Molecular and biochemical analysis of 8 kDa calcium-binding protein in *Clonorchis sinensis*

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Molecular and biochemical analysis of 8 kDa calcium-binding protein in *Clonorchis sinensis*

Directed by Professor Tai-Soon Yong

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Molecular and biochemical analysis of 8 kDa calcium-binding protein in *Clonorchis sinensis*

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Clonorchis sinensis is a parasitic helminth distributed through East Asia including Korea, Japan, China, Taiwan, and Vietnam. The infection with the flukes induces biliary cirrhosis followed by fibrosis of surrounding tissues, and even occasionally cholangiocarcinoma. Calcium-binding protein is common, and pivotal protein which is implicated in the muscle contraction and signal transduction in most of organisms. However, calcium-binding protein of *C. sinensis* has not reported thus far. In this study, a cDNA clone encoding a calcium-binding protein was isolated from *C. sinensis* and entitled CsCaBP. Deduced amino acid sequence of CsCaBP showed around 50% of sequence homologies with calcium-binding proteins of the other trematodes. The Pfam database search showed that the CsCaBP sequence has two

canonical EF hands commonly seen in calmodulin. To understand the structural basis of CsCaBP, homology modeling was performed by Geno3D and SWISS-MODEL. Both of calcium-binding site I and II were located on the surface of CsCaBP. Estimated structure of calcium-loaded state was different from that of apo-state. The recombinant CsCaBP (rCsCaBP) was produced in *Escherichia coli* and purified by Ni-NTA affinity chromatography to homogeneity. Molecular mass of rCsCaBP confirmed by SDS-PAGE was 12 kDa as predicted by sequence analysis. Native gel mobility shift assay performed with rCsCaBP incubated with metal ions showed calcium-specific binding affinity of rCsCaBP. The rCsCaBP-immunized mouse sera reacted with rCsCaBP and native CsCaBP in adult *C. sinensis* worm extract. Distribution of native CsCaBP in adult *C. sinensis* worm was visualized by immunostaining. The most of CsCaBP was found in oral and ventral suckers, vitelline follicles and subtegumental regions.

Collectively, the CsCaBP might be concerned with parasite's movement and muscle contraction of suckers for the attachment to its habitat in the host. The present study provides structural and biological basis for the development of new anthelmintic molecules.

Key words : *Clonorchis sinensis*, calcium-binding protein, EF hand, homology modeling, immunolocalization, suckers, subtegumental regions, vitelline follicles

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Ⅰ. INTODUCTION

Clonorchis sinensis, the Chinese liver fluke, is a parasitic platyhelminth widely distributed in East Asia including Korea, Japan, China, Taiwan, and Vietnam[1]. The natural final host of *C. sinensis* is freshwater fish-eating animals. Eggs are produced by adult worms in the final host, and released via feces. Once an egg has hatched in fresh water, a miracidium is released, and ingested by first intermediate snail hosts including *Alocinma longicornis, Parafossarulus* spp., and *Bithynia* spp. Miracidia develop into cercariae within the snail, and then cercariae leave snail. Released cercariae penetrate the skin of a second intermediate host, the freshwater fish family Cyprinidae,

and encyst as metacercariae in the flesh of the fish[2,3]. When the final host eats the metacercariae-infected fish, the metacercariae excyst in duodenum of host. The excysted juvenile worm moves to bile ducts and become adult worms.

Human can be infected by the ingestion of raw, inadequately cooked, or even dried, salted, or pickled flesh of infected carp-like freshwater fish[1,2]. Adult *C. sinensis* lives in the intrahepatic bile ducts and occasionally the pancreatic ducts[2]. In the case of light infections (<100 flukes), humans are in general asymptomatic or have few clinical signs such as diarrhea and abdominal pain[3]. But moderate or chronic infection might cause inflammatory injury, tissue/organ damages, edema occurred by the parasite movement, impairment followed by the attachment of toxic/allergic metabolites[4]. Chronic inflammation and infection with the flukes induce biliary cirrhosis followed by fibrosis of surrounding tissues, and occasionally cholangiocarcinoma by persistent stimuli and damages[2,3,4]. Major causes of dysfunction are a mechanical obstruction of the biliary tract by the worms, a congestion of bile, and the effects of soluble substances of metabolites released from the flukes into the ducts and surrounding tissues[2]. The severity of the dysfunction depends on the number of worms and the period of infection[2]. Recently, International Agency for Research on Cancer (IARC) classifies *C. sinensis* as a probable carcinogen (group 2A)[5,6].

Calcium ion, an intracellular messenger, has been known as a principal regulator of the cell life cycle including cell development/differentiation, and

apoptosis[7,8]. Calcium ion performs its diverse functions by binding to a calcium-binding protein or calcium receptor. The EF-hand domain has been found in more than 50% of various calcium-binding proteins[8]. Until now, the protein family having EF-hands has been reported to include 66 subfamilies[8,9] including troponin C, myosin light chain, parvalbumin[10], calmodulin[11,12], and S100[8,9,13]. The EF-hand in the sequence varies from two to eight according to the kind of proteins[13]. The calcium-binding loop of the EF-hand, called EF-hand motif, binds directly to calcium and magnesium ions[9]. Binding affinity of the calcium-binding loop to calcium ion is much higher than to magnesium ion[14]. Studies on the structure of calcium-binding protein have been reported that the conformational change is induced when calcium-binding proteins bind to these ions[11,15].

The calcium-binding protein have been studied particularly in parasitic trematodes such as *Schistosoma japonicum*[16,17], *S. mansoni*[18], *Opisthorchis viverrini*[19], *Fasciola hepatica*[20,21,22] and *F. gigantica*[23]. Among the two calmodulin-like proteins (FhCaM1, FhCaM2) from *F. hepatica*, FhCaM1 had a higher similar structure to other calmodulins, whereas surface charge of FhCaM2 was higher than FhCaM1[21]. These differences may reflect a specialized role for FhCaM2 in *F. hepatica*. Biochemical characteristics and the structure of the other calcium-binding protein of *F. hepatica*, FH8 with two EF-hand motifs, were examined[20]. The FH8 playing as a sensor protein, had low affinity to calcium ion, but was stabilized by binding calcium ions. It

was reported that a 8 kDa *S. japonicum* calcium-binding protein was comprised of two EF-hand motifs and specifically expressed in transforming cercariae/schistosomula[17]. The study of *S. mansoni* calmodulins suggests a potentially important role of calmodulin during early larval development[18].

The calcium-binding protein is common in various organisms, and is a pivotal protein implicated in muscle contraction and signal transduction.

Although studies about the calcium-binding proteins might help development of new anthelmintic drugs, the calcium-binding proteins of *C. sinensis* have not been reported thus far. In this study, a calcium-binding protein of *C. sinensis* was newly cloned, expressed, and characterized structurally and biochemically.

Ⅱ. MATERIALS AND METHODS

1. Molecular cloning of CsCaBP cDNA

New Zealand white Rabbits were infected orally with 50 *C. sinensis* metacercariae obtained from infected freshwater fishes (*Pseudorasbora parva*) caught at Shenyang, Liaoning Province, China. Twelve weeks after the infection, they were euthanized and adult *C. sinensis* worms were obtained from bile ducts of the liver. Total RNA of *C. sinensis* was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's manual. First strand cDNA of CsCaBP were synthesized by RT-PCR from isolated total RNA.

EST clone ID Cs296 (NCBI acc. No. AF527456) which is similar to calcium-binding protein of schistosomes was selected in *C. sinensis* adult worm Expressed Sequence Tag (EST) database of NCBI[24] and named as CsCaBP. PCR primers for cloning of CsCaBP were designed (Forward primer, 5'-ACTCCGATTTGGCTCTCGT-3'; Reverse primer, 5'-TCGGGTAAAATAAGGCAACG-3') on the basis of this EST clone sequence. The PCR reaction mixture was made by adding 5 μl of cDNA, 2 μl of 10 μM primers, 5 μl of 10X Taq buffer, 1 μl of 10 mM dNTP, 0.25 μl of Taq DNA polymerase (Solgent, Daejeon, Korea), and 36.75 μl of the

distilled water. The 30 cycles of PCR were executed after initial denaturation for 5 min at 95℃. Each cycle was performed under the following conditions: 20 sec at 95℃, 40 sec at 60℃, and 1 min at 72℃. The final extension was carried out at 72℃ for 5 min. The PCR products were loaded in 1% agarose gel and purified using an GeneAll Expin^{TM} Gel SV Kit (GeneAll Biotechnology, Seoul, Korea).

2. Preparation of recombinant CsCaBP and Cs crude extracts

Amplified CsCaBP cDNAs were inserted to the bacterial expression plasmid vector pEXP5-NT/TOPO (Invitrogen). *Escherichia coli* TOP10 was transformed by heat shock with CsCaBP-cloned pEXP5-NT/TOPO. Bacterial colonies in which plasmid contained in frame-inserted CsCaBP cDNA were screened with colony PCR. Plasmid DNA was isolated from the positive bacterial culture. Nucleotide sequence of CsCaBP was determined at Genotech. *E. coli* BL21(DE3)pLysS was transformed with CsCaBP-cloned pEXP5-NT/TOPO plasmid. Recombinant CsCaBP (rCsCaBP) was overexpressed by adding 0.5 mM of IPTG to the transformed *E. coli* BL21(DE3)pLysS culture. Overexpressed rCsCaBP was purified using Ni-NTA affinity chromatography under denaturation condition and analyzed by 16% SDS/Tricine gel.

For the preparation *C. sinensis* crude extracts (Cs crude extracts), adult worms were homogenized in PBS (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4). Insoluble materials were pelleted by centrifugation at 15,000 ×g for 1 hr, and the supernatant was stored at -70℃ until use.

The concentrations of crude extracts and purified proteins were determined using Bradford assay kit (Bio-Rad Laboratories, Reagent Park, Australia).

3. Sequence analysis and homology modeling

Sequences of calcium-binding proteins from *S. mansoni, S. japonicum,* and *F. hepatica* were aligned with CsCaBP sequence using ClustalW. Sequence analysis of CsCaBP was performed using CLC Main Workbench 6.5. Phylogenetic tree was made by neighbor-joining method with 100 replicates. Hydropathy plots were obtained by Kyte and Doolitle scale with window size of 5. Pfam/Prosite database were searched in their websites (http://pfam.sanger.ac.uk and http://prosite.expasy.org). Helical wheel images were obtained in the website offered by Kael Fischer. (http://kael.net/helical.htm)

Homology modeling of the CsCaBP was carried out by SWISS-MODEL[25] and Geno3D[26] based on the structure of calcium-loaded calmodulin (Protein Data Band code: 3B32, 1FW4, 1F55) and apo-calmodulin (Protein Data Bank code: 1QX5, 1CMF, 1F54) from *Rattus norvegicus, Bos taurus*, and *Saccharomyces cerevisiae*. Pymol program was used to visualize the resulted models.

4. Mouse anti-rCsCaBP sera production

Fifty μg of rCsCaBP mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) was injected into Balb/c mice intra-peritoneally. After two weeks, 50 μg of rCsCaBP mixed with Freund's incomplete adjuvant (Sigma-Aldrich) was injected by the identical method. The immunization was finally boosted by injecting 25 μg of rCsCaBP to the mice via tail veins. Blood was collected by heart puncture at 3 days after the boosting, and the serum was clotted at 4℃, overnight. Immune sera were separated by centrifugation at 5,000 ×g and stored at -70℃ until use.

5. Western blotting

One μg of purified rCsCaBP was separated in 16% SDS/Tricine acrylamide gel, transferred onto nitrocellulose membrane by semi-dry blotting. Blotted membrane was incubated overnight with 3% skim milk in TBST (20 mM Tris, 150 mM NaCl, pH 7.4 with 0.05% Tween 20) at 4℃. After the washing 3 times of with TBST, the membrane was stripped and incubated with 1:30,000-diluted rCsCaBP-immunized mouse sera, normal mouse sera or 1:1000-diluted anti Penta-His mouse monoclonal IgG (QIAGEN, Valencia, CA, USA) in TBST containing 1% of BSA. Then, the membrane strips were washed 3 times with TBST and incubated at RT for 1 hr with alkaline phosphatase conjugated anti-mouse IgG (Sigma-Aldrich) diluted with TBST containing 1% of BSA (1:5,000). After the 3 times of washing with TBST, color on the membrane was developed by incubating with BCIP/NBT as a substrate (Promega, Madison, WI, USA).

6. ELISA using rCsCaBP-immunized mouse sera

Microplates were coated with 5 μg/ml of rCsCaBPs and *C. sinensis* crude extract at 4℃, overnight, washed with PBS-T (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4 with 0.05% Tween 20), and

incubated with 3% skim milk in PBST at 37℃ for 1 hr. Following 3 times of washing with PBS-T, plates were incubated at 37℃ for 1 hr 30 min with rCsCaBP-immunized mouse sera or normal mouse sera diluted with PBST containing 1% BSA (1:300). After 3 times of washing, plates were incubated at 37℃ for 1 hr with 1:5,000-diluted alkaline phosphatase-conjugated anti-mouse IgG (Sigma-Aldrich). Finally washed plates were incubated in dark with OPD substrate (Dako, Glostrup, Denmark) at RT for 10 min. The reaction was stopped by adding 3 M NaOH. Absorbances at 405 nm of wave length were measured using Sunrise microplate reader (TECAN, Salzburg, Austria)

7. Mobility shift assay

Purified rCsCaBPs were refolded using protein refolding kit (Novagen, Merck, Biosciences, Darmstadt, Germany) according to the manufacturer's instruction. Concentration of the refolded protein was determined by Bradford assay. Calcium-binding activity was analyzed by 15% non-denaturing PAGE in the presence of 2 mM EDTA and 2 mM $CaCl_2$, 2 mM $MgCl_2$ or deionized DW. BSA was used as a negative control.

8. Immunolocalization

The paraffin-embedded sections were obtained from *C. sinensis* adult worms fixed with 10% formalin over 24 hr. Tissue sections were deparaffined, rehydrated and retrieved. After blocking with 1% BSA in PBST, tissue sections were incubated with rCsCaBP-immunized mouse sera or normal mouse sera diluted by 1:30,000 with PBST containing 1% BSA at RT for 1 hr. Tissue sections were washed 3 times with PBST, incubated with 1:200-diluted HRP-conjugated anti-mouse IgG at RT for 1 hr, reacted with DAB reagent, and then counter-stained with Mayer's hematoxylin. Finally, tissue sections were mounted and observed through light microscope (Nikon, Tokyo, Japan)

For immunofluorescence, tissue sections reacted with normal mouse sera or rCsCaBP-immunized mouse sera, were incubated with Alexa Fluor 488 goat anti-Mouse IgG (Invitrogen) diluted with 1% BSA in PBST (1:200) at RT for 1 hr, and washed 3 times. The sections were then mounted using VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). Images were examined by Confocal microscopy LSM700 (Carl Zeiss, Oberkochen, Germany)

Ⅲ. RESULTS

1. Sequence analysis

Deduced amino acid sequence of CsCaBP showed 55% of identity with *F. hepatica* putative calcium-binding protein, 49% with *S. mansoni* calcium-binding protein, and 48% with the *S. japonicum* 8 kDa calcium-binding protein (Figure 1A). The sequences of these calcium-binding proteins had two calcium-binding sites. The calmodulin sequences of *R. norvegicus, B. Taurus,* and *S. cerevisiae* which were chosen as templates for the homology modeling of the CsCaBP, contained identical canonical calcium-binding sites and showed 20~30% of sequence identities with each other (Figure 1B).

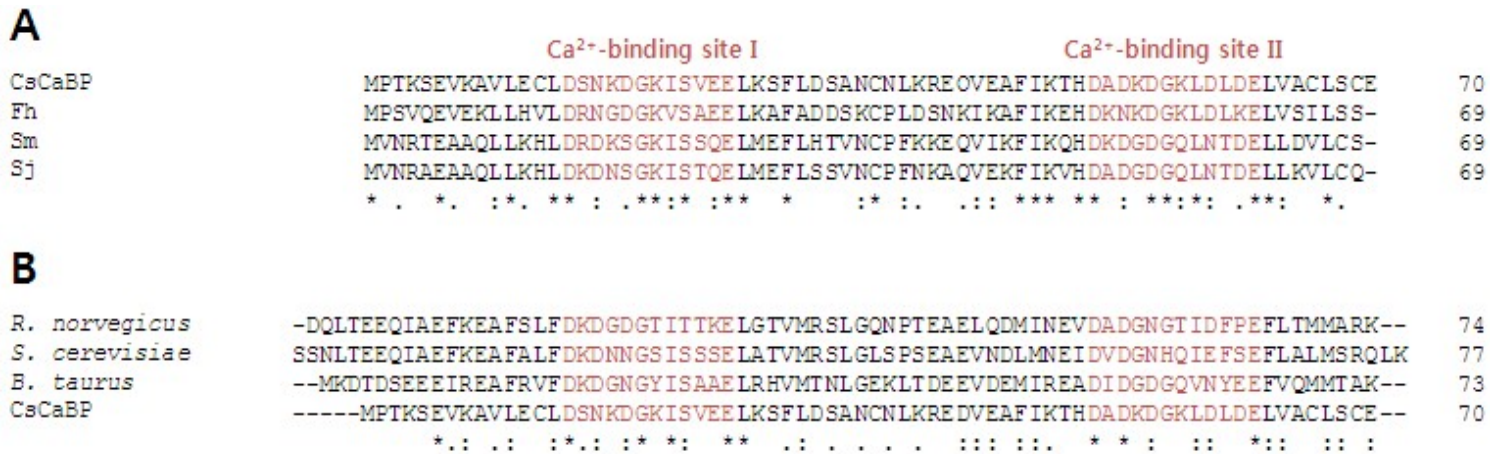


Figure 1. Multiple sequence alignment of CaBPs. CsCaBP sequence was aligned with calcium-binding proteins of trematodes (A) and other animals (B). Calcium-binding sites are red-colored. The symbol '*' indicates identical in all sequences in the alignment, ':' indicates conserved substitutions and '.' indicates semi-conserved substitutions.

Phylogenetic tree made on the basis of calcium-binding sequence in other parasitic helminthes showed that calcium-binding proteins of cestodes and nematodes were clustered as different clades. CsCaBP was largely clustered with calcium-binding proteins of trematodes (Figure 2).

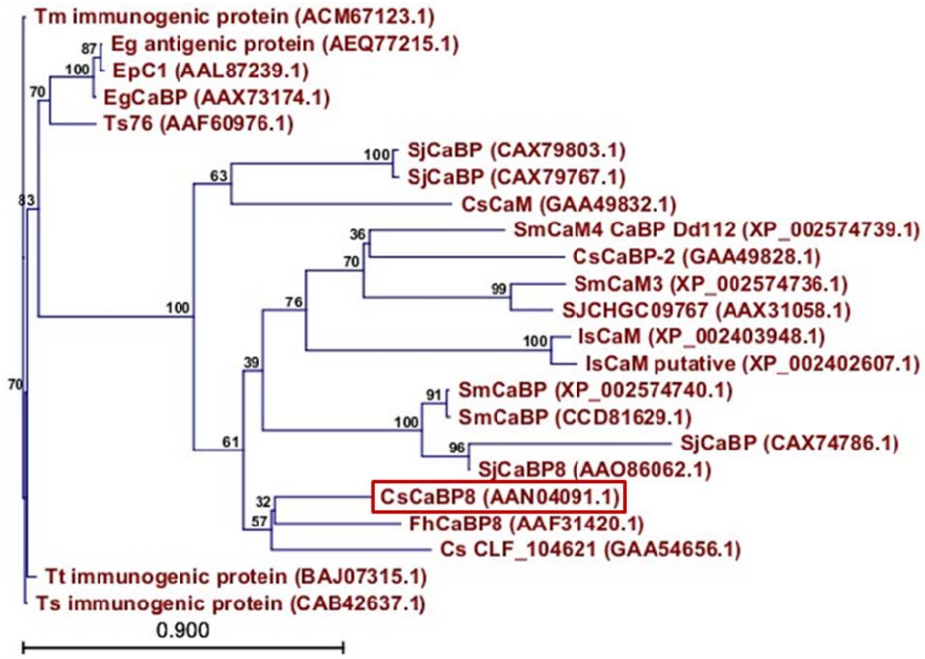


Figure 2. Phylogenetic tree of trematodaes' CaBPs. The phylogenetic tree was constructed using CLC Main Workbench 6.5 program. Bootstrap values were obtained by the neighbor-joining method using 100 replications. The red box indicates CsCaBP.

Secondary structure prediction using Pfam database search showed that the CsCaBP had two EF hand domains (Pfam accession No. PF00036, residues 6-34, 42-70) with the helix-loop-helix structure (Figure 3). Twelve residues in canonical EF hand motif (the loop region in the EF-hand domain) commonly compose X-Y-Z-(-Y)-(-X)--(-Z) pattern order[8,9]. CsCaBP had the amino acid sequences which were identical to canonical EF hand motif on the Prosite database search. There is a hydrophobic amino acid next to -Y position[20]. In CsCaBP, Ile22 and Leu58 were next to -Y position (Lys21 and Lys57).

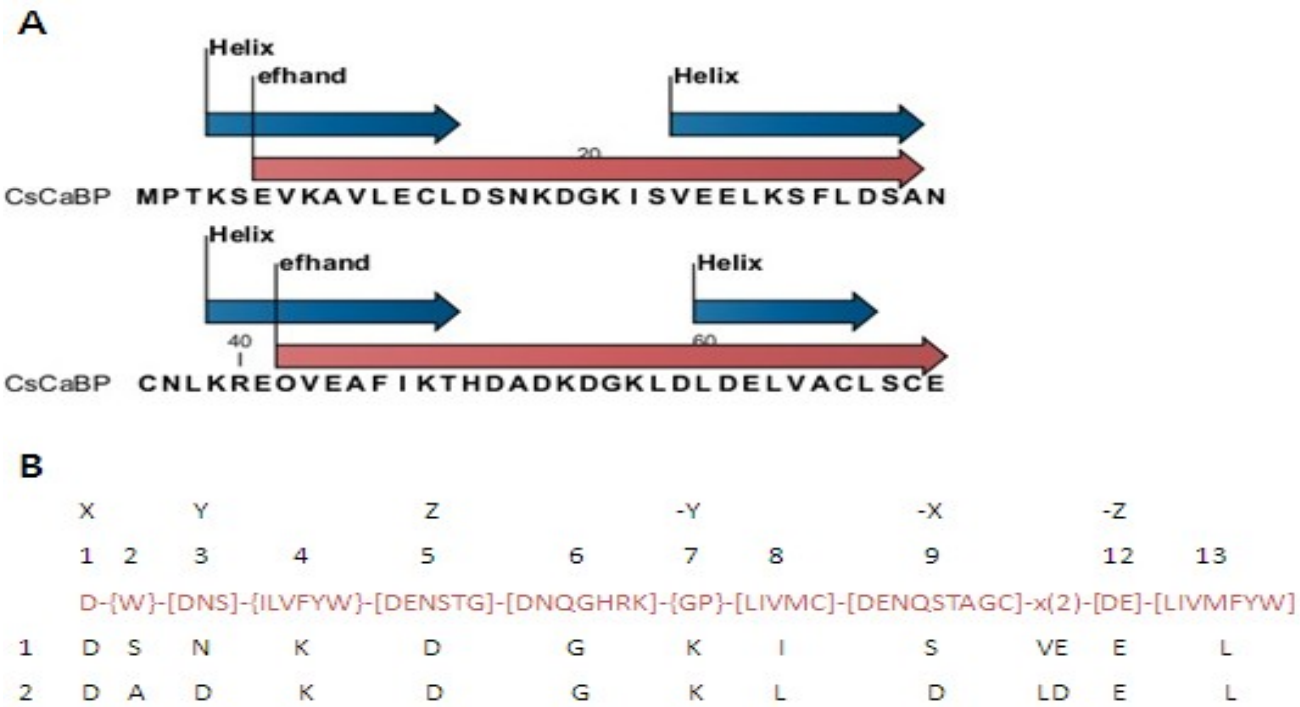


Figure 3. Sequence analysis of CsCaBP using Pfam/Prosite database search. (A) Pfam database search was executed using CLC Main Workbench 6.5 program. (B) Comparison between EF-hand consensus motif (red) of the Prosite database (pattern PS00018) and the two calcium-binding sites of CsCaBP. (x, any amino acid; [], acceptable amino acid for the position; {}, not acceptable amino acids at the position)

Hydrophobicity (hydropathy) plot of CsCaBP sequence was obtained by Kyte-Doolittle scale which has been used to estimate hydrophobic character of proteins (Figure 4). In order to estimate regions exposed to the surface of CsCaBP, the plot obtained by window size 5. The EF-hand motifs in the CsCaBP were located in front of the second and fourth helix, and supposed to be exposed on the surface of the molecule because hydrophobicity values of most EF-hand motif residues were below 0.

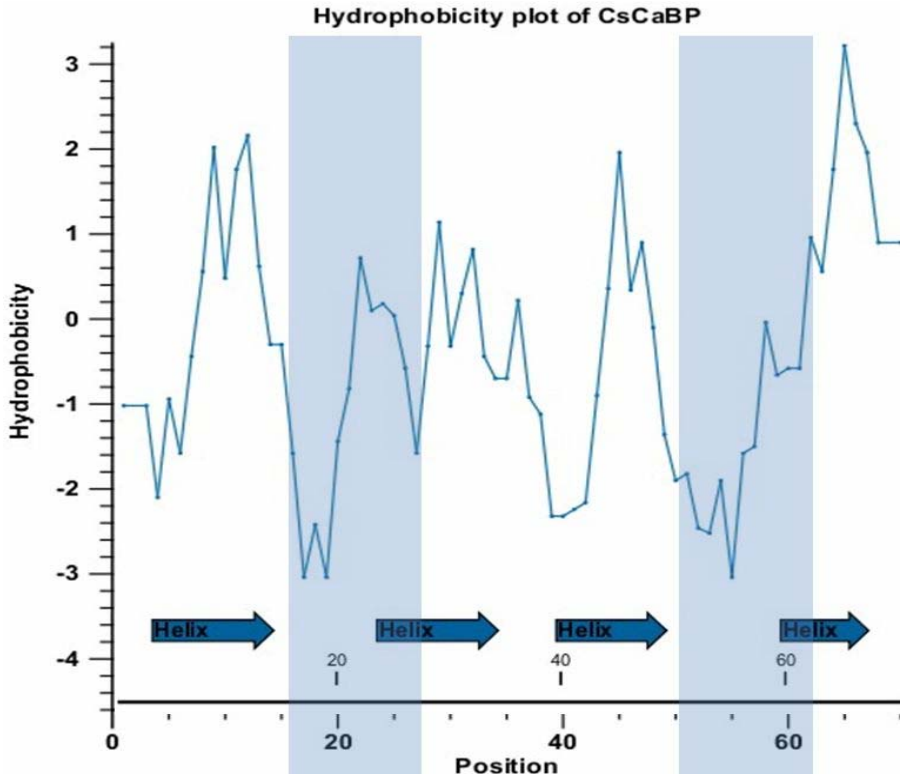


Figure 4. Hydropathy plot of CsCaBP was obtained by Kyte-Doolittle scale. EF-hand motifs were showed by blue highlight. Regions of which hydrophobicity values are above 0 are considered to be hydrophobic.

2. Protein structure prediction

The cartoon ribbon structures and statuses of molecular surface of CsCaBP were estimated by using calcium-loaded calmodulin (Protein Data Bank code: 3B32, 1FW4, 1F55) and apo-calmodulin (Protein Data Bank code: 1QX5, 1CMF, 1F54) of *R. norvegicus, B. taurus,* and *S. cerevisiae* as templates (Figure 5A, B). Calcium-binding residues were located protein surface (Figure 5C, D). The conformational change by binding to calcium ion was predicted. Although CsCaBP has closed conformation in apo-state, the protein has open conformation in calcium-loaded state. Calcium-binding residues in X, Y, Z, -Y, -X, -Z position of CsCaBP were dispersed in apo-state, but collected in calcium-binding state.

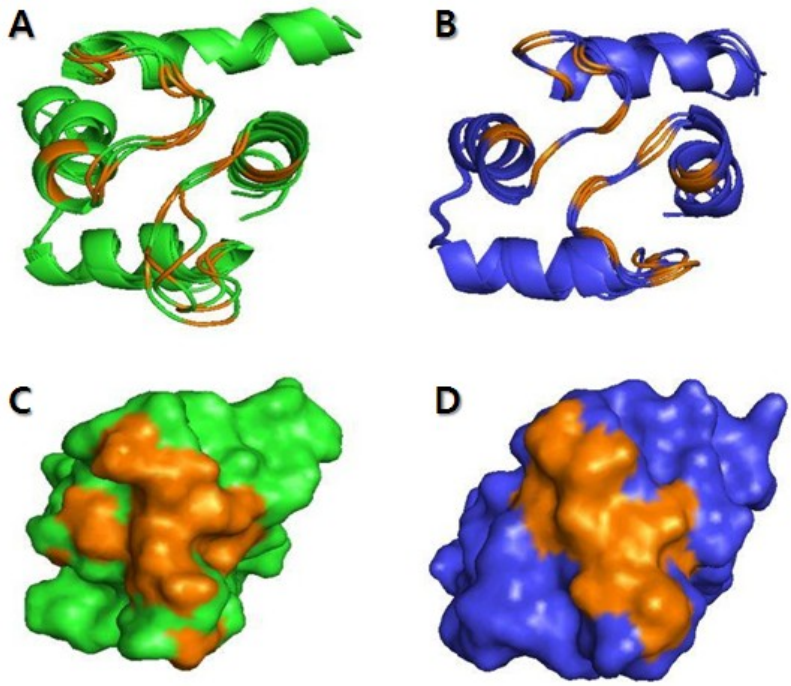


Figure 5. The modeled structures of CsCaBP. The modeled structures of CsCaBP in the apo (A) and calcium-loaded (B) states represented by cartoon ribbons. The molecular surface of CsCaBP in the apo (C) and calcium-loaded (D) states. Amino acid residues in X, Y, Z, -Y, -X, -Z positions in EF-hand motifs were indicated orange. The molecular models were obtained by homology modeling using the SWISS-MODEL and Geno3D. The figures were prepared with PYMOL program.

Helical wheel projection showed that four helices in the CsCaBP were amphipatic (Figure 6A). These helices were generally consisted of hydrophobic and non-polar residues on one sides of the helix and hydrophilic and polar residues on the other sides. In the cartoon structure obtained through homology modeling (Figure 6B), most of hydrophobic residues in four helices were located inward.

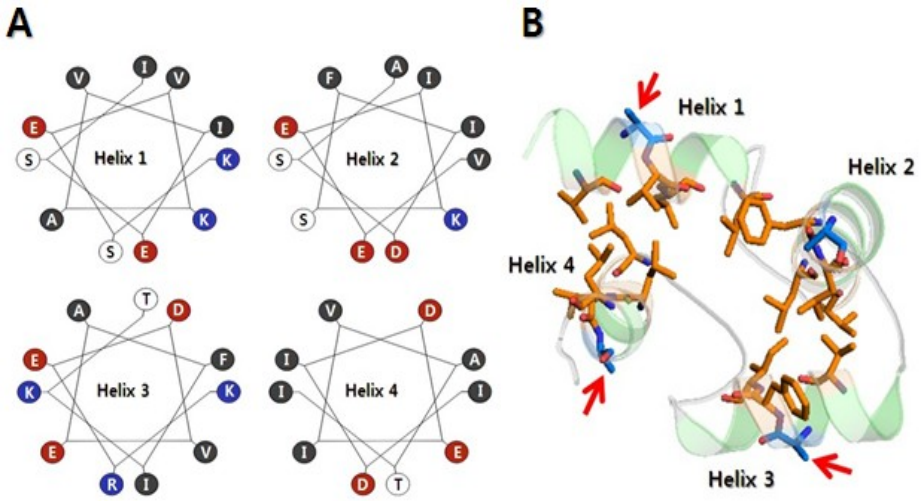


Figure 6. Helical wheel images of CsCaBP. Four helices of CsCaBP are amphipatic helices. (A) Helical wheel images (http://kael.net/helical.htm). Colors indicate characters of amino acid (Gray, hydrophobic; Red, acidic; Blue, basic; White, polar). (B) The hydrophobic residues of amphipatic helices were indicated by colored sticks in 3D cartoon ribbons. Red arrows indicate alanine residues located in hydrophilic side. (Orange, hydrophobic amino acid; Blue, Alanine)

3. Purification of rCsCaBP

rCsCaBP was produced bacterially and purified as a 11.7 kDa protein band by Ni-NTA affinity chromatography (Figure 7A). The purified rCsCaBP was confirmed by western blotting with mouse anti-His IgG, as well as with mouse sera immunized with rCsCaBP (Figure 7B). A 20 kDa protein band appeared in the blot with rCsCaBP-immunized mouse sera might be dimeric form of the rCsCaBP.

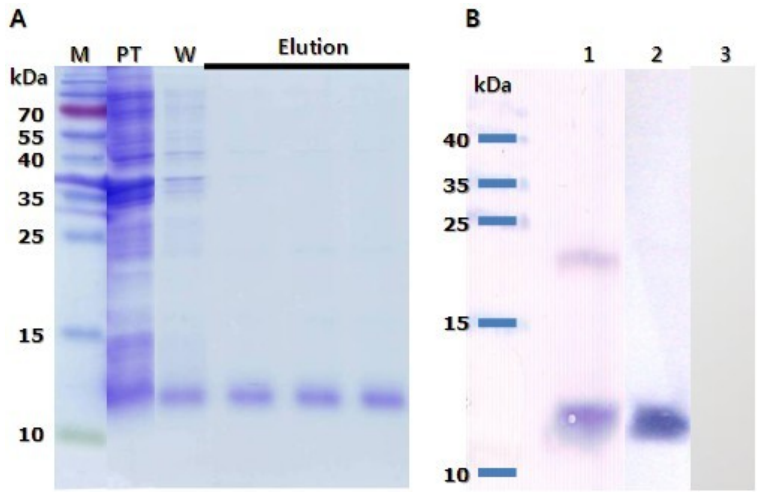


Figure 7. Recombinant CsCaBP showed by SDS-PAGE and the binding activity of the anti-rCsCaBP antibody. The purified rCsCaBP was showed as single band using 16% SDS-PAGE (A). M, size maker; PT, pass through; W, washing. Western blots of rCsCaBP (B). lane 1: rCsCaBP-immunized mouse sera, lane 2: mouse anti-His antibody, lane 3: normal mouse serum.

4. Reactivity of anti-rCsCaBP mouse sera

To identify whether anti-rCsCaBP antibody specifically recognize rCsCaBP and native CsCaBP in *C. sinensis* crude extract, ELISA was performed (Table 1). Absorbance values of rCsCaBP or *C. sinensis* crude extract by the reaction with rCsCaBP-immunized sera were higher than the reaction with normal mouse sera.

Table 1. Reactivity of rCsCaBP-immunized mouse sera by ELISA.

Sera	Antigen	
	Recombinant CsCaBP	*C. sinensis* crude extract
Normal mouse sera	0.141 ± 0.011	0.235 ± 0.020
rCsCaBP-immunized mouse sera	1.762 ± 0.061	0.535 ± 0.049

5. Calcium binding property of rCsCaBP

Sequence analysis indicated that CsCaBP sequence contained EF hand motifs which could bind to the calcium ion. Hence, whether rCsCaBP can bind calcium ion was examined using mobility shift assay. The mobility of the protein band was reduced on the native gel when the rCsCaBP was loaded with $CaCl_2$. However, rCsCaBP loaded with $MgCl_2$ showed fast mobility similar to rCsCaBP loaded with DW.

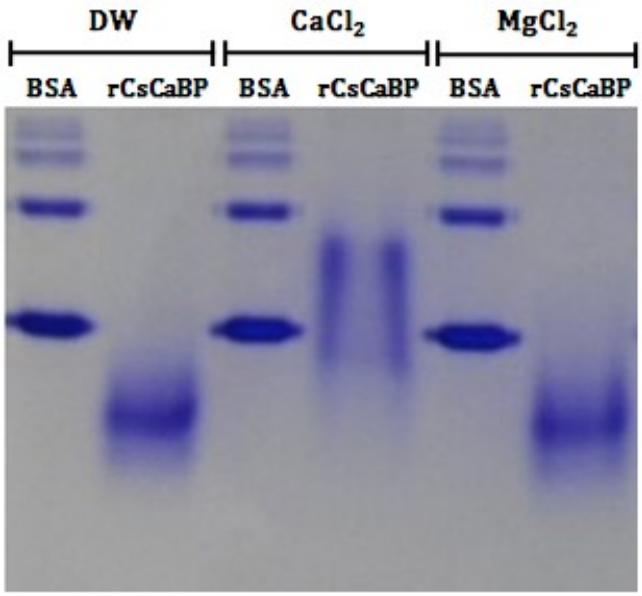
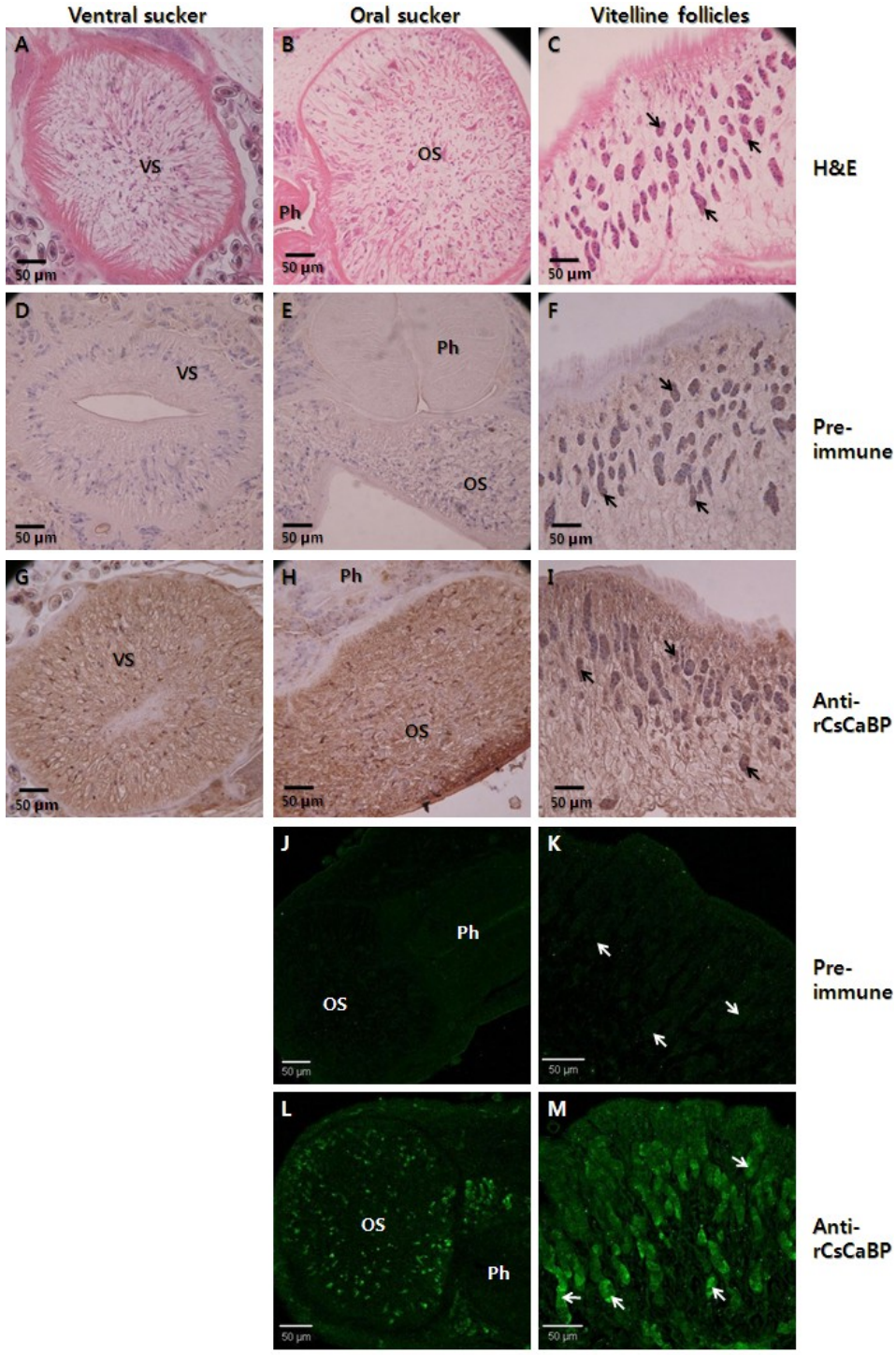


Figure 8. The calcium binding property of the rCsCaBP. The mobility shift assay was performed by adding 2 mM EDTA to all protein samples loaded on 10% PAGE in the presence/absence of 2 mM $MgCl_2$ or 2 mM $CaCl_2$. BSA was used as control.

6. Immunolocalization

Native CsCaBP was localized in adult *C. sinensis* worms by immunofluorescence and immunohistochemistry (IHC) using rCsCaBP-immunized mouse sera. Distribution of native CsCaBP in oral and ventral sucker, vitelline follicles, and subtegumental region in the worm was found by IHC (Figure 9D-I). Immunofluorescence showed that CsCaBPs were distributed in oral sucker, around the pharynx and vitelline follicles of the worm (Figure 9J-M).

Figure 9. Immunolocalization of CsCaBPs in adult worms. Slide sections were reacted with the normal mouse sera or the rCsCaBP-immunized mouse sera. The normal mouse sera were not reacted with any tissues (D-F, J and K). Parts detected with the rCsCaBP-immunized mouse sera were showed green in immunofluorescence (L and M) or red in IHC (G-I). (VS, ventral sucker; OS, oral sucker; Ph, pharynx; arrows indicate vitelline follicles)


Ventral sucker
Oral sucker
Vitelline follicles
A
B
C
VS
OS
Ph
50 μm
H&E
D
E
F
Pre-immune
G
H
I
Anti-rCsCaBP
J
K
Pre-immune
L
M
Anti-rCsCaBP

Ⅳ. DISCUSSION

Until now, helminthic calcium-binding proteins have been characterized mainly in schistosomes and *Fasciola* spp. In this study, molecular structures, the biochemical property and distribution in the adult worm of a calcium-binding protein newly identified from *C. sinensis* were examined.

The CsCaBP sequence was composed of the amino acid patterns X-Y-Z-(-Y)-(-X)--(-Z), which included into canonical EF hand proteins[14,22]. Seven oxygen atoms in this pattern can bind to a calcium ion. A calcium ion binds to each an oxygen ion of amino acid residue in X, Y, Z position, a carbonyl oxygen ion of main chain in -Y position, and a water ion indirectly in case of -X position. Particularly, two oxygen ions of Glu or Asp residue in -Z position bind to a calcium ion[14,20,22]. The hydropathy plot indicated that the amino acid sequences in calcium-binding sites of the CsCaBP have low hydrophobicity, and are exposed to the surface of the protein. When the surface structure of the CsCaBP was anticipated using homology modeling, two calcium-binding sites of the CsCaBP were localized in the outside of the protein. Calcium ion may bind to CsCaBP more easily owing to these exposed calcium-binding sites.

Calcium-binding protein can be categorized as calcium buffer and calcium sensor. In contrast to calcium buffers such as parvalbumin, calcium

sensors (e.g., calmodulin, troponin C) change their conformation upon calcium binding, and thereby enable their interaction with the target[27-29]. The conformational changes according to calcium-binding indicate that the CsCaBP plays a role as a calcium sensor. Such a conformational change was also observed in the FH8 of *F. hepatica*[20] which is phylogenetically closest to CsCaBP (Figure 2).

In the homology modeling and helical wheel images, hydrophobic amino acid residues in four helices of the CsCaBP were located at interior parts of the protein. Therefore, the CsCaBP might exist as a cytosolic soluble form, but not a membrane-embedded form in the cells.

The binding affinity of rCsCaBP to calcium and magnesium ions was tested through the mobility shift assay in the present study. The rCsCaBP bound only to calcium ions. This indicates that the CsCaBP has not only calcium affinity but also selectivity for calcium over magnesium. Similar selectivity for calcium observed in the study on a calcium-binding protein of *O. viverrini*[19]. Calmodulins of *F. hepatica*, however, bind to both calcium and magnesium ions[21]. These differences were considered to be caused by various molecular size and biological roles of each protein.

In the reaction using rCsCaBP as an antigen rCsCaBP-immunized mouse sera showed over 10 times higher absorbance value than normal mouse sera. However only over two times higher absorbance values were obtained in the reaction of rCsCaBP with *C. sinensis* crude extract. This result implies

that the overall quantity and antigenicity of native CsCaBP is quite lower than rCsCaBP, and a little cross reaction was occurred.

Since the sera of mouse immunized with rCsCaBP reacted with crude extract of *C. sinensis* in ELISA, the sera were used for the immunohistochemical localization of native CsCaBP in adult *C. sinensis* worms. The native CsCaBPs were mainly localized in muscular tissues such as oral and ventral suckers, and subtegumental regions. Vitelline follicles were also found to contain the native CsCaBPs. Hence, it is considered that the CsCaBP might be involved in the muscle contraction for the movement attachment of the worm to the host tissue for the intake of nutrients, and also concerned with the egg production[30].

Great variety was found in calcium-binding proteins of trematodes. They have diverse size, and were distributed in various sites in the worms. The 8 kDa calcium-binding protein from *S. japonicum* (SjCa8) was localized to head gland, and penetration glands where calcium was abundant, and the cercarial tegument and body-tail junction[17]. In the case of SmCaM from *S. mansoni*, intense anti-SmCaM reactivity was observed in not only the cilia, epidermal plates and multiciliated sensory papillae in the miracidia but also the tegumental layer in 3-day in vitro cultured sporocysts[18].

When the immunolocalization was performed in an adult worm of liver flukes, the *O. viverrini* CaBP was located in gut epithelium, miracidia in eggs[19]. The *F. gigantica* CaBP1 (FgCaBP1) was observed in the syncytial

tegument and the tegument type layers of the pharynx[23]. In addition to that, mouse anti-rFgCaBP1, 3, 4 antisera were strongly reactive with antigens in tegumental type tissues[31]. The epithelia of intestinal tract and excretory system were stained using anti-FgCaBP1 and 4 antisera. These antisera were also reactive with vitelline follicles and proximal uterus. In *C. sinensis*, the 21.6 kDa tegumental protein (CsTegu21.6) was reported. A calcium-binding EF-hand domain at the N terminus and a dynein light chain domain at the C terminus were found in CsTegu21.6. This protein was specifically observed on the tegument of adult worms[32].

Praziquantel has been used in control platyhelminths, but it is ineffective against other parasites such as nematodes. Because of this selectivity, the molecular target of the drug might be expected to be limited to trematodes[33]. Praziquantel increases the permeability of calcium ions of the worm via an unknown mechanism[34]. Many studies reported that the praziquantel also influences the muscle contraction, the tegument disruption and reproductive systems[33,35]. The drug also induces contraction of the *C. sinensis* worms, resulting in paralysis[36]. Praziquantel have showed significant tolerance in case of *S. japonicum* of some regions[37], and hypersensitivity to praziquantel in a patient infected with *C. sinensis* has been reported[38]. Necessity for the development of advanced drug is increased. Studies on the specific inhibitor of CsCaBP might contribute the creation of new and innovative drug for the disease which can substitute praziquantel.

The present study provides a basic understanding on the characteristics of CsCaBP. This could contribute the studies on the development of new anthelminthic drugs based on the specific inhibitor of proteins necessary for the survival of the helminth.

Ⅴ. CONCLUSION

Nucleotide sequence of CsCaBP, a *Clonorchis sinensis* calcium-binding protein, contained canonical EF hands, and two calcium-binding sites which was identical to the sequences of typical calcium-binding proteins.

The CsCaBP had open conformation in calcium-loaded state, but closed conformation in apo-state. Hydrophobic amino acid residues of CsCaBP were located in internal parts of protein indicating that CsCaBP may exhibit cytosolic distribution in the cells.

The rCsCaBP bound to only calcium ions but not magnesium ions. This result indicates that the CsCaBP has high selectivity as well as high affinity to calcium ions.

The native CsCaBPs were mainly located in and around vitelline follicles, pharynx and oral/ventral suckers. It is assumable that the CsCaBP participates in muscle contraction and egg production in *C. sinensis* adult worms.

As a conclusion, the present study revealed newly identified CaBP from *C. sinensis*. Characterization of the CsCaBP provided in this study can contribute the development of new anthelminthic drugs.

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ABSTRACT (IN KOREAN)

간흡충 8 kDa 칼슘 결합 단백질의 분자·생화학적 분석

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간흡충 (*Clonorchis sinensis*)은 한국, 일본, 중국, 타이완, 베트남을 포함한 동아시아 지역에 널리 분포하고 있는 기생충으로서 인체에 감염될 경우 주변 조직의 섬유화에 의해 간경변이 발생하며 만성감염이 될 경우 담관암까지 일으킬 수 있는 것으로 알려져 있다. 칼슘 결합 단백질은 모든 생명체 내에 존재하며 근수축과 신호전달에 관여하는 중요한 단백질이나 간흡충의 칼슘 결합 단백질에 대해서는 지금까지 보고된 바가 없다. 이 논문에서는 간흡충 EST를 검색하여 다양한 생물 종에 존재하는 칼슘 결합 단백질과 유사한 cDNA 클론을 찾아내었고 이를 '간흡충 칼슘 결합 단백질 (CsCaBP)'로 칭하였다. EF hand는 칼모듈린 (calmodulin)과 같은 칼슘 결합 단백질 종류에서 발견되는

helix-loop-helix 구조의 도메인을 통칭한다. 간흡충 칼슘 결합단백질의 아미노산 서열은 다른 흡충류의 칼슘 결합 단백질들과 약 50% 일치하였으며 간흡충 칼슘 결합 단백질의 아미노산 서열은 일반적으로 칼모듈린에서 보이는 canonical EF hand를 두 개 가지고 있는 것으로 보여졌다. 간흡충 칼슘 결합 단백질의 구조적인 특성을 알아보기 위해 homology 모델링을 수행하였다.

칼슘 결합 부위 I과 II는 모두 간흡충 칼슘 결합 단백질의 표면에 위치하였으며 칼슘이 결합한 상태의 구조와 결합하지 않은 상태는 다른 것으로 나타났다. 간흡충 결합 단백질의 실질적인 역할과 특성을 알아보기 위해서 *E. coli*에서 발현시킨 재조합 간흡충 결합 단백질을 Ni-NTA 크로마토그래피로 정제하였다. 정제한 재조합 간흡충 칼슘 결합 단백질의 분자량을 SDS-PAGE로 확인했을 때 서열분석을 통해 예상한 것과 동일한 12 kDa이었다. Native gel mobility shift assay를 수행한 결과 재조합 간흡충 칼슘 결합 단백질은 마그네슘 이온과는 결합하지 않고 칼슘 이온에만 특이적으로 결합한다는 사실을 확인하였다. 재조합 간흡충 칼슘 결합 단백질을 면역한 마우스의 혈청은 간흡충 성충내의 native 간흡충 칼슘 결합 단백질과 재조합 간흡충 칼슘 결합 단백질과 반응하였다. 간흡충 성충 내에 분포하는 native 간흡충 칼슘 결합

단백질의 분포는 면역조직염색, 면역형광염색을 통해 시각적으로 확인하였다. 간흡충 칼슘 결합 단백질의 대부분은 난황소, 구흡반, 복흡반 내부와 그 주변부에서 발견되었다. 이를 보았을 때, 간흡충 칼슘 결합 단백질은 충란 형성과 근수축에 관여하는 것으로 보여진다.

이 논문에서는 간흡충 칼슘 결합 단백질의 생화학적 분석과 면역염색을 통하여 이 단백질이 새로운 구충제 개발이나 기생충/숙주의 상호작용을 이해하기 위한 구조적, 생물학적 기반을 제공하였다.

핵심되는 말 : 간흡충, 칼슘 결합 단백질, EF hand, homology 모델링, 면역염색, 흡반, 난황소