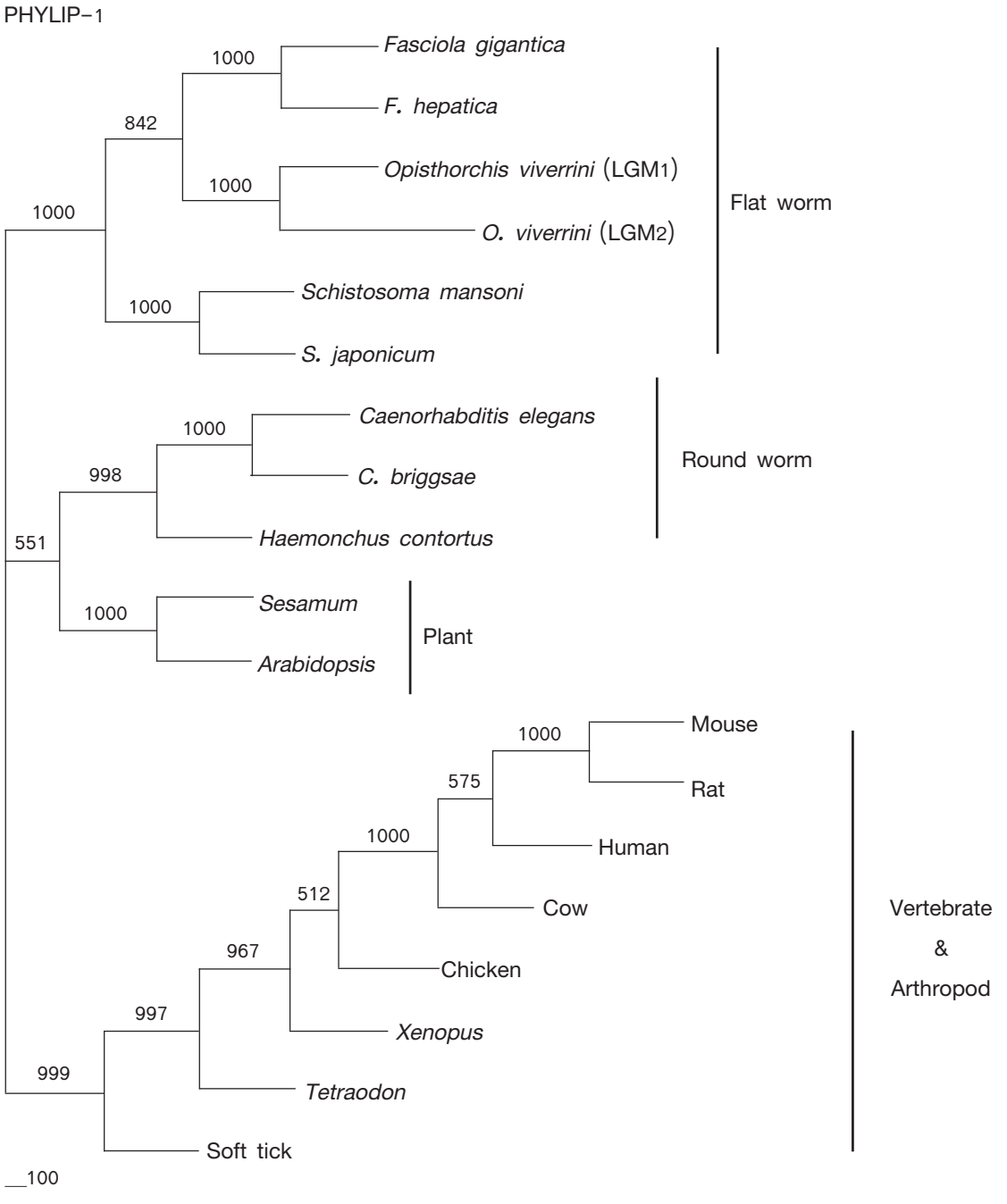

Figure 3 Phylogenetic tree of the evolutionary relationship of OV legumain with others legumain from animal and plant kingdom. The accession numbers of legumain sequences used in the phylogenetic analysis were ABD64147 (*O. viverrini*), ABQ02437 (*Fasciola gigantica*), CAC85636 (*F. hepatica*), P09841 (*Schistosoma mansoni*), P42665 (*S. japonicum*), CAB01126 (*Caenorhabditis elegans*), CAE75506 (*C. briggsae*), CAJ45481 (*Haemonchus contortus*), AAF89679 (Sesamum), AAM60827 (Arabidopsis), CAA04439 (Mouse), AAH87708 (rat), CAG33687 (*human*), AAI11118 (*cow*), XP__421328 (chicken), AAH56842 (Xenopus), CAG13252 (Tetraodon) and AAS94231 (soft tick).

```
1   ATAGTCTCTGCAGTACACAATGAAGCCAATCTGTCTTCTGTTGGTGGGTTTAGTGTCCAT      60
                          M  K  P  I  C  L  L  L  V  G  L  V  S  I
61  CTCACTTACCAGTGGTTACAAACGAGGCTACAAATTTGGCTTGGGAGAAGGAAGACTTGC     120
      S  L  T  S  G  Y  K  R  G  Y  K  F  G  L  G  E  G  R  L  A
121 AACGGGACGCTTTTACAGGGGCGGCTACGGAGATGCCACTGGAAACGAAGTGTCCGGATA    180
      T  G  R  F  Y  R  G  G  Y  G  D  A  T  G  N  E  V  S  G  Y
181 TGACTATGATTTGGAAGGTGAGTTGAGCGGAAGCGGCAGTTCCACCCACGCCGGCCGATT    240
      D  Y  D  L  E  G  E  L  S  G  S  G  S  S  T  H  A  G  R  F
241 TGGAAATCAGAGACACGAGGAGGATGATGGATTCTACACACAAGGCGGGAGTTACTACAT    300
      G  N  Q  R  H  E  E  D  D  G  F  Y  T  Q  G  G  S  Y  Y  M
301 GAGTGGTAAGGCTCGCCGTGACGATGATTATGGCATCACCGCTGGTCTGAAGGCCAAAGG    360
      S  G  K  A  R  R  D  D  D  Y  G  I  T  A  G  L  K  A  K  G
361 AAATTTCTACGGCACTGGTACTGAGGGCGAAGGAAGCCAATATGAGCAAGTGACCACTTT    420
      N  F  Y  G  T  G  T  E  G  E  G  S  Q  Y  E  Q  V  T  T  F
421 CGTCGTGGCGGCGGACACGACACAAAAGGGAAAAAGAAGCACTACAACGAGTACGACAG     480
      R  R  G  G  G  H  D  T  K  G  K  K  K  H  Y  N  E  Y  D  S
481 TTACGGCCAGGCCATGAAATACGGTGACAAGAAGGTTGCCAACAACTTCAACCTTCGCGG    540
      Y  G  Q  A  M  K  Y  G  D  K  K  V  A  N  N  F  N  L  R  G
541 CTCACTGAAAGCCAAGGGCAAGTTTGATGGTTACGGCAAGAGTGACGTGTCCAGTGAATT    600
      S  L  K  A  K  G  K  F  D  G  Y  G  K  S  D  V  S  S  E  F
601 TGACAAATACGGCAAGTATGGATATTCTGGCAGTTCCAAAGGTTATGGAGGTCGTGATGT    660
      D  K  Y  G  K  Y  G  Y  S  G  S  S  K  G  Y  G  G  R  D  V
661 CTACGGAAAACTTAAGGGCAAAAGCGAGTATGACGCCTATGGCAAGCTAAAGGGTTACGG    720
      Y  G  K  L  K  G  K  S  E  Y  D  A  Y  G  K  L  K  G  Y  G
721 TGCACAGAACGACTACTCCAAATACGGTCGTCATGCCGACTACGACACTCTCGGTTATTA    780
      A  Q  N  D  Y  S  K  Y  G  R  H  A  D  Y  D  T  L  G  Y  *
781 GATCACTTATTGTCCGGAGTTGTTGTAACAAAACTAATAGGTAGTTGAAAATTGGAAAAT    840
841 TAAATGTATTATCTC                                                 855
```

Figure 4 Nucleotide and deduced amino acid sequences of the full length of Egg protein encoded gene of OV.

on the carboxyl side of asparagine residues. These enzymes are often referred to 'legumain-like' because this enzyme was first identified and characterized from seed of leguminous plant, *Canavalia ensiformis* or jackbean.[13]

In this present study, legumain is the novel gene isolated from OV and its amino acids sequence revealed high degree homology with blood fluke, *Schistosoma* and liver fluke, *Fasciola* and contains highly conserve of active sites, Histidine and Cysteine of lugumain. Signal peptide analysis reveals that OV legumain is a secreted protein that has a predicted cleavage site between amino acid residues 17 and 18. Legumain is the enzyme that is important for helping worms to survive in their environment. Previous studies showed that the legumain has been definitively

```
1    ACTCACAGTCGCTTTCGTTTCGCGCACAAAACAACCGAATCGGACAGGGATAAAACTGAC   60
61   AAATGGCGGACGAAGAAGTTCAGGCCCTGGTTGTGGACAATGGTTCGGGAATGTGCAAAG  120
       M  A  D  E  E  V  Q  A  L  V  V  D  N  G  S  G  M  C  K  A
121  CTGGTTTCGCTGGTGACGATGCGCCTCGTGCGGTCTTCCCCTCAATTGTTGGACGTCCAC  180
       G  F  A  G  D  D  A  P  R  A  V  F  P  S  I  V  G  R  P  R
181  GTCACCAAGGTGTGATGGTCGGTATGGGACAAAAGGATAGCTACGTTGGTGATGAAGCCC  240
       H  Q  G  V  M  V  G  M  G  Q  K  D  S  Y  V  G  D  E  A  Q
241  AGTCCAAACGAGGTATCCTCACCCTCAAATACCCAATCGAACACGGCATCGTGACCAACT  300
       S  K  R  G  I  L  T  L  K  Y  P  I  E  H  G  I  V  T  N  W
301  GGGATGATATGGAGGAGATCTGGCATCACACTTTCTACAATGAGTTGCGAGTCGCGCCCG  360
       D  D  M  E  E  I  W  H  H  T  F  Y  N  E  L  R  V  A  P  E
361  AAGAGCACCCCGTTCTGCTCACCGAAGCCCCGTTGAATCCGAAAGCCAACCGAGAGAAGA  420
       E  H  P  V  L  L  T  E  A  P  L  N  P  K  A  N  R  E  K  M
421  TGACACAGATCATGTTCGAAACATTCAACTCACCAGCCATGTATGTGGGCATCCAGGCTG  480
       T  Q  I  M  F  E  T  F  N  S  P  A  M  Y  V  G  I  Q  A  V
481  TGCTGTCCCTGTACGCGTCGGGTCGTACGACCGGTATCGTGCTGGACTCTGGTGACGGTG  540
       L  S  L  Y  A  S  G  R  T  T  G  I  V  L  D  S  G  D  G  V
541  TCACACACGCAGTCCCAATCTATGAGGGTTACGCGCTTCCTCACGCCATCCTCCGTCTCG  600
       T  H  A  V  P  I  Y  E  G  Y  A  L  P  H  A  I  L  R  L  D
601  ATCTGGCTGGTCGTGATCTGACTGACTACCTGATGAAAATCCTGACCGAGCGTGGTTACA  660
       L  A  G  R  D  L  T  D  Y  L  M  K  I  L  T  E  R  G  Y  S
661  GTTTCACGACAACGGCCGAGCGAGAAATCGTGCGTGATATCAAGGAGAAGTTGTGCTACG  720
       F  T  T  T  A  E  R  E  I  V  R  D  I  K  E  K  L  C  Y  V
721  TGGCTCTGGACTTCGAGCAGGAGATGGCGACCGCGGCATCGAGCTCCTCACTGGAGAAGA  780
       A  L  D  F  E  Q  E  M  A  T  A  A  S  S  S  S  L  E  K  S
781  GCTACGAACTGCCTGATGGTCAGGTGATCACGATTGGTAACGAGCGGTTCCGTTGTCCCG  840
       Y  E  L  P  D  G  Q  V  I  T  I  G  N  E  R  F  R  C  P  E
841  AGGCTCTGTTCCAACCGAGCTTCTTGGGTATGGAATCTGCCGGTATCCATGAGAGCACGT  900
       A  L  F  Q  P  S  F  L  G  M  E  S  A  G  I  H  E  S  T  F
901  TCACTCGATCATGAAGTGCGATGTGATATCCGCAAGGATTTGTACGCGAACACCGTGTGT  960
       T  R  S  *
961  CTGGTGGTACGACCATGTTCCCTGGTATTGCGGATCGTATGCAGAAGGAGATCACTGCGT  1020
```

Figure 5 Nucleotide and deduced amino acid sequences of the full length of actin encoded gene of OV.

characterized in blood feeding helminthes *S. mansoni* and recently, it has been characterized in *Haemonchus contortus* and *Paragonimus westermani.*[14,15] In *S. mansoni* and *H. contortus*, legumain has been implicated in the activation of cysteine peptidases such as cathepsin B which are thought to help to degrade the bloodmeal in blood-feeding heiminths.[16] In *P. westermani*, legumain would digest the host RBC for nutrition especially amino acids and iron that are important for worm development.[15] Legumain is attractive for chemotherapeutic intervention because it is likely to effect a number of downstream

pathways where peptidases trans-processing and activation is inhibited. Legumain is also well known as an immunodiagnosis tool in schistosomiasis. Antigen Sm32 which is legumain has been used as antigen for immunodiagnosis in schistosomiasis.[17] OV legumain is probably useful for immunodiagnosis of opisthorchisis.[18] For OV legumain, it would be useful to study the role and the function of this enzyme to host. The study of gene structure and function will give important information to understand the cause of harmful pathology in this parasitic disease. So, our further works are producing recombinant protein legumain from OV and investigate the protein activity.

The full length of the egg protein or vitelline precursor proteins and actin encoded gene from OV were isolated and characterized here. In trematodes, vitelline precursor proteins are required for eggshell formation.[19] Recombinant protein of egg protein was used for immunodiagnosis in clonorchiasis.[9] This would be an advantage for the investigation function of egg protein in egg formation and application in immunodiagnosis for opisthorchiasis.

Acknowledgment

The authors would like to thank Miss Nonglack Keawgrai for technical laboratory support. The work was supported by the Thailand Research Fund (TRF) grant number MRG4880045.

References

1. Schistosomes, liver flukes and *Helicobacter pylori.* IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. IARC Monogr Eval Carcinog Risks Hum 1994;61:1-241.
2. Harinasuta T, Riganti M, Bunnag D. *Opisthorchis viverrini* infection: pathogenesis and clinical features. Arzneimittelforschung 1984;34(9B):1167-9.
3. Thamavit W, Bhamarapravati N, Sahaphong S, Vajrasthira S, Angsubhakorn S. Effects of dimethylnitrosamine on induction of cholangio-carcinoma in *Opisthorchis viverrini* infected Syrian golden hamsters. Cancer Res 1978;38(12):4634-9.
4. Vatanasapt V, Sriamporn S, Kamsa-ard S, Suwanrungruang K, Pengsaa P, Charoensiri DJ, et al. Cancer survival in Khon Kaen, Thailand. IARC Sci Publ 1998(145):123-34.
5. Loilome W, Yongvanit P, Wongkham C, Tepsiri N, Sripa B, Sithithaworn P, et al. Altered gene expression in *Opisthorchis viverrini* associated cholangiocarcinoma in hamster model. Mol Carcinog 2006;45(5):279-87.
6. Thuwajit C, Thuwajit P, Uchida K, Daorueang D, Kaewkes S, Wongkham S, et al. Gene expression profiling defined pathways correlated with fibroblast cell proliferation induced by *Opisthorchis viverrini* excretory/secretory product. World J Gastroenterol 2006;12(22):3585-92.
7. Sripa B, Kaewkes S. Relationship between parasite-specific antibody responses and intensity of *Opisthorchis viverrini* infection in hamsters. Parasite Immunol 2000;22(3):139-45.
8. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22(22):4673-80.
9. Lee M, Chung YB, Lee SK, Chung BS, Li S, Choi MH, et al. The identification of a Clonorchis sinensis gene encoding an antigenic egg protein. Parasitol Res 2005;95(3):224-6.
10. Lee JS, Lee J, Kim SH, Yong TS. Molecular cloning and characterization of a major egg antigen in Paragonimus westermani and its use in ELISA for the immunodiagnosis of paragonimiasis. Parasitol Res 2007;100(4):677-81.

11. Fagotti A, Panara F, Di Rosa I, Simoncelli F, Gabbiani G, Pascolini R. Actin expression in some Platyhelminthe species. J Submicrosc Cytol Pathol 1994;26(4):545–51.

12. Stitt AW, Fairweather I, Trudgett AG, Johnston CF, Anderson SM. Localisation of actin in the liver fluke, Fasciola hepatica. Parasitol Res 1992;78(2): 96–102.

13. Sajid M, McKerrow JH. Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 2002;120(1):1–21.

14. Oliver EM, Skuce PJ, McNair CM, Knox DP. Identification and characterization of an asparaginyl proteinase (legumain) from the parasitic nematode, *Haemonchus contortus*. Parasitology 2006;133 (Pt 2):237–44.

15. Choi JH, Lee JH, Yu HS, Jeong HJ, Kim J, Hong YC, et al. Molecular and biochemical characterization of hemoglobinase, a cysteine proteinase, in *Paragonimus westermani.* Korean J Parasitol 2006;44(3):187–96.

16. Caffrey CR, McKerrow JH, Salter JP, Sajid M. Blood 'n' guts: an update on schistosome digestive peptidases. Trends Parasitol 2004;20(5):241–8.

17. Chappell CL, Hackel J, Davis AH. Cloned *Schistosoma mansoni* proteinase (hemoglobinase) as a putative serodiagnostic reagent. J Clin Microbiol 1989;27(1):196–8.

18. Klinkert MQ, Bommert K, Moser D, Felleisen R, Link G, Doumbo O, et al. Immunological analysis of cloned *Schistosoma mansoni* antigens Sm31 and Sm32 with sera of schistosomiasis patients. Trop Med Parasitol 1991;42(4):319–24.

19. Tang Y, Cho PY, Kim BS, Hong SJ. Molecular cloning and characterization of vitelline precursor protein B1 from *Clonorchis sinensis.* J Parasitol 2005;91(6):1374–8.