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Cloning, expression, and characterization of a novel *Opisthorchis viverrini* calcium-binding EF-hand protein

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Abstract

A novel 22.8 kDa of *Opisthorchis viverrini* (*Ov*) calcium-binding EF-hand protein (*Ov* CaBP) was identified and isolated from an immunoscreening of the adult stage *Ov* cDNA library by using a human cholangiocarcinoma (CCA) serum. This protein was related to other calcium binding proteins and conserved among the trematodes. *Ov* CaBP shared 98% amino acid identity to 22.8 kDa of *Clonorchis sinensis* CaBP and both were classified as a new group of CaBP EF-hand protein by multiple sequence alignment and phylogenetic tree analysis. The open reading frame of *Ov* CaBP was 585 bp which encoded for 194 amino acids. The N-terminal part is composed of two calcium-binding EF-hand motifs whereas the C-terminal part contains a dynein light chain motif (DLC). In addition, transcription analysis by RT-PCR revealed that it was constitutively transcribed in all stages, including metacercariae, juvenile, and adult. Furthermore, recombinant *Ov* CaBP protein (r*Ov* CaBP) was expressed as a soluble protein and antibody generated against this r*Ov* CaBP protein was capable of detecting *Ov* CaBP in the *Ov* somatic extracts but not in *Ov* ES products. This anti-r*Ov* CaBP serum was also used to localize *Ov* CaBP in *Ov* infected hamster's liver sections which the distribution of *Ov* CaBP was located in gut epithelium, miracidia in eggs and slightly in parenchyma. Moreover, r*Ov* CaBP protein showed a calcium binding property in non-denaturing gel mobility shift assay.

Keywords

Opisthorchis viverrini; calcium-binding EF-hand; protein expression; gel mobility shift assay; immunolocalization

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1. Introduction

Opisthorchis viverrini (*Ov*), a human carcinogenic liver fluke, is a major health problem especially in the Northeast of Thailand. A Thailand wide survey showed infection in this area declined to 15.7% in 2001 [1], but in the local endemic areas of Khon Kaen province, in Northeast Thailand, the prevalence can still be as high as 70% [2]. *Ov* infection is associated with hepatobiliary diseases including hepatomegaly, cholangitis, fibrosis of the periportal system, cholecystitis, gallstones and are major aetiological agents of bile duct cancer (CCA). Moreover, *Ov* and *C. sinensis* are classified as Group 1 carcinogens—metazoan parasites that are carcinogenic to humans—by the International Agency for Research on Cancer, World Health Organization (WHO) [3]. The highest incidence of the liver cancer in the world has been reported in the liver fluke endemic area of Khon Kaen province, Northeast Thailand [4]. In our laboratory, the adult stage *Ov* cDNA was constructed for molecular study of interesting *Ov* genes [5].

Calcium binding EF-hand protein (CaBP) was first discovered and termed by R.H. Kretsinger in his research on the carp muscle, parvalbumin, a small Ca^{2+}-binding protein which had a helix-loop-helix Ca^{2+}-binding structure [6]. At present, there are more than 3,000 calcium-binding EF-hand related sequences in the NCBI Reference Sequences Data Bank [7]. The CaBP EF-hand proteins can be classified into at least 66 subfamilies [6, 8, 9]. While the functions of EF-hand CaBP protein can be divided into three categories: sensor proteins (calmodulin [10]), buffer proteins (parvalbumin [6]), and Ca^{2+}-stabilized proteins (thermolysin [11]). In trematodes, the CaBP EF-hand containing dynein light chain (DLC) protein motif was parasite specific and was grouped into nematode calcium-binding protein subfamily. Although the function of CaBP EF-hand protein in trematode is still unknown several have shown calcium binding properties in gel mobility shift assays [12, 13, 14]. In this study, *Ov* CaBP was cloned, expressed, and characterized, for transcriptional patterns and protein properties as well as immunolocalization.

2. Materials and Methods

2.1. Parasites and parasite proteins preparation

Ov metacercariae were obtained naturally from infected cyprinoid fish in an endemic area of Khon Kaen province, Thailand while juvenile and adult worms were obtained after infecting hamsters with metacercariae as described [15]. *Ov* excretory/secretory (ES) products were collected from *Ov* culture as previously described [16]. *Ov* somatic extracts of adult stage were prepared as described [17]. All parasite proteins, ES and somatic, were concentrated by Amicon ultra centrifugal filter devices with a cut-off size of 10 kDa (Millipore, MA, USA) and concentration measured by ND-1000 Spectrophotometer (NanoDrop®, Thermo Fisher Scientific Inc., MA, USA).

2.2. Immunoscreening and sequence analysis

A serum from CCA patient R-091 was used for screening the adult stage of *Ov* cDNA library. Positive plaques were selected and cDNA inserts were determined by restriction analysis and sequencing in both directions. Similarity searches were done by NCBI-BLAST. Several proteins were identified including 22.8 kDa *Ov* CaBP. Multiple sequence and amino acid sequence alignments of the homologs of CaBP from other parasites were analyzed by using ClustalX program (Version 1.81). Phylogenetic tree were generated using Phylogeny.fr program for non-specialist [18]. The characteristic of *Ov* CaBP protein was analyzed by using BioEdit 7.0.1 and Expasy (http://au.expasy.org/tools/). Pfam database search was performed to determine the domain families [19].

2.3. Cloning, expression and purification

The *Ov CaBP* was amplified using *Ov* cDNA library as a template, *Ov CaBP* specific primers (sense primer, 5′-CAGCACCATATGAGCTACACC-3′; antisense primer 5′-GAGGGATCCTCAGTTAAATGAATC-3′) that introduced *Nde*I and *Bam*HI restriction sites into sense primer and antisense primer (underlined letters), respectively, and Phusion DNA polymerase (Finnzymes, Thermo Scientific, MA, USA). The PCR profile was initial denaturation at 98°C for 30 sec followed by 35 cycles of 98°C for 10 sec, 55°C for 30 s, 72°C for 20 sec and final termination at 72°C for 5 min. The PCR product was then digested with *Nde*I-*Bam*HI and purified before subcloning into *Nde*I-*Bam*HI site of pET-15b expression vector. The recombinant plasmid was confirmed by restriction analysis and sequencing. After transforming the recombinant plasmid into *Escherichia coli* BL21 (DE3), the *Ov* CaBP protein was expressed as the N-terminus 6×His-tagged when induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). A Ni-NTA column (Ni sepharose™ 6 Fast Flow, GE Healthcare, UK) was used to purify the fusion *Ov* CaBP protein under native conditions. Eluted fractions of purified recombinant *Ov* CaBP protein (r*Ov* CaBP) was analyzed by 12.5% SDS-PAGE.

2.4. Transcription analysis of each stage of Ov CaBP

Total *Ov* RNA from each stage was extracted using TRIzol reagent (Invitrogen Corporation, CA, USA) and reverse transcribed to cDNA using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Thermo Scientific, MA, USA). The cDNA of each stage served as template for PCR amplification of *Ov CaBP* gene with specific primers as described in section 2.3 and β-actin gene with β-actin primers (sense primer, 5′-AGCCAACCGAGAGAAGATGA-3′; antisense primer, 5′-ACCTGACCATCAGGCAGTTC-3′) as an internal control using the same profile of initial denaturation at 95°C for 2 min followed by 40 cycles of 95°C for 1 min, 55°C for 30 s, 72°C for 1 min and final termination at 72°C for 4 min. PCR were performed using Go*Taq*®colorless DNA polymerase (Promega Corporation, WI, USA). PCR products were determined by 1% agarose gel electrophoresis.

2.5. Anti-sera preparation

ICR mice were immunized subcutaneously with 100 μg of purified r*Ov* CaBP emulsified with Freud's complete adjuvant (GIBCO BRL, Gel Company, CA, USA) in the first shot. After two weeks, mice were boosted three times at one week intervals with 50 μg of purified r*Ov* CaBP proteins emulsified with Freud's incomplete adjuvant (GIBCO BRL, Gel Company, CA, USA). Antisera were obtained one week after the last shot.

2.6. Western blotting

Western blot analysis was performed to determine r*Ov* CaBP protein from the purified fraction and to analyze the presence of *Ov* CaBP protein in crude *Ov* somatic extracts as well as in *Ov* ES products. Proteins were separated on 12.5% SDS-PAGE as described previously [20] and transferred onto nitrocellulose membrane (Amersham™ Hybond™-ECL, GE Healthcare, UK) by semi-dry blotting (Trans-Blot®, Bio-Rad Laboratories, CA, USA). Membrane was blocked with 5% skim milk in Phosphate buffered saline with 0.1% Tween 20 (PBS-T) at room temperature (RT) for 1 hr. and washed three times with PBS-T before incubation at RT for 1 hr with primary antibody diluted in 2% skim milk in PBS-T (1:1,000 for anti-6×His-tag and 1:5,000 for anti-r*Ov* CaBP). Then membrane was washed three times with PBS-T and incubated at RT for 1 hr. with secondary antibody, anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Zymed Laboratories Inc., CA, USA), diluted in PBS (1:1,000). After washing with PBS-T two times, membrane was rinsed with

PBS and detected by using diaminobenzidine (DAB) reagent (Sigma-Aldrich Co., MO, USA).

2.7. Gel mobility shift assay

Eluted fraction of purified r*Ov* CaBP protein had the buffer exchanged to PBS while concentrating by using amicon ultra centrifugal filter devices. Dithiothreitol (DTT) was added to a final concentration of 10 mM. Calcium binding activity was analyzed by loading of r*Ov* CaBP protein on 12.5% PAGE (without SDS) in the presence of 20 mM ethylenediaminetetraacetic acid (EDTA) and (a) 1 mM $MgCl_2$ (b) 1 mM $CaCl_2$ or without $MgCl_2$ and $CaCl_2$. Bovine serum albumin (BSA) was used as control.

2.8. Immunolocalization of Ov CaBP

The paraffin-embedded sections (4 μm) of an *Ov* infected hamster's liver was obtained as previously described [21]. The native *Ov* CaBP was localized by immunohistochemistry as described previously [16]. In brief, tissue sections were deparaffined, rehydrated, and retrieved antigen before destroying the endogenous peroxidase. After blocking non-specific binding with normal serum, tissue sections were incubated overnight at 4°C with mouse preimmune serum (negative control) and anti-r*Ov* CaBP serum (1:2,000). The sections were then washed with PBS and subsequently incubated at RT for 1 hr with HRP-conjugated anti-mouse IgG (1:300). After washing with PBS, the sections were detected using DAB reagent, counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene, and mounted in Permount®. Mounted sections were examined under light microscope and photographed.

3. Results

3.1. Immunoscreening and characterization of the Ov CaBP sequence

Ov CaBP cDNA was isolated in an immunoscreen of adult stage *Ov* cDNA library with human CCA serum. The result from NCBI-BLAST (tblastn) showed that *Ov* CaBP had 98%/99% amino acid identity/similarity to CaBP of *C. sinensis* 22.8 kDa (GenBank accession no. EU290671). Furthermore, BLAST searches within expressed sequence tags (EST) database of *Ov* adult stage showed that both *Ov* and *Cs* CaBPs were 100% and 99% compared to four sequences (OvAE3718, OvAE3719, OvAE3720, and OvAE1788) at the amino acid level, respectively. On the other hand, the CaBPs of the *Fasciola* group; *F. hepatica* 22.2 kDa (AJ003821), *F. gigantica* 22.1 kDa (DQ115800), and *F. hepatica* 22 kDa (AJ003822), shared more than 45%/69% amino acid identity/similarity to *Ov* CaBP. However, when these *Fasciola* CaBPs sequences were blasted within the *Ov* EST databases, different sequences with 97% amino acid similarity were hit from those described above. While the *Schistosoma* CaBPs, *S. mansoni* 22.6 kDa (DQ059818), *S. japonicum* 22.6 kDa (AB006459), *S. bovis* 22.6 kDa (EU595756), *S. haematobium* 22.6 kDa (AY851615), *S. japonicum* 21.7 kDa (AF048759), and *S. mansoni* 21.7 kDa (U30663) had more than 41%/57% amino acid identity/similarity to *Ov* CaBP, no sequence was hit within the *Ov* EST database. Whereas the other size of CaBPs from *Clonorchis sinensis (Cs)*: 20.8 kDa (DQ317450) and 22.3 kDa (EF077216) had less than 32%/50% amino acid identity/similarity to the *Ov* CaBP protein. The phylogenetic classification showed that the *Ov* and *Cs* CaBP 22.8 kDa proteins were considered as a new group of CaBP protein (Fig. 1). The *Ov* CaBP cDNA was 585 bp and encoded for a putative protein of 194 amino acids. Its molecular weight and theoretical isoelectric point predicted from ProtParam program were 22.82 kDa and 5.11, respectively. The *Ov* CaBP protein was predicted not to contain a signal peptide and transmembrane helix via SignalP 3.0 and DAS program, respectively. Besides, the amino acid sequence of 22.8 kDa *Ov* CaBP was not found when searching within the secreted (ES products) and surface (tegument) proteomic database of *Ov* adult stage (personal communication with Dr. Jason Mulvenna) [22]. In addition, the TargetP

program predicted that this protein is present in cytoplasm (http://www.cbs.dtu.dk/services/Target). Pfam database search showed that the N-terminus domain of *Ov* CaBP protein contained two putative EF-hand calcium-binding motifs (Pfam accession no. PF00036, residues 14–72) whereas the C-terminus domain was similar to dynein light chain type 1 (Pfam accession no. PF01221, residues 97–186) (Fig. 2).

3.2. Transcription analysis of each stage of Ov CaBP

RNA of each stage (metacercariae, 2-week, 3-week, 1-month-old juvenile, 2-month-old adult worm) was extracted and reversed transcribed to cDNA for transcriptional analysis. The RT-PCR product of *Ov CaBP* gene in all stages showed a single band of 585 bp (Fig. 3a). The PCR product of β-actin gene (internal control) showed a single band of 430 bp in all stages and in equal quantity (Fig.3b). Consequently, the transcripts of *Ov CaBP* were present in all intra-mammalian developmental stages.

3.3. Expression, purification, and western blotting analysis of rOv CaBP

The recombinant plasmid pET15b-*Ov* CaBP was expressed in *E. Coli* BL21 (DE3) as a soluble protein with 6×His-tag at the N-terminus under IPTG induction at 25°C. When expressed at 37°C the majority of expressed r*Ov* CaBP protein was found in the inclusion body. A Ni-NTA column was used to purify the fusion r*Ov* CaBP protein under non-denaturing conditions (Fig. 4a). The multimeric forms of r*Ov* CaBP in the eluted fraction of purified protein were observed and confirmed by western blotting with anti-His-tag antibody (Fig. 4b). The purified 6×His-tagged-r*Ov* CaBP protein was used to immunize mice and antibody raised against this fusion r*Ov* CaBP protein was able to detect 22.8 kDa of *Ov* CaBP in *Ov* somatic extracts (Fig. 4c) but not in *Ov* ES products (data not shown).

3.4. Analysis of rOv CaBP calcium-binding activity

Initially the mobility shift of r*Ov* CaBP protein was not observable. But when EDTA was added to the protein sample at various concentrations (1 mM – 20 mM) before performing 12.5% PAGE, a retardation effect occurred without adding $CaCl_2$ (Fig. 5a). That suggested that Ca^{2+} ion was already present and saturating the system, probably via the *E. coli* culture medium. So, without a divalent cation chelating agent such as EDTA we could not observe the mobility shift of r*Ov* CaBP. However, the retardation effect was clearly seen when adding 20 mM EDTA (final concentration) to the protein sample and in the presence of 1 mM $CaCl_2$ while BSA protein used as a control did not undergo a mobility shift (Fig. 5b). Moreover the retardation effect of r*Ov* CaBP protein was not observed in the presence of 1 mM $MgCl_2$ (Fig. 5b).

3.5. Immunolocalization of Ov CaBP

Mouse polyclonal anti-r*Ov* CaBP serum was used to localize the native *Ov* CaBP in paraffin-embedded *Ov* infected hamster's liver sections which revealed that *Ov* CaBP was located in gut epithelium, miracidia in eggs, and slightly in parenchyma but not in epithelium grand, mature sperm, and tegument while the preimmune serum showed negative staining to all tissues (Fig. 6).

4. Discussion

In this study, a novel 22.8 kDa of *Ov* CaBP EF-hand protein was identified from *Ov* cDNA library with a CCA serum. Moreover, the *Ov* CaBP protein had 98% amino acid identity to 22.8 kDa of *Cs* CaBP protein which is found up-regulated when exposing *Cs* metacercariae to gamma irradiation [23]. However, the 22.8 kDa of *Cs CaBP* sequence reported in GenBank is a partial mRNA sequence. In contrast, the 22.8 kDa *Ov* CaBP sequence

obtained from the *Ov* cDNA library was a complete mRNA sequence. From the identity alignment, we concluded that 22.8 kDa of *Ov* CaBP and *Cs* CaBP were orthologs while the different MW size of CaBPs from *Clonorchis, Fascisola*, and *Schistosoma,* arose from distinct genes which had an amino acid identity less than 46%. In addition, the result from phylogenetic tree analysis classified these two CaBPs 22.8 kDa of *Ov* and *Cs* as a new group of CaBP proteins. However, more than one size of CaBP EF-hand proteins is found in any given species [12, 24–28]. In the case of *Ov* CaBP protein, the homology search within the *Ov* EST database by NCBI-BLAST using the other size of CaBP sequences from the other parasites (*Cs* 20.8; DQ317450, *Fh* 22.2; AJ003821, and *Cs* 22.3; EF077216) showed that there were at least four sequences of *Ov* CaBP with more than 88% amino acid identity (20.8, 22.2, 22.3, and 22.8 kDa). On the other hand, the *Ov* CaBP of 21.7, 22.6 and 31.8 kDa were not found in the *Ov* EST database when using the sequences from *Sm* 21.7 (U30663), *Sj* 22.6 (AB006459), and *Cs* 31.8 (EF077217) searching.

The 6×His-tagged-r*Ov* CaBP protein was expressed as a soluble protein at 25°C and able to be purified by Ni-NTA column. However, we found the r*Ov* CaBP protein preferred to homodimerize as multimeric forms as observed in SDS-PAGE. This property was also observed in the CaBP proteins from other parasites [12, 13, 14]. The homodimerization of CaBP proteins occurred due to the presence of Cys residues in the CaBPs which form disulfide bonds between inter/intramolecule of proteins. In the case of *Ov* CaBP protein, there were two Cys residues located on the N-terminus and three residues located on the C-terminus. Nevertheless, only a monomeric form of r*Ov* CaBP protein was observed when 10 mM DTT was added to the protein sample before performing SDS-PAGE (data not shown) as previously described for *Fg* CaBP protein [13].

Some of the parasite CaBP EF-hand proteins showed a Ca^{2+} binding property [12, 13, 14, 29]. Binding of calcium to CaBP protein occurs in the loop region between helix-loop-helix motifs of EF-hand structure via an ionic interaction. Consequently, amino acid residues in the loop region at positions 1, 3, 5, and 12 were found to be a conserved negative charge that will bind to the positive charge of Ca^{2+} [30, 31]. Our *Ov* CaBP protein is composed of two EF-hand motifs (EF-hand 1 and EF-hand 2) at the N-terminus and both EF-hand motifs showed the conserved negative charged amino acids at positions 1, 3, 5, and 12 which were Asp, Asn, Asp, and Asp, respectively. A loop residue at position 6 provided loop bending and had a highly conserved Gly residue that was found in EF-hand 2 but not EF-hand 1 of *Ov* CaBP. Whereas a loop residue at position 8 showed a conserved hydrophobic amino acid that provided the β–sheet structure between the pair of EF-hands with Val and Ile residues found in EF-hand 1 and 2 of *Ov* CaBP, respectively [30]. Moreover, r*Ov* CaBP showed the Ca^{2+} binding property in non-denaturing gel mobility shift assay in the presence of $CaCl_2$ as well as 8 kDa *Sj* CaBP, 8 kDa *Fh* CaBP, 22 kDa *Fh* CaBP, and 22.1 kDa *Fg* CaBP [12, 13, 14, 29] whereas *S. mansoni* 20.8 and 21.7 kDa calcium-binding proteins failed in Ca^{2+}-binding assays [25, 27]. Unlike *Fg* CaBP, the r*Ov* CaBP did not bind to Mg^{2+} ion, such that a retardation effect was not observed on PAGE in the presence of $MgCl_2$ [13].

Based on recent literature, some of CaBP EF-hand containing DLC motifs in trematodes were found to be located in the syncytial tegument membrane and/or tegument cell bodies [13, 24, 26–28, 32]. The other two small sizes of 8 kDa *Sj* and *Fh* CaBPs, however, were located on the surface of the parasites during cercarial stage [14, 33]. On the contrary, native *Ov* CaBP could be localized by the anti-r*Ov* CaBP serum in gut epithelium, miracidia in eggs, and slightly in parenchyma (Fig. 6). According to this finding, the 22.8 kDa *Ov* CaBP might have a different function from the other sizes of trematode CaBPs. Although some of trematode CaBPs including *Ov* CaBP had more than 30% amino acid similarities in calcium-binding EF-hand motifs to calmodulins of other species [12, 14], the function of CaBP EF-hand proteins in trematodes is still unknown. Furthermore, the 8 kDa *Fh* CaBP

which shared 27%/48% amino acid identity/similarity to *Ov* CaBP calcium-binding motif has been categorized into a sensor protein like calmodulin [14]. Nevertheless, trematode CaBPs had one pair EF-hand less than calmodulin and they were composed of two motifs, Ca^{2+} binding at N-terminus and DLC at C-terminus. These motifs were parasite specific and highly conserved among trematode CaBPs. However, the small size 8 kDa trematode CaBP contained only one Ca^{2+} binding motif. Both calmodulin and trematode CaBPs were multifunctional proteins [34, 35]. The speculated function of trematode CaBPs was based on their sequences and localization. In this study, *Ov* CaBP could be predicted to be a sensor protein, in which its binding to Ca^{2+} induced the conformational change in order to bind to its target protein. Furthermore, *Ov* CaBP was an abundant somatic antigen, highly conserved and diverged in parasite specific CaBP subfamily that could be found in every development stage as a soluble protein and was located mainly in gut epithelium as well as miracidia in eggs. Therefore, *Ov* CaBP might play roles in nutrient uptake and/or miracidia transformation. Basic knowledge on the *Ov* CaBP will increase understanding the structure-function relationship as well as to develop novel anti-parasitic drugs, vaccines, and/or to be a potential protein for immunodiagnostic detection of the parasitic disease in the future.

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Research highlights

A novel 22.8 kDa *Ov* CaBP that reacted with cholangiocarcinoma serum was identified > It was transcribed in all stages and had functional calcium-binding property > *Ov* CaBP was categorized into a sensor protein like calmodulin based on sequences > This somatic protein was localized mainly in gut epithelium and miracidia in eggs > It might play role in nutrient uptake and/or miracidia transformation

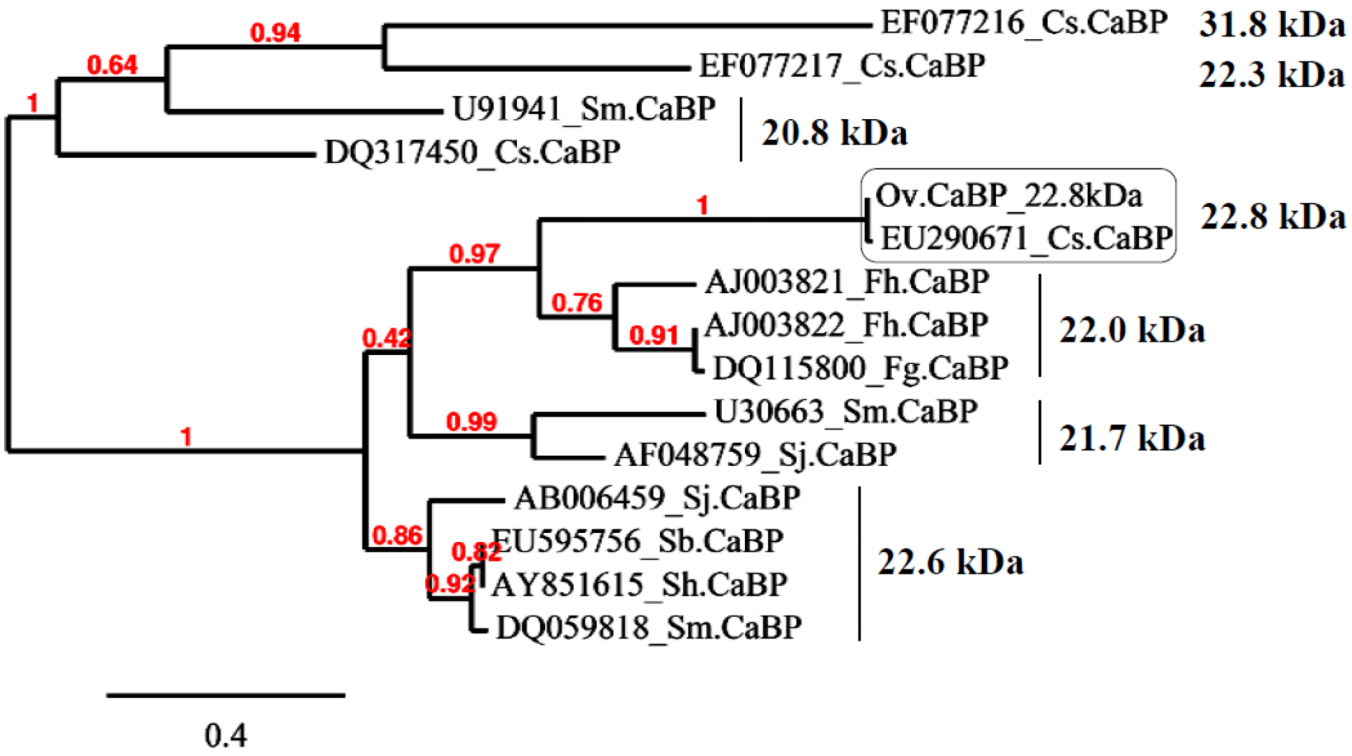

Fig. 1.

The phylogenetic tree of *Ov* CaBP with homologs from other parasites. GenBank accession number is given followed by abbreviation of species name and the right hand side indicates the molecular weight of CaBPs.

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                                        * * *  I   *                          * * *  I   *
Ov.CaBP 22.8    : ---MSYTPSQLERLILKFLELDTNRDEVVDRRELKHAWLNDGITEDEVSHWLDKYDLNGDGNITLDEFCHALGLK :  72
EU290671-Cs22.8: ---.................................Y..................................... :  72
AJ003821-Fh22.2: --MAATKLHD..S..EW.........A..A...TGY.RK...P.EK.KN..EQ..T.K..K...M...RG.... :  73
DQ115800-Fg22.1: -MTTA.KLHD..S..DW.....K.N..A......TAY.KSK...DSK.NE..EH....S..K...M...RG.... :  74
AJ003822-Fh22.0: -MTTA.KLHD..S..DW.....K.N..A......TAY.KSK...DSK.NE..EH..A.S..K...M...RG.... :  74
AB006459-Sj22.6: MATTE.RL.L..QF.RA.....K.N......Q..TKYCQQ.Q.DMK..DP..A...T.K..K...E...RGF... :  75
EU595756-Sb22.6: -MSTETKL.S..EF.RA.....A.N......Q..IKYCQK.R.DMRL.DP..A...T.K.NK...E...RGF... :  74
AY851615-Sh22.6: -MSTETRL.S..EF.RA.....A.N......Q..IKYCQK.R.DMRL.DP..A...T.K.NK...E...RGF... :  74
DQ059818-Sm22.6: -MATETKL....EF.RA.....A.S......Q..IKYCQKYR.DMKL.DP..A...T.K.NK...E...RGF... :  74
AF048759-Sj21.7: ------MA.T..QF.TA..T.YR.G.........IDYCRKEK.DMKM.DT..SH....K.NR...E...RG.... :  69
U30663-Sm21.7   : ------MD.P...F.QT..T.LR.G..T.ETSK.SESCRKEK.DMK..NE..AL....K.QK..FE...RG.... :  69
U91941-Sm20.8   : ----------..PF.QV.FA..R.GT.T.TVE...KYVAE.K.DDMM..K.KSL..PK.T.R..FKT..DV...S :  65
DQ317450-Cs20.8: ----------.DSF.NI..S..K.GTN..SYP..EQYVAE.N.DPSM.EK.KQL..P.NT.S...ET..SK.... :  65
EF077216-Cs22.3: ---.CALS...DAF.EA.FA..A.NR...SL.D..SYSQE.N.E.GFPET..RV..T.NT.Q.......KT...I :  72


Ov.CaBP 22.8    : CEEMRIERYERQRERDGFAKVLNPDVSIIASTMSLDKQVDITNKFVELL----KETSGRPEDLNEVAKNLKDYLD : 143
EU290671-Cs22.8: ..............E.....................................----..................... : 143
AJ003821-Fh22.2: VD.....QK...V..Q.K.PT.ST..E........P...E..E..KS..----TAHN..D.E.KD..Q....... : 144
DQ115800-Fg22.1: ID.....QK..AL..A.T.PA.GT..E.......QP...E..E..K...----TAHNATD.E.KD..HE..T... : 145
AJ003822-Fh22.0: ID.....QK.PPL..A.T.PA.GT..E.......QP...E..E..KS..----TAHN..D.E.KD..HE..T... : 145
AB006459-Sj22.6: VW...R..E.LK.D.E.KVST.PL..Q...A...KA..Y..CC..K...----DK..RTGDE.RA..N...A... : 146
EU595756-Sb22.6: VS...R..E.LK.....KVSK.P...E...A...KT..Y..CHQ.K.Y.----DN..RTNN..K...NK..TL.. : 145
AY851615-Sh22.6: VS...R..E.LK.....KVSK.P...E...A...KT..Y..CYQ.K.Y.----DN..RTNK..K...NK..TL.. : 145
DQ059818-Sm22.6: VS...R..D.LK.....KFPK.P...E...A...KT..YE.CCQ.K.Y.----DN..RTGN..R...NK..SL.. : 145
AF048759-Sj21.7: AN......NHIKAVQS.RVPK.PDG.E......P.P...E..QL.KD..----PK.G.HEA..RK..N.F.IK.E : 140
U30663-Sm21.7   : QN......NHIKTVQS.REQS.PEG........PKP...E..QL.KD.Y----N.VK-.DP...K.V.TF.SE.E : 139
U91941-Sm20.8   : PA.AVAM.T.H---QQASSLK.H...V..YEQ.P.....A....A....----TS.K-.FDEK..AVQ-..Q... : 131
DQ317450-Cs20.8: PA..IDF.E..G--LHAAPPS.P.E.I..SAN...ED..K.ARET.P.A----PGAQ-TS.E.GR.TE...S.A. : 133
EF077216-Cs22.3: PK.A.EF.RR.TL.LIEALT--PS..E..HDD.DQEIK.K.LQM..DD.REAG.A.NVDA.L...S.QK..Q..E : 145


Ov.CaBP 22.8    : KQYGRVWQTVLVAGSYWMKFSHEPFMSLQFKCGPHICLVWRTPCIERDSFN : 194
EU290671-Cs22.8: ..................................................... : 194
AJ003821-Fh22.2: EK......C...T.....H............Y.RY...A....RG------ : 189
DQ115800-Fg22.1: DT......C...T.....Q............Y.R....A....RG------ : 190
AJ003822-Fh22.0: DT......C...T.....Q............Y.RY...A....RG------ : 190
AB006459-Sj22.6: S.......V...T.....N............YSNY.........SS------ : 191
EU595756-Sb22.6: NT......V...T.....N............YNNY...A....SQ------ : 190
AY851615-Sh22.6: NT......V...T.....N............YNNY...A....SQ------ : 190
DQ059818-Sm22.6: NT......V...T.....N............YNNY...A....SQ------ : 190
AF048759-Sj21.7: ER.E....V...T.....N.....LQ.....VDKN.I.A....NN------ : 185
U30663-Sm21.7   : .R......VNA.TH...AS......Q....QYDNK.I.A....SN------ : 184
U91941-Sm20.8   : IT...A.HI...K....SS...SANKCFI..VRDVSY......DE.IT.A- : 181
DQ317450-Cs20.8: .T..GC..V...D.....TQ.FV.N..F..ELYNRAY.F.Q.SED.VALAQ : 184
EF077216-Cs22.3: QR...T.HI..SINQQLAW..YC.GYMFH.CL.RFAV......W.------ : 190
```

Fig. 2.

Multiple sequence alignment between *Ov* CaBP and the homologs from *Clonorchis, Fasciola*, and *Schistosoma*. GenBank accession numbers are given followed by abbreviation of species name and molecular weight of CaBPs. The underlined sequences indicate the loop region between helix-loop-helix motif of EF-hand 1 and 2. The (*) represents the amino acid residues interacting with calcium while (I) represents the conserved hydrophobic residue within loop whereas the underlined italic letters indicated the DLC part of CaBP.

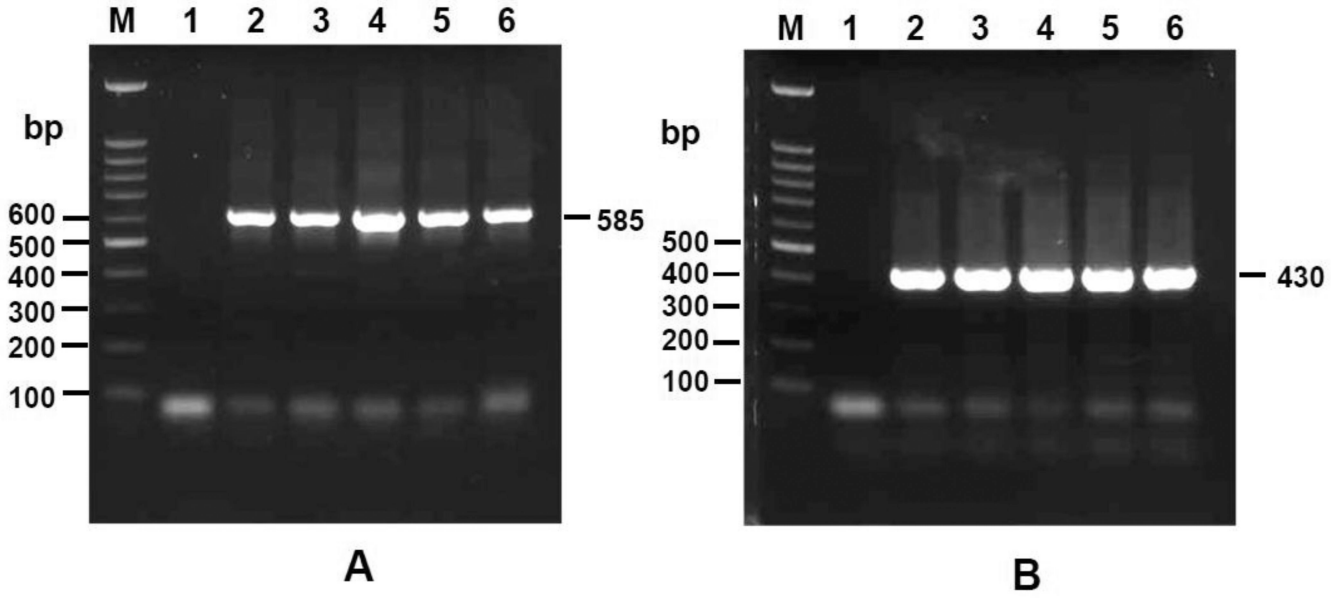

Fig. 3.

Transcriptional analysis of stage specific expression of *Ov CaBP* gene by RT-PCR. Lane M, 1, 2, 3, 4, 5, and 6 show a molecular weight marker, negative control (without template), metacercariae, 2-week-old juvenile, 3-week-old juvenile, 1-month-old juvenile, and 2-month-old adult worm, respectively. Panel **A** shows the RT-PCR amplification of *Ov CaBP* gene in each stage whereas panel **B** shows the amplification of actin gene in each stage as an internal control. Electrophoresis was performed on 1% agarose gel.

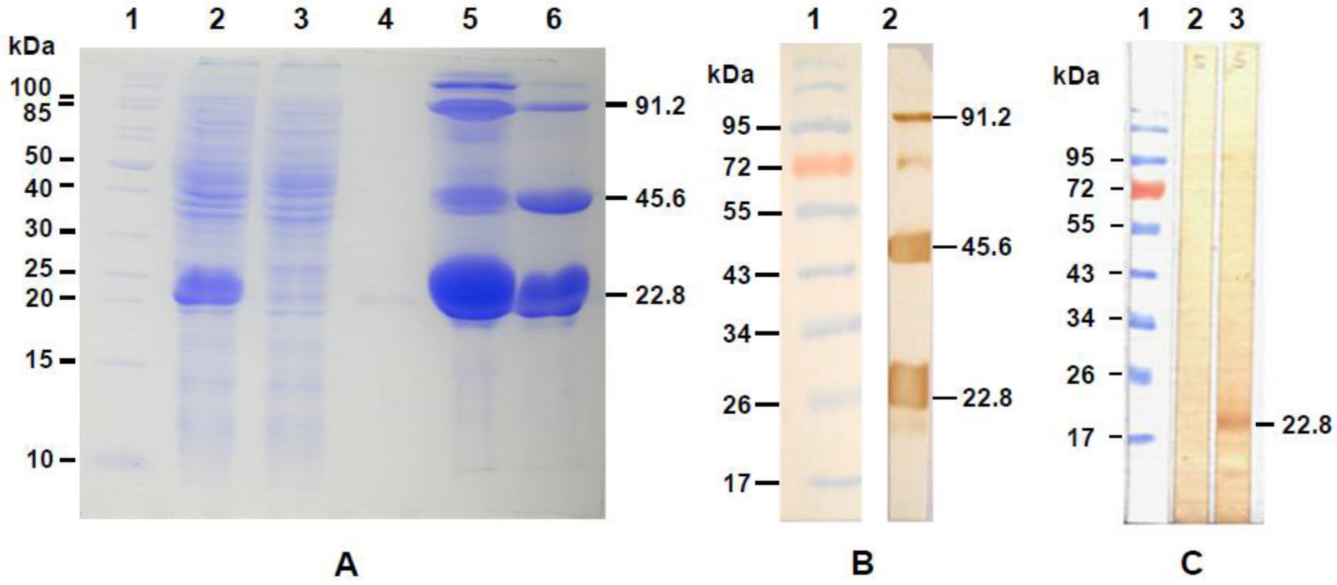

Fig. 4.

SDS-PAGE of r*Ov* CaBP and western blot analysis of the purified r*Ov* CaBP protein and native *Ov* CaBP in *Ov* somatic extracts. In panel **A**, r*Ov* CaBP protein was purified under the native condition and loaded on 12.5% SDS-PAGE. Lane 1: molecular weight marker, Lane 2: *E. coli* lysate, Lane 3: flow through, Lane 4–6: eluted fraction 1, 2, and 3 of r*Ov* CaBP protein, respectively. The multimeric forms of r*Ov* CaBP protein were observed and the sizes are shown by arrows. In panel **B**, western blot was performed to verify the multimer of r*Ov* CaBP protein in the eluted fraction 3 using anti-His tag antibody. Lane 1: molecular weight marker, Lane 2: eluted fraction 3 of purified r*Ov* CaBP protein. In panel **C**, western blot analysis of native *Ov* CaBP protein in *Ov* somatic extracts probed with sera from mice. Lane 1: molecular weight marker, Lane 2: *Ov* somatic extracts reacted with mouse preimmune serum, Lane 3: *Ov* somatic extracts reacted with serum from r*Ov* CaBP protein immunized mouse.

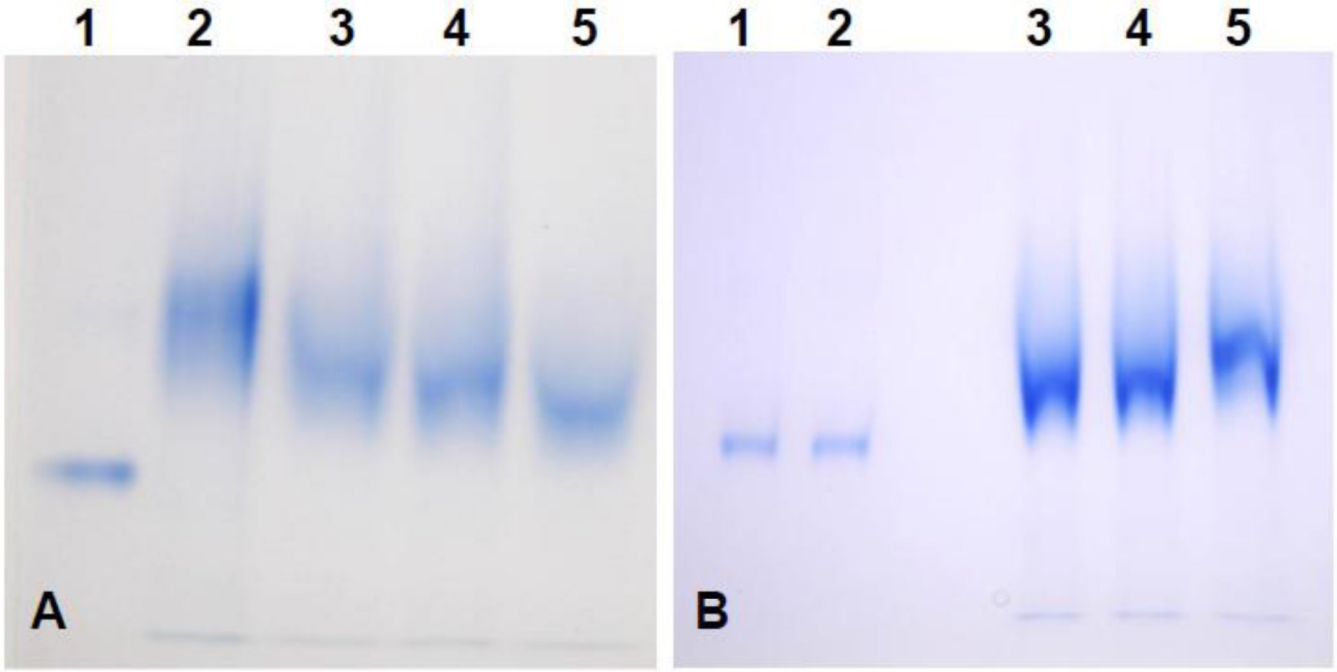

Fig. 5.

Analysis of r*Ov* CaBP calcium-binding property by gel mobility shift assay (12.5% PAGE). In panel **A**, a retardation effect was observed before adding $CaCl_2$ to the protein sample due to the contamination of Ca^{2+} ion in system, consequently, various concentration of EDTA was added to protein sample for chelating out Ca^{2+}. Lane 1: BSA served as control, Lane 2: r*Ov* CaBP without adding EDTA, Lane 3: r*Ov* CaBP with 1 mM EDTA, Lane 4: r*Ov* CaBP with 5 mM EDTA, Lane 5: r*Ov* CaBP with 20 mM EDTA. In panel **B**, the mobility shift assay was performed by adding 20 mM EDTA to all protein samples loaded on 12.5% PAGE in the presence/absence of 1 mM $MgCl_2$ or 1 mM $CaCl_2$. Lane 1: BSA with 1 mM $MgCl_2$, Lane 2: BSA with 1 mM $CaCl_2$, Lane 3: r*Ov* CaBP as negative control, Lane 4: r*Ov* CaBP with 1 mM $MgCl_2$, Lane 5: the retardation effect of r*Ov* CaBP with 1 mM $CaCl_2$.

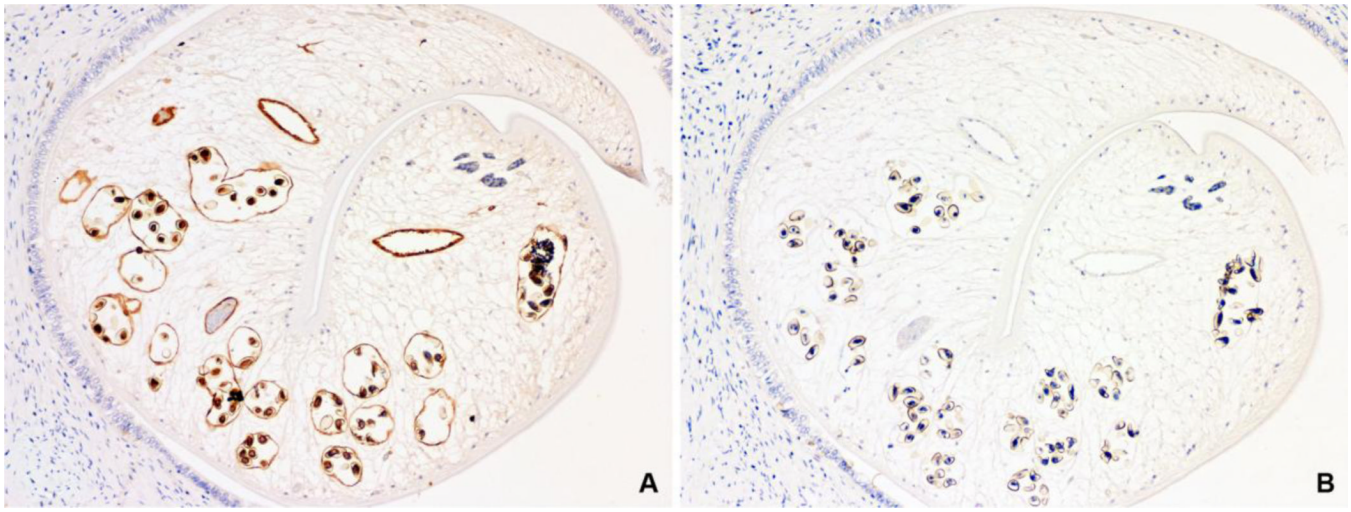

Fig. 6.

Immunolocalization of native *Ov* CaBP in tissue section of *Ov* infected hamster's liver by mouse anti-r*Ov* CaBP serum. Positive immunoperoxidase staining was observed in gut epithelium, miracidia in eggs, and slightly in parenchyma (A), while negative control with mouse preimmune serum showed no staining (B). (Immunoperoxidase, original magnification, ×100)