Immunological characterization of *Clonorchis sinensis* venom allergen-like protein 13

Hea Sun Woo

Department of Medical Science

The Graduate School of Yonsei University

Immunological characterization of *Clonorchis sinensis* venom allergen-like protein 13

Directed by Professor Tai-Soon Yong

The Master's Thesis

submitted to the Department of Medical Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

Master of Medical Science

Hea Sun Woo

December 2014

This certifies that the Master's Thesis

of Hea Sun Woo is approved.

Thesis Supervisor: Tai-Soon Yong

Thesis Committee Member#1: Hyoung-Pyo Kim

Thesis Committee Member#2: Jun-Young Seo

The Graduate School

Yonsei University

December 2014

Acknowledgements

어느덧 대학원 졸업을 앞두고 있습니다. 처음 대학원에 진학했을 때는 무엇을 해야 하나 막하기만 했지만, 지금에서야 연구에 대한 눈이 떠지기 시작합니다.
제가 대학원 선택에 방황하던 시절 저를 밝게 받아 주신 용태순 교수님. 바쁘신 와중에도 기생충 진단 실험을 팔 걷어 부치시며 함께 하자는 교수님의 모습이 아직도 아련합니다. 석사는 경험이 중요하다며 많은 학술적인 경험을 해외에서 쌓게 힘 써주셨습니다. 또한 2014년 멕시코 ICOPA 학회에서 2018년 대구 ICOPA 개최 확정의 기쁨을 함께 할 수 있어서 영광이었습니다. 지금까지 저를 성장 할 수 있도록 도와주신 교수님께 깊이 감사 드립니다.
그리고 심사 과정 동안 제 논문의 부족한 점을 살펴주신 김형표 교수님 그리고 서준영 교수님께도 감사 드립니다.

수년간 함께 해온 선생님들의 인연을 너무 소중하게 생각하고 있으며 저를 도와주신 분 들게 쑥스럽지만 다시 한번 고개 숙여 말씀 드리고 싶습니다. 과학에 대한 지식과 이해가 부족한 저에게 실험에 대한 맥락을 잡아 주시고 물고기를

낚아 주시는 것 보다 낚는 방법을 알려주신 김태윤 선생님, 동물실험에 대한 이해나 테크닉을 알려주시고 진로에 대한 비전 및 전망에 대해서 조언 해주신 이인용 선생님, 석사로서의 갖추어야 할 기본 지식 및 실험에 대한 원리를 가르쳐 주신 김충렬 선생님, 학술 프레젠테이션에 관련된 테크닉에 대해서 가르쳐 주신 이명희 누나, 곤충에 관한 모든 지식을 전해주려고 저에게 힘쓴 남성현 형, 실험실 들어와서 처음부터 끝까지 희로애락을 같이 한 곽유신, 항상 안부를 물어 주며 격려해준 콜린 그리고 김주영, 수업과 시험공부를 함께 했던 김민정 선생님 그리고 노현진 선생님.

마지막으로 학위과정을 무사히 마칠 수 있도록 지원해주신 사랑하는 부모님에게 감사하다는 말을 전하고 싶습니다.

2014년12월

우해선

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Immunological characterization of *Clonorchis sinensis* venom allergen-like protein 13

Hea Sun Woo

Department of Medical science

The Graduate School, Yonsei University

(Directed by Professor Tai-Soon Yong)

The SCP/TAPS (sperm coating protein/Tpx-1/Ag5/PR-1/Sc7) proteins are multifunctional protein found in eukaryotes. Venom allergen-like (VAL) proteins, members of the SCP/TAPS protein superfamily, have been reported from several parasitic helminths. However, little is known about their biological and immunological function. In this study, a VAL protein of the Chinese liver fluke *Clonorchis sinensis* was cloned and characterized. A cDNA encoding 25 kDa protein was identified from EST database of *C. sinensis*. A BLAST search revealed that the protein shares 46% sequence identity with *Schistosoma mansoni* VAL 13 protein, and

thus, the protein was named CsVAL13. Multiple sequence alignment indicated that SCP/TAPS domain of CsVAL13 shared 39~46% sequence identity with VAL proteins from parasitic helminths. His and Tyr residues, which help to stabilize protein structure, were highly conserved across the VAL protein sequences. Phylogenetic analysis showed that SCP/TAPS domain of CsVAL13 sequence clusters together with other group 2 VAL protein sequences. In the homology-modeled structure of CsVAL13, an α-β-α sandwich structure and residues for a putative active site were highly conserved. PCR-amplified cDNA sequence of CsVAL13 was subcloned into pET32a bacterial expression plasmid vector. Recombinant CsVAL13 protein was produced bacterially and purified by Ni-NTA affinity chromatography. Immune sera were obtained from BALB/c mice immunized with the recombinant CsVAL13 protein. ELISA value of IgG1 in the immune sera against the recombinant CsVAL13 protein was six-times higher than that of IgG2a suggesting that CsVAL13 can induce Th2-polarized immune response. Immunohistochemical localization using the immune sera revealed that CsVAL13 was distributed mainly in the tegument and intrauterine eggs of adult *C. sinensis*. These findings suggest that CsVAL13 may be involved in host-parasite interactions and immune stimulation on the surrounding host environments.

Key words: *Clonorchis sinensis*, venom allergen-like protein, SCP/TAPS protein, intrauterine eggs, tegument

Immunological characterization of *Clonorchis sinensis* venom allergen-like protein 13

Hea Sun Woo

Department of Medical science

The Graduate School, Yonsei University

(Directed by Professor Tai-Soon Yong)

Ⅰ. INTRODUCTION

Clonorchis sinensis, the Chinese liver fluke, is the causative agent of clonorchiasis[1,2,3,4]. It is widely distributed in Korea, China, Taiwan, Japan, eastern Russia and northern Vietnam. About 20 million people are estimated to be infected globally with *C. sinensis*. Humans can be infected with *C . sinensis* after consumption of raw or undercooked freshwater fish[3]. Eggs produced by adult worms residing bile ducts of its final hosts, mammalians eating raw fishes, are released via feces. First intermediate host of *C. sinensis* is freshwater snails of *Bithynia* spp., *Alocinma* spp. and *Parafossarulus* spp.. From the eggs eaten by the snails, miracidia hatch out in the intestinal tract and develop to sporocysts and then to rediae. Cercariae produced from

rediae leave the snail and invade the second intermediate hosts, cyprinid fishes. In the fishes, cercariae encyst to form metacercariae, which are infective forms to the final host. When the final host ingests the metacercariae-infected fish, the metacercariae excyst in the duodenum, migrate toward bile ducts and develop into adult worms[4].

People infected with a little number of *C. sinensis* worms (<100) are usually asymptomatic or show few clinical signs[1]. Moderate number of worms (<1,000) can cause similar or more pronounced clinical signs. Fever, chills, anorexia, weight loss, colic, fatigue, and abdominal distension may occur. When infected with a higher number of worms (1,000-25,000), people present these signs more prominently with an acute pain in the right upper quadrant of the abdomen. Chronic heavy infection with *C. sinensis* can result in biliary stones, gastrointestinal symptoms, cholangitis, jaundice and possibly cholangiocarinoma[5]. Recently, *C. sinensis* was classified as a group I carcinogen by the International Agency for Research on Cancer (IARC) as well as other parasitic trematodes, *Opisthorchis viverrini* and *Schistosoma haematobium*[6].

The sperm coating protein/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) superfamily is a large group of proteins containing a distinctive 3-layer alpha-beta-alpha sandwich structure domain called SCP/TAPS domain[7]. The SCP/TAPS proteins are present throughout the eukaryotic kingdom[8]. Although the SCP/TAPS domain has yet to be ascribed its activity, several superfamily members have provided strong evidence for the importance of these proteins in a wide range of biological processes[7].

The pathogenesis-related 1 (PR-1) family of the plant could be upregulated in response to infection with tobacco mosaic virus[8]. Mammalian SCP/TAPS domain are associated with biological processes such as lung development (rat lgl)[9], immune response (human CRISP-3)[10] and testis/sperm development[11]. Antigen 5 (Ag5) proteins, one of the 3 major allergens in hornet and yellow jacket venoms, was the most frequently studied SCP/TAPS protein of arthropods[12]. Other SCP/TAPS proteins were identified from the salivary gland of *Aedes aegypti* (yellow fever vector)[13], *Glossina morsitans* (sleeping sickness vector)[14], and *Anopheles gambiae* (malaria vector)[15]. SCP/TAPS proteins are also present in nematodes[7]. A number of parasitic nematodes from different taxonomic clades are known to secrete SCP/TAP proteins into the host during infection[7]. Several of these proteins possess immunomodulatory effects such as platelet aggregation inhibition (*Ancylostoma caninum* HPI)[16], neutrophil chemotaxis alteration (*Necator americanus* ASP-2)[17], and angiogenesis stimulation (*Onchocerca volvulus* ASP-1)[18]. SCP/TAPS proteins identified from nematodes and platyhelminths were named venom allergen-like (VAL) proteins, as there is a wide range of naming conventions for SCP/TAPS proteins depending on the species discussed[7].

McCrisp1, 2, 3 and 4 of a cestode, *Mesocestoides corti*, were the first reported platyhelminth VAL family members[19]. Among them, the full-length sequence was determined only in McCrisp2. McCrisp2 gene encoded a protein containing a signal peptide and complete SCP/TAPS domain. McCrisp2 expression in the apical region (where the frontal gland develops) in tetrathyridia of *M. corti* suggested that cestode

VALs could be involved in host/parasite inter-relationships[19]. From the trematode *Schistosoma mansoni* genome, 28 VAL proteins (SmVAL1 to 28) have been identified, which encode complete SCP/TAPS domains[20]. Combination of genomic, transcriptomic, phylogenetic and tertiary structural analyses discovered 2 distinct types of SCP/TAPS proteins, group1 and 2. Group 1 VAL proteins contain a signal peptide, 3 conserved disulfide bonds and an extended first loop region. Group 2 VAL proteins do not possess these features but contain highly conserved His and Tyr residues. These conserved amino acids are thought to help stabilize helices of SCP/TAPS domains by intramolecular hydrogen bond formation[20]. Upregulation of SmVAL gene family in the infective stage of *S. mansoni* suggests that they may be involved in the establishment of chronic host/parasite interactions[20]. Excreted/secreted SmVAL9 protein is known to be involved in tissue reorganization/extracellular matrix remodeling during intra-mammalian egg translocation, intra-molluscan sporocyst development/migration and miracidia infection[21]. Until now, 14 different VAL proteins of *C. sinensis* were found by intensive genomic search[7]. However, only a few studies on the *C. sinensis* VAL proteins were performed. A fragment of a VAL protein screened by *C. sinensis* EST database search showed an anti-allergic effect suggesting that the protein is a promising effector for the inhibition of allergic and inflammatory diseases[22].

In these respects, studies on the structural and immunological bases of *C. sinensis* VAL proteins are important. In this study, a VAL protein from *C. sinensis* was cloned.

To provide some insight into its biological role, tissue localization and immunological characterization were performed as well as structural analysis.

Ⅱ. MATERIALS AND METHODS

1. Parasite and animals

Rats were orally infected with 200 *C. sinensis* metacercariae, which were obtained from *Pseudorasbora parva* caught in Jinju, Gyeongsangnam-do, Korea. Six weeks after infection, adult *C. sinensis* worms were harvested from the bile ducts of the rats and stored at -70℃ or used immediately.

2. RNA extraction and cDNA construction

Total RNA was isolated from adult *C. sinensis* worms using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. First-strand cDNA was synthesized by reverse transcription-PCR.

3. Molecular cloning

The open reading frame (ORF) of CsVAL13 was amplified by polymerase chain reaction (PCR) using the indicated primers (Table 1). The PCR was performed with an initial denaturation at 94℃ for 5 min, 30 cycles of denaturation for 1 min at 94℃, annealing for 1 min at 60℃, extension for 1min at 72℃, and a final extension for 5 min at 72℃. The PCR products were electrophoresed on 1.2 % agarose gel and

purified. Purified PCR product was cloned into pCR4-Topo plasmid vector (Invitrogen), and sequenced (Genotech, Daejeon, Korea). After sequencing, the plasmid was digested by *Bam*HI and *Xho*I. Resulting fragment was cloned into prokaryotic expression vector pET-32a(+) (Merck KGaA, Darmstadt, Germany) (Fig. 1).

Table 1. Primers for cloning of CsVAL13

Primer	Sequence
CsVAL13 (Forward)	5'-GGATCCACCGTATTAAATAACGAAGGAATT-3'
CsVAL13 (Reverse)	5'-CTCGAGCTTTTTATTTCCGCCTTTC-3'

Restriction enzyme sites are underlined. *Bam*HI (GGATCC) and *Xho*I (CTCGAG)

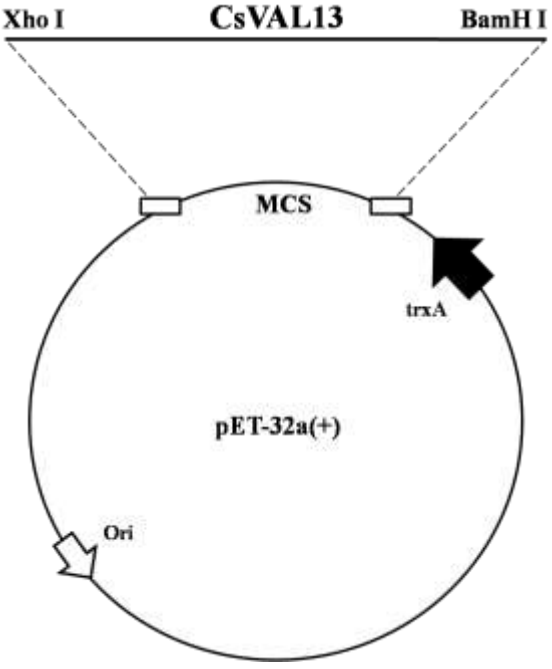
4. Sequence analysis and homology modeling

The sequences of various VAL proteins were obtained by the GenBank. Multiple sequence alignment of CsVAL13 was performed using a ClustalW 2.0. CLC Main Workbench 6.5 (CLC bio, Aarhus, Denmark) was used for drawing a phylogenetic tree. The homology model of CsVAL13 was generated in SWISS-MODEL using Golgi-associated pathogenesis-related protein 1 (GAPPR1) of humans (PDB ID 4aiw) as a template for modeling.

5. Production of recombinant protein

pET-32a(+) plasmids containing CsVAL13 were introduced into *Escherichia coli* BL21[DE3]. Overexpression of a recombinant protein (rCsVAL13) was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to the bacterial culture for 4 h at 37℃. Overexpressed rCsVAL13 was purified by Ni-NTA affinity chromatography (Qiagen, Hilden, Germany) under denaturation condition. The quality of the purified rCsVAL13 was examined by 10% SDS-PAGE.

Fig 1. Cloning strategy for bacterial expression of recombinant CsVAL13 protein.



6. Production of mouse immune sera

Six-week old female BALB/c mice were immunized intraperitoneally with 50 μg of rCsVAL13 mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Two weeks later, the mice were immunized again intraperitoneally with 50 μg of rCsVAL13 mixed with Freund's incomplete adjuvant (Sigma-Aldrich). Two weeks later, immunization was boosted by injecting 20 μg of rCsVAL13 into the tail vein of the mice. Three days later, the mice were sacrificed and immune sera against rCsVAL13 were harvested.

7. Immunolocalization of CsVAL13

Adult *C. sinensis* worms were washed extensively with phosphate buffered saline (PBS), fixed with 10% formalin, and embedded in paraffin blocks. Slide sections mounted onto glass slides were deparaffinized and rehydrated. The glass slides were blocked with 5% BSA in PBS-tween20 solution (PBST) for 1 h, and then incubated with rCsVAL13-mmunized mouse sera or normal mouse sera at the indicated dilution at room temperature for 1 h. After washing 3 times with PBST, sections were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Dako, Carpinteria, CA, USA) at room temperature for 1 h. The slides were washed 3 times with PBST, immunoreactivity was visualized using DAB reagent (Dako, Carpinteria,

CA, USA), and sections were then counterstained with hematoxylin. Color development on the slides was examined by light microscopy (Nikon, Tokyo, Japan).

8. Western blot analysis.

To determine the antigenic properties of CsVAL13, western blot analysis was performed using sera from rats infected with *C. sinensis* (n=10) experimentally. Two μg of purified rCsVAL13 was separated by 10% SDS-PAGE and subsequently transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane were cut into strips and blocked overnight with 5% 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 with TBST, the membrane was incubated with 1:4,000-diluted pooled sera from rats infected with *C. sinensis*, normal healthy controls (n=10) or 1:1,000-diluted anti-His antibody (Qiagen) in TBST containing 5% skim milk at room temperature for 1 h. After washing 3 times with TBST, the membrane strips were incubated with 1:20,000-diluted alkaline phosphatase (AP)-conjugated anti-rat IgG (Sigma-Aldrich) or 1:20,000- diluted AP-conjugated anti-mouse IgG (Sigma-Aldrich). After 3 times of washing, colors on the membrane strips were developed by incubating with BCIP/NBT as a substrate (Promega, Madison, WI, USA).

9. IgG-ELISA

The wells of a microplate were coated with 2 μg/ml of rCsVAL13 at 4℃ overnight, washed with PBST (135mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4mM KH_2PO_4, pH 7.4 with 0.05% Tween 20) containing 3% skim milk. The plate incubated at 37℃ for 1 h with sera of patients with clonorchiasis (n=10) and normal human sera (n=10) with PBS-T containing 3% skim milk (1:200) as a primary antibody. After the microplate were washed, the secondary antibody, 1:10,000-diluted AP-conjugated anti-mouse IgG (Sigma-Aldrich) was added to the wells. After washing with PBS-T containing 3% skim milk, P-nitrophenyl phosphate substrate (Sigma-Aldrich) was incubated at room temperature for 10 min. The reaction was stopped by adding 3 M NaOH, and then absorbance values of each well were measured at wavelength of 405 nm by using a microplate reader (Tecan, Salzburg, Austria).

Ⅲ. RESULTS

1. Cloning and sequence analysis

The Coding region of CsVAL13 cDNA was amplified by PCR and electrophoresed (Fig.2A). A single band of 670 bp appeared by the electrophoresis. The PCR product was purified and sequenced (Fig. 2B). Deduced sequence of CsVAL13 was composed with 223 amino acids. Its calculated molecular mass was 25.1 kDa. Isoelectric point of CsVAL13 protein was estimated to 9.602. Putative SCP/TAPS domain was found in the sequence.

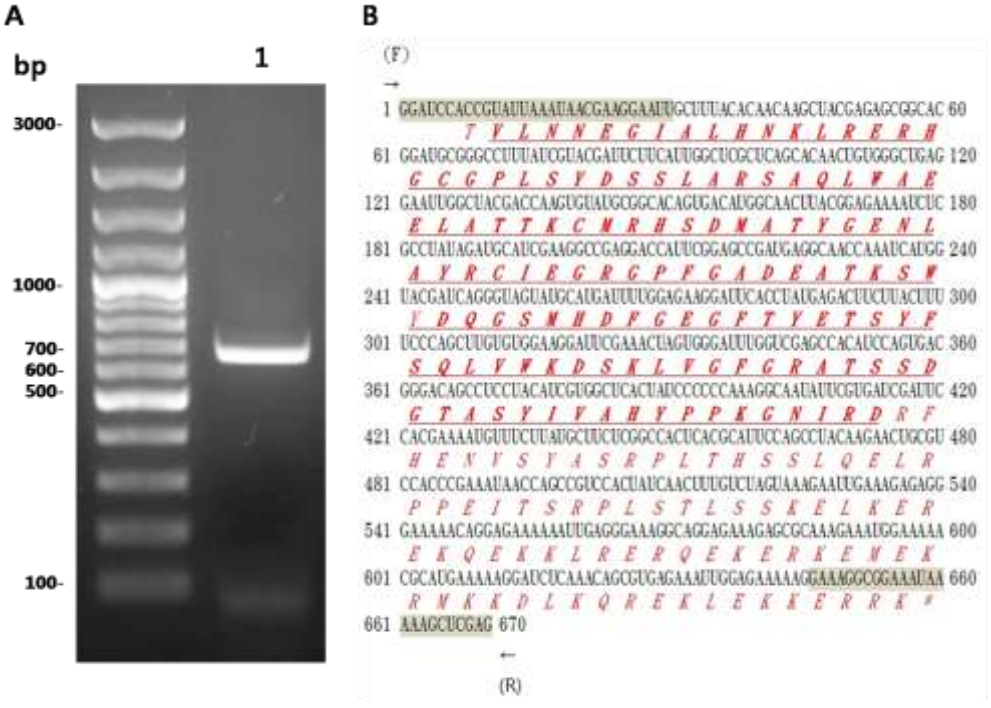


Fig. 2. PCR and sequence analysis of CsVAL13. 1.2% agarose gel electrophoresis of the PCR product (A). Sequence alignment of the cloned CsVAL13 (B). SCP/TAPS domain is underlined, the shades indicate forward and reverse primers.

There is no known signal peptide in the CsVAL13 sequence (Fig. 3A). Transmembrane domain was not shown in the CsVAL13 sequence. All the residues of CsVAL13 were predicted to be located outside of the cell membrane (Fig. 3B).

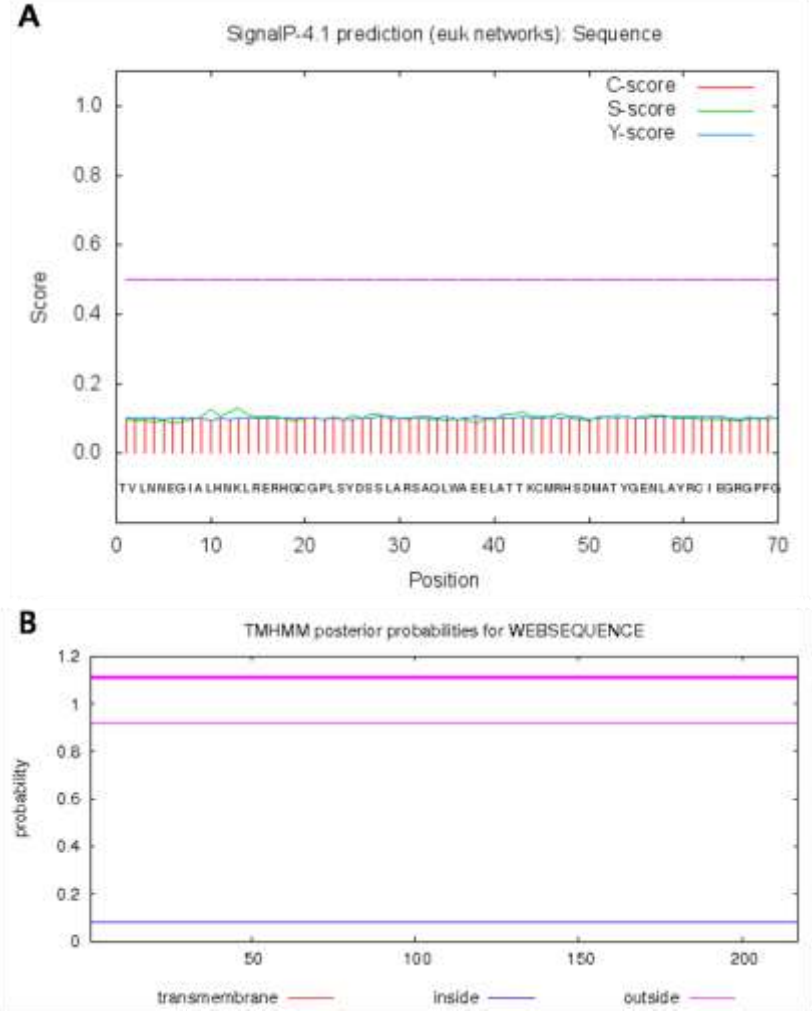


Fig. 3. Sequence analysis of CsVAL13 using TMHMM and SignalP 4.1 server. Transmembrane domain was predicted using TMHMM 2.0 server (A). Signal peptide was predicted using SignalP 4.1 server (B).

The open reading frame of CsVAL13 SCP/TAPS domain encodes 139 amino acids (Fig. 4). The CsVAL13 SCP/TAPS domain sequence shares 42–61% identity with group 2 VAL proteins in platyhelminths. His and Tyr residues known to contribute structural stability were highly conserved. Among the residues of putative active site in SCP/TAPS domain, Glu84 and His100 were replaced by Gln and Tyr, respectively.

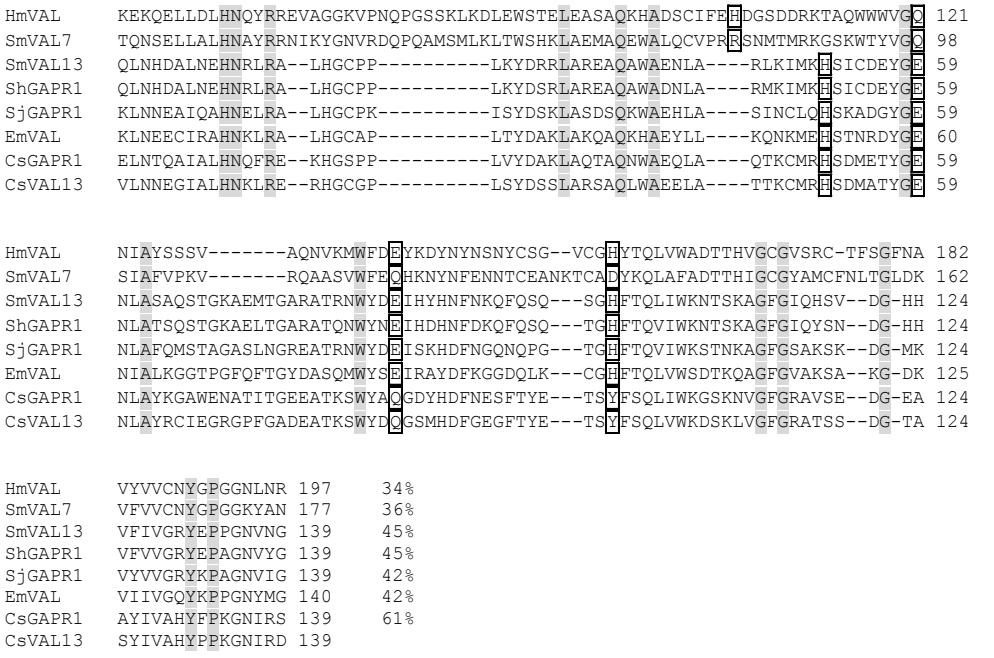


Fig. 4. Multiple sequence alignment of SCP/TAPS domains of various helminth VAL proteins. HmVAL *Hymenolepis microstoma* venom allergen protein (CDS32832); SmVAL17 *S. mansoni* VAL 7 protein (AAZ04924), SmVAL13 *S. mansoni* VAL 13 protein (CCD74793), ShGAPR1 *S. haematobium* Golgi-associated plant pathogenesis-related protein 1 (KGB36935), SjGAPR1 *S. japonicum* Golgi-associated plant pathogenesis-related protein 1 (CAX74107), EmVAL *Echinococcus multilocularis* venom allergen protein (CDJ02618), CsGAPR1 *C. sinensis* Golgi-associated plant pathogenesis-related protein 1 (GAA29512). Conserved amino acids are shaded. Four residues comprising the putative SCP/TAPS domain of the active site are boxed.

2. Phylogenetic analysis

A phylogenetic tree was constructed with the same sequences by the NJ method and revealed that CsVAL13 was clustered with CsGAPR1 (Fig. 5). These were classified as group 2 VAL proteins along with EmVAL, ShGAPR1, SmVAL13, and SjGAPR1. SmVAL7 and HmVAL were classified as Group group 1 VAL proteins.

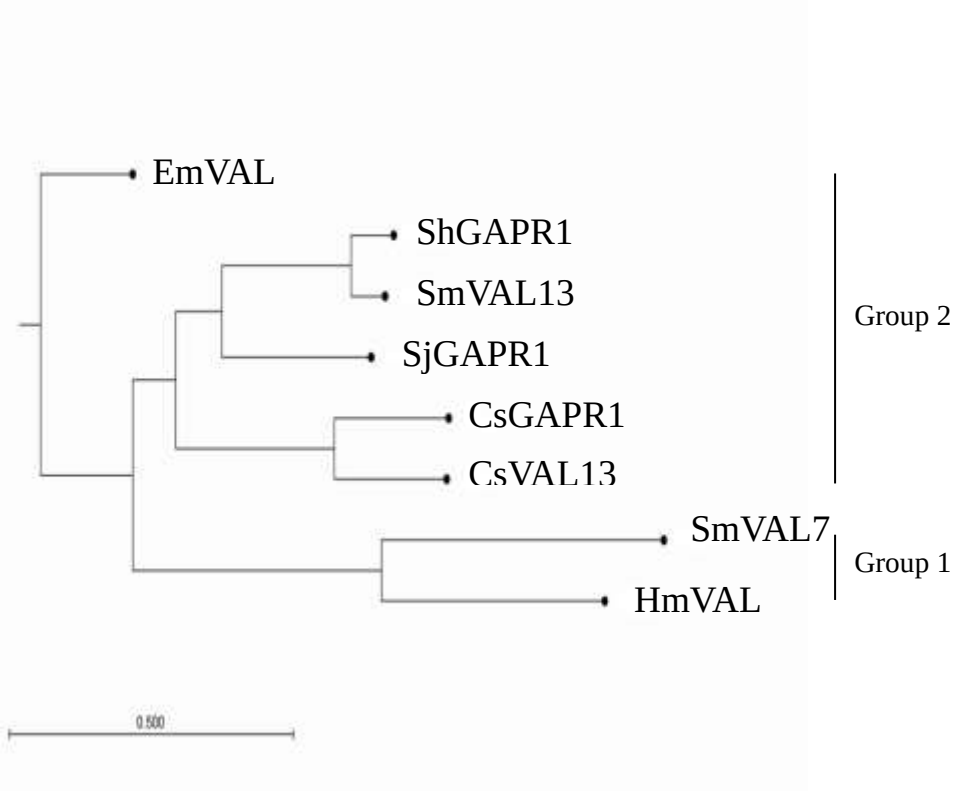


Fig. 5. Phylogenetic analysis of SCP/TAPS domain sequences of CsVAL13 and other VAL proteins. The phylogenetic tree was constructed using CLC Main Workbench 6.5 program. Bootstrap values were obtained with 100 replications.

3. Molecular modeling of CsVAL13

Homology model of CsVAL13 represented characteristic α-β-α sandwich structure of SCP/TAPS domain in the molecule. The length of N-terminal α helix was shorter than that of human GAPPRP1 used as template for modeling (Fig. 6A and B). Four residues (His51, Glu59, Gln84, and Tyr100) known to comprise putative active site of SCP/TAPS domain were closely associated and located on the surface of the main cleft (Fig. 6C).

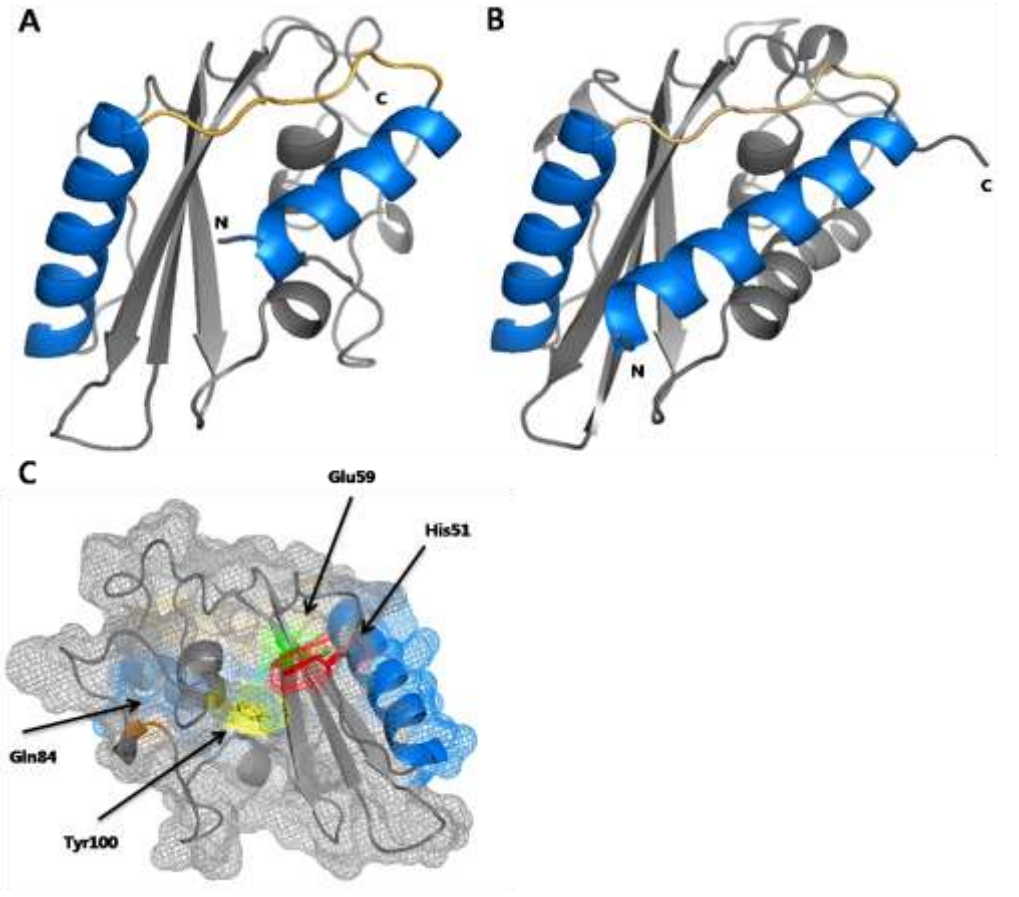


Fig. 6. Homology modeling of CsVAL13. Cartoon representations of CsVAL13 (A) and human GAPPRP1 (B). α-β-α sandwich structures were represented blue and yellow colors. N- and C-terminus of the sequence are indicated by N and C, respectively. Meshed strcuture of CsVAL13 (C) represented the suface localization of the four residues (His51, Glu59, Gln84, and Tyr100).

4. Production of a recombinant protein

The recombinant CsVAL13 protein (rCsVAL13) was produced bacterially and purified. The purified rCsVAL13 showed a single band of 41.9 kDa on a 12 % SDS-PAGE (Fig. 7A). Reactivity of rCsVAL13 to anti-His antibody was proved by western blot (Fig. 7B).

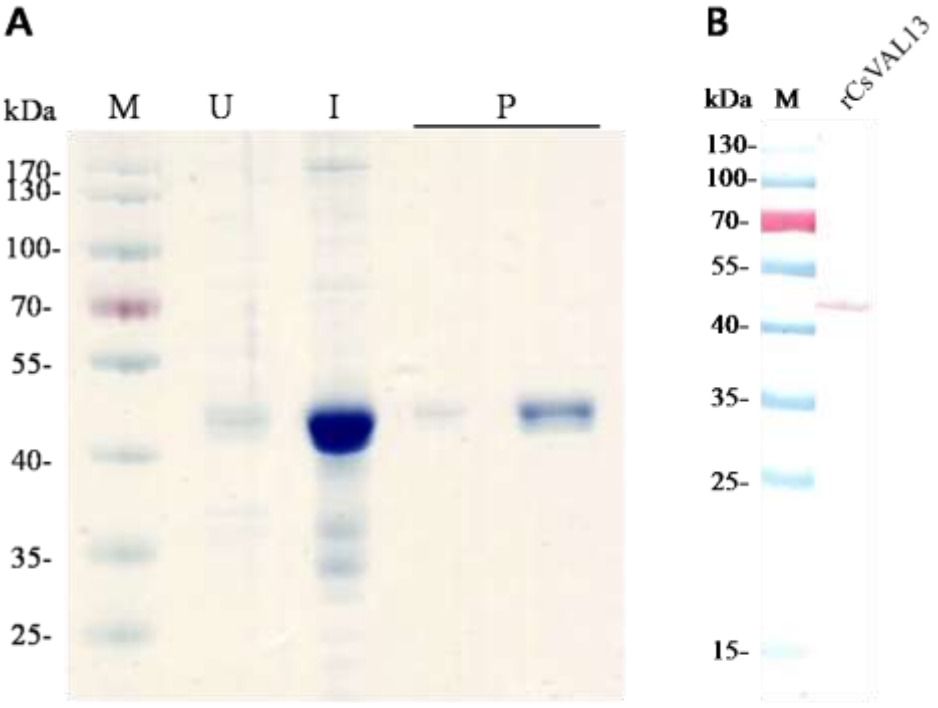


Fig. 7. Purification of rCsVAL13. 12% SDS-PAGE of the protein fractions collected during purification (A). M, pre-stained protein size marker; U, uninduced bacterial lysate; I, induced bacterial lysate; P, purified recombinant protein. Western blot analysis showing purified rCsVAL13 with anti-His antibody (B). M, pre-stained protein size marker.

5. Antigenicity of CsVAL13

Mouse immune sera were generated by immunizing BALB/c mice with purified rCsVAL13. The mouse immune sera reacted with rCsVAL13, whereas normal mouse sera had no reactivity (Fig. 8).

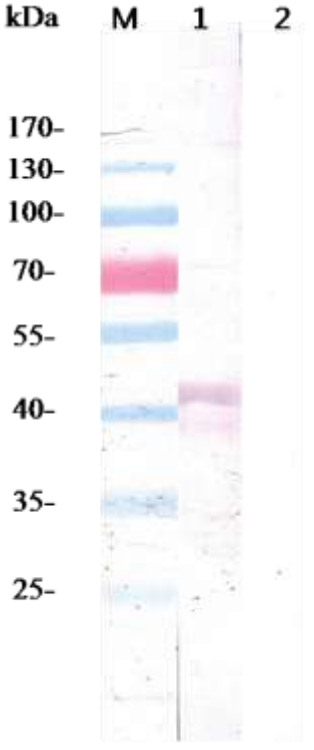


Fig. 8. Western blot analysis of rCsVAL13 with rCsVAL13-immunized mouse sera. Lane 1, rCsVAL13-immunized mouse sera; lane 2, normal mouse sera.

To examine antigenicity of rCsVAL13, western blot was performed with *C. sinensis*-infected rat sera (Fig. 9). rCsVAL13 reacted with anti-His antibody as well as with rCsVAL13-immunized mouse sera, but not with *C. sinensis*-infected rat sera.

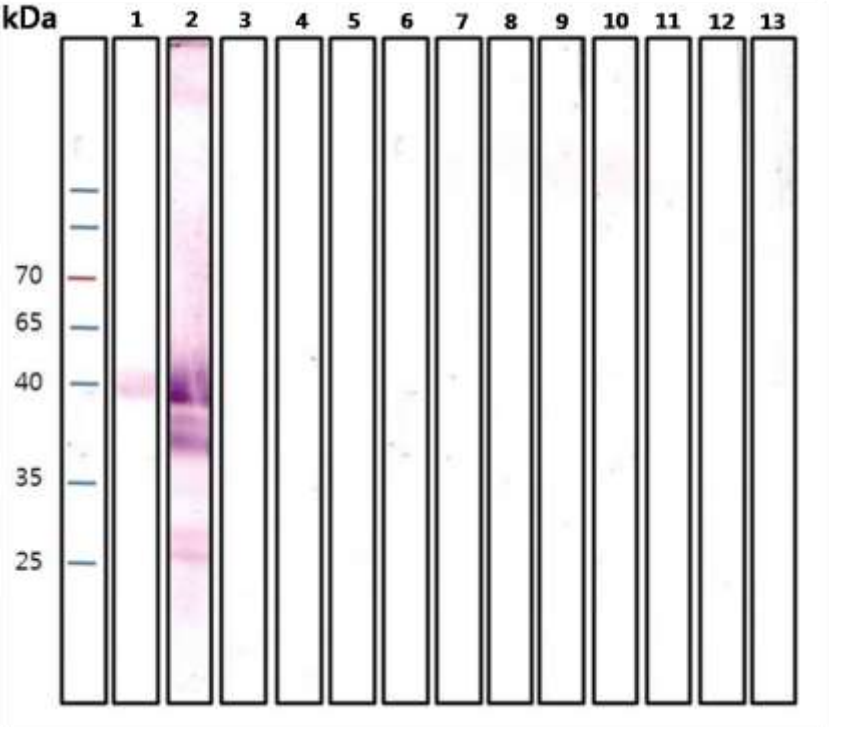


Fig. 9. Western blot analysis of rCsVAL13. Lane 1, anti-His antibody; lane 2, rCsVAL13-immunized mouse sera; lane 3~12, sera from each rat infected with *C. sinensis*; lane 13; sera from healthy rats.

ELISA values of rCsVAL13 against 10 *C. sinensis*-infected human sera and 10 normal human sera were 0.10 ± 0.00 and 0.10 ± 0.02, respectively (Fig. 10). With these results, rCsVAL13 was supposed to have no or little antigenicity.

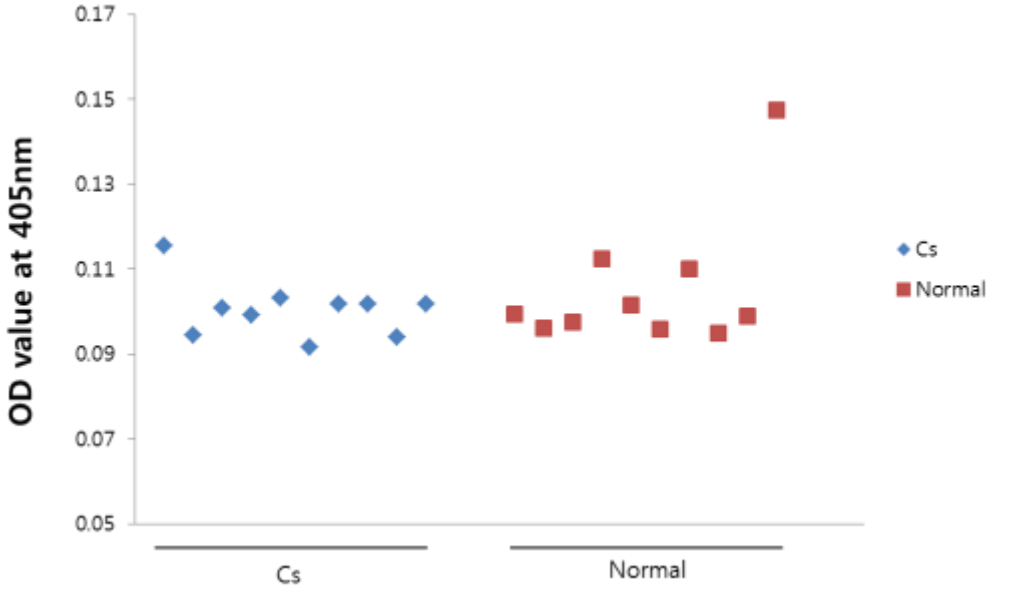


Fig. 10. ELISA of rCsVAL13 with human sera. Blue diamonds, sera from people infected with *C. sinensis* (n=10); red squares, sera from healthy people (n=10).

6. Immune responses of *C. sinensis*-immunized mice to CsVAL13

Levels of IgG1 and IgG2a in rCsVAL13-immunized mouse sera were examined by ELISA. ELISA value of IgG1 and IgG2a in the immunized sera were 0.73 ± 0.04 and 0.12 ± 0.00 , respectively. (Fig. 11). This result indicates that CsVAL13 may induce predominated Th2 immune responses in the host.

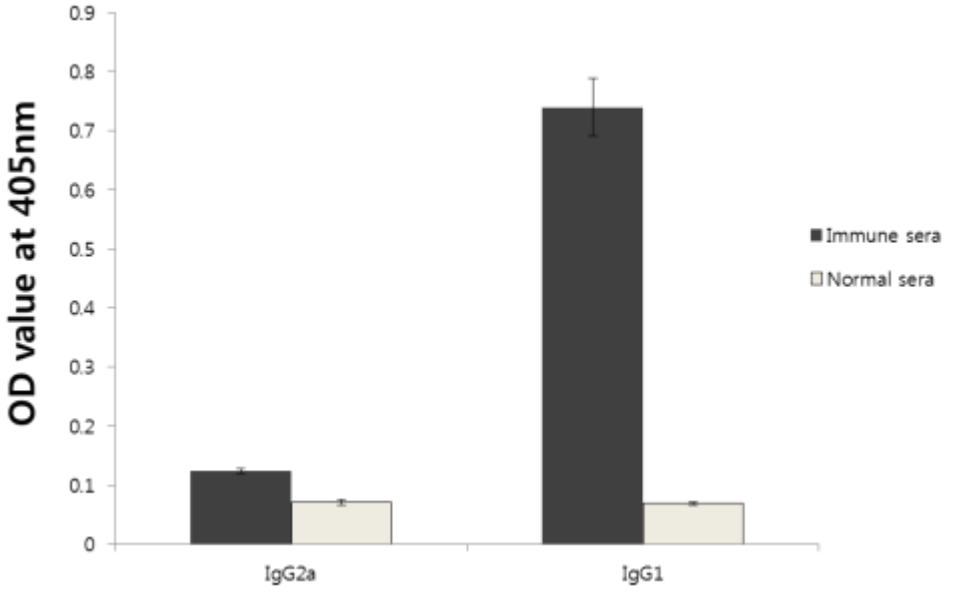


Fig. 11. IgG1 and IgG2a levels in rCsVAL13-immunized mouse sera.

7. Immunohistochemical localization

To localize native CsVAL13 protein in adult *C. sinensis* worm, immunohistochemistry using rCsVAL13-immunized mouse sera was performed. Brown colors were observed in the tegument and intrauterine eggs of an adult *C. sinensis* worm stained with rCsVAL13-immunized mouse sera (Fig. 12B and D). Normal mouse sera showed no colored signals (Fig. 12A and C).

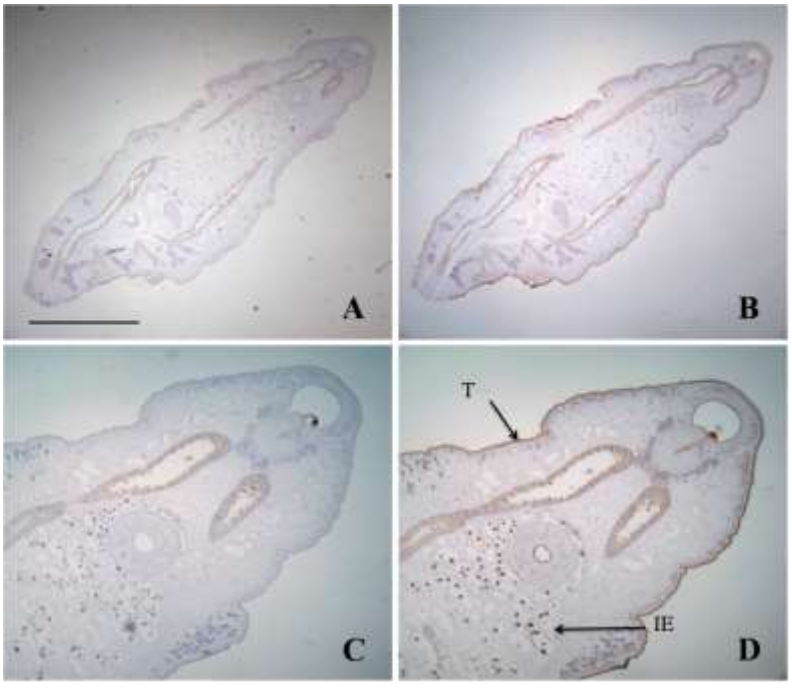


Fig. 11. Immunohistochemical localization of CsVAL13. Adult *C. sinensis* specimens were stained and visualized under a light microscope (**a** and **b**, ×40; **c** and **d**, ×100). **a** and **c**, specimen reacted with normal mouse sera; **b** and **d**, specimen reacted with rCsVAL13-immunized mouse sera. *Scale bar* 10 mm

Ⅳ. DISCUSSION

Studies on VAL proteins of trematodes have been mainly focused to *S . mansoni*[7] and *S. japonicum*[24]. In this study, molecular structures and distribution in the adult worm of the CsVAL13 protein, a newly identified VAL protein from *C. sinensis,* were examined.

The CsVAL13 sequence had no known signal peptides and transmembrane domain. However, its location was predicted to be outside of cell membrane (Fig. 3). Therefore, the CsVAL13 might be secreted to the outside of cells by a non-classical pathway like a secretion of nitric oxide synthase-interacting protein from *C. sinensis*[23].

SCP/TAPS domain sequence of CsVAL13 had conserved His and Try residue at its N-and C-terminus, respectively (Fig. 4). These conserved residues are supposed to be associated with stabilizing SCP/TAP domains by hydrogen bond formation as referred in SCP/TAPS domain of *S. mansoni* VAL proeins[20]. SmVAL13 protein has four active site residues (His51, Glu59, Glu84, and His100) in its SCP/TAPS domain[20]. In the CsVAL13 sequence, Glu84 and His100 were substituted by Gln and Tyr, respectively, which had similar biochemical characteristics. As shown in homology modeling, the active site residues were closely located on the surface of main cleft in the molecule (Fig. 6), suggesting Ca^{2+} chelation activity[25] are conserved.

SCP/TAPS domain of CsVAL13 was phylogenetically categorized as group 2 VAL protein (Fig. 5). Among the 14 VAL proteins found by intensive genomic search in *C. sinensis*, 6 VAL proteins were estimated as group 2 proteins[7].

rCsVAL13 did not reacted with *C. sinensis*-infected rat sera in the western blot (Fig. 9). This result indicated that the molecular proportion and/or antigencity of CsVAL13 in the adult *C. sinensis* worm may relatively low (Fig. 9). This low antigeneicty was also observed in the ELISA using *C. sinensis*-infected human sera (Fig. 10) implying there is no potential as a diagnostic antigen.

IgG1 level in the rCsVAL13-immunized mouse sera was higher that IgG2a level suggesting that CsVAL13 protein induce polarized Th2 immune responses in the host (Fig. 11). VAL-1 protein of *S. japonicum* also increased Th2 responses[24]. Th2-polarized immune responses are common in parasitic infection. However, further immunological characterization of VAL proteins is required to provide information on the helminth antigens contributing Th2 immune responses.

Native CsVAL13 proteins were found mainly in tegument and intrauterine eggs (Fig. 12). Tegument-specific localization implies that they might have a role in interaction with its surrounding environment. Excretion of CsVAL13 protein with eggs from adult worms may contribute immunological stimulation. McCrsp2 of *M. corti* was expressed in the apical region in tetrathyridia of *M. corti*, suggesting that the cestode VALs could be involved in host-parasite relationships[19]. VAL-1 protein of *S. japonicum* was secreted by eggs, head glands and penetration glands of cercariae suggesting its role in interaction with a host[24].

Until now, only a few studies on the *C. sinensis* VAL protein have been performed. There is no evidence that VAL proteins of trematodes including *C. sinensis* can modulate the host immune response as observed for the nematode VAL proteins. The

present study provides information regarding the biological role of *C. sinensis* VAL protein.

Ⅴ. CONCLUSION

1. Sequence of CsVAL13 had no known signal peptides and transmembrane domain. CsVAL13 was predicted to be located outside of cell membrane indicating that it may be translocated by non-classical pathways.

2. CsVAL13 protein was classified as a group 2 VAL protein.

3. CsVAL13 protein has four known putative active site residues located on the main cleft in the molecular surface.

4. CsVAL13 protein induced polarized Th2 immune responses in the host, since IgG1 level in the rCsVAL13-immunized mouse sera was higher than IgG2a level.

5. The native CsVAL13 protein was mainly located in tegument and intrauterine eggs suggesting that they might interact with environmental host tissues and stimulate immune responses.

Collectively, CsVAL13 is the first cloned *C. sinensis* VAL protein of which localization is egg and tegument-specific.

REFERENCES

1. Lun ZR, Gasser RB, Lai DH, Li AX, Zhu XQ, Yu XB, et al. Clonorchiasis: a key foodborne zoonosis in China. Lancet Infect Dis. 2005;5(1):31-41.

2. Hong ST, Fang Y. *Clonorchis sinensis* and clonorchiasis, an update. Parasitol Int. 2012;61(1):17-24.

3. Zhou J, Sun J, Huang Y, Zhou C, Liang P, Zheng M, et al. Molecular identification, immunolocalization, and characterization of *Clonorchis sinensis* calmodulin. Parasitol Res. 2013;112(4):1709-17.

4. Liang P, Zhang F, Chen W, Hu X, Huang Y, Li S, et al. Identification and biochemical characterization of adenylate kinase 1 from *Clonorchis sinensis*. Parasitol Res. 2013;112(4):1719-27.

5. Rim HJ. Clonorchiasis: an update. J Helminthol. 2005;79(3):269-81.

6. Shin HR, Oh JK, Masuyer E, Curado MP, Bouvard V, Fang YY, et al. Epidemiology of cholangiocarcinoma: An update focusing on risk factors. Cancer Sci. 2010;101(3):579-85.

7. Chalmers IW, Hoffmann KF. Platyhelminth Venom Allergen-Like (VAL) proteins: revealing structural diversity, class-specific features and biological associations across the phylum. Parasitology. 2012;139(10):1231-45.

8. van Loon LC, Gerritsen YA, Ritter CE. Identification, purification, and characterization of pathogenesis-related proteins from virus-infected Samsun NN tobacco leaves. Plant Mol Biol. 1987;9(6):593-609.

9. Oyewumi L, Kaplan F, Sweezey NB. Lgl1, a mesenchymal modulator of early lung branching morphogenesis, is a secreted glycoprotein imported by late gestation lung epithelial cells. Biochem J. 2003;376(Pt 1):61-9.

10. Udby L, Calafat J, Sørensen OE, Borregaard N, Kjeldsen L. Identification of human cysteine-rich secretory protein 3 (CRISP-3) as a matrix protein in a subset of peroxidase-negative granules of neutrophils and in the granules of eosinophils. J Leukoc Biol. 2002;72(3):462-9.

11. Fitzpatrick JM, Johnston DA, Williams GW, Williams DJ, Freeman TC, Dunne DW, et al. An oligonucleotide microarray for transcriptome analysis of *Schistosoma mansoni* and its application/use to investigate gender-associated gene expression. Mol Biochem Parasitol. 2005;141(1):1-13

12. LuG, Villalva M, Coscia MR, Hoffman DR, King TP. Sequence Analysis and Antigenic Cross-reactivity of a Venom Allergen, Antigen 5, from Hornets, Wasps, and Yellow Jackets. J Immunol. 1993;150(7):2823-30.

13. Valenzuela JG, Pham VM, Garfield MK, Francischetti IMB, Ribeiro JMC. Toward a description of the sialome of the adult female mosquito *Aedes aegypti*. Insect Biochem Mol Biol. 2002;9(32):1101-22.

14. Francischetti IMB, Valenzuela JG, Pham VM, Garfield MK, Ribeiro JMC. Toward a catalog for the transcripts and proteins (sialome) from the salivary gland of the malaria vector *Anopheles gambiae*. J Exp Biol. 2002;205(Pt 16):2429-51.

15. Li S, Kwon J, Aksoy S. Characterization of genes expressed in the salivary glands of the tsetse fly, *Glossina morsitans morsitans*. Insect Mol Biol. 2001;10(1):69-76.

16. Del Valle A, Jones BF, Harrison LM, Chadderdon RC, Cappello M. Isolation and molecular cloning of a secreted hookworm platelet inhibitor from adult *Ancylostoma caninum*. Mol Biochem Parasitol. 2003;2(129):167-77.

17. Bower MA, Constant SL, Mendez S. *Necator americanus*: The Na-ASP-2 protein secreted by the infective larvae induces neutrophil recruitment in vivo and in vitro. Exp Parasitol. 2008;118(4):569-75.

18. Tawe W, Pearlman E, Unnasch TR, Lustigman S. Angiogenic activity of *Onchocerca volvulus* recombinant proteins similar to vespid venom antigen 5. Mol Biochem Parasitol. 2000;109(2);91-9.

19. Britos L, Lalanne AI, Castillo E, Cota G, Señorale M, Marín M. *Mesocestoides corti* (syn. vogae, cestoda): Characterization of genes encoding cysteine-rich secreted proteins (CRISP). Exp Parasitol. 2007;116(2):95-102.

20. Chalmers IW, McArdle AJ, Coulson RM, Wagner MA, Schmid R, Hirai H, et al. Developmentally regulated expression, alternative splicing and distinct sub-groupings in members of the *Schistosoma mansoni* venom allergen-like (SmVAL) gene family. BMC Genomics. 2008;9:89.

21. Yoshino TP, Brown M, Wu XJ, Jackson CJ, Ocadiz-Ruiz R, Chalmers IW, et al. Excreted/secreted *Schistosoma mansoni* venom allergen-like 9 (SmVAL9) modulates host extracellular matrix remodelling gene expression. Int J Parasitol. 2014;44(8):551-63.

22. Jeong YI, Kim YJ, Ju JW, Hong SH, Lee MR, Cho SH, et al. Identification of anti-allergic effect of *Clonorchis sinensis*-derived protein venom allergen-like proteins (CsVAL). Biochem Biophys Res Commun. 2014;445(3):549-55.

23. Bian M, Li S, Wang X, Xu Y, Chen W, Xhou C, et al. Identification, immunolocalization, and immunological characterization of nitric oxide synthase-interacting protein from *Clonorchis sinensis*. Parasitol Res. 2014;113(5):1749-57.

24. Chen J, Hu X, He S, Wang L, Hu D, Wang X, et al. Expression and immune response analysis of *Schistosoma japonicum* VAL-1, a homologue of vespid venom allergens. Parasitol Res. 2010;106(6):1413-8.

25. Cantacessi C, Campbell BE, Visser A, Geldhof P, Nolan MJ, Nisbet AJ, et al. A portrait of the "SCP/TAPS" proteins of eukaryotes-developing a framework for fundamental research and biotechnological outcomes. Biotechnol Adv. 2009;27(4):376-88.

ABSTRACT (IN KOREAN)

간흡충 venom allergen-like protein 13 의 면역학적 특성

< 지도교수 용태순 >

연세대학교 대학원 의과학과

우 해 선

SCP/TAPS (sperm coating protein/Tpx-1/Ag5/PR-1/Sc7) 단백질은 진핵생물에서 발견되는 다기능 단백질이다. Venom allergen-like (VAL) 단백질은 주로 기생 연충에서 보고된 SCP/TAPS 단백질로서 그 생물학적, 면역학적 특성은 많이 알려지지 않았다. 본 연구에서는 간흡충의 VAL 단백질 한 종류의 유전자를 클로닝하고 특징을 알아보았다. 간흡충 EST 데이터베이스로부터 25 kDa 단백질을 코딩 하는 cDNA를 찾아냈다. 이 단백질은 BLAST 검색에서 만손주혈흡충의 VAL13 단백질과 46%의 서열유사성을 보였기에 CsVAL13이라 명명하였다. Multiple sequence alignment 결과 CsVAL13 단백질서열은 기생 연충의 VAL 단백질서열과 39~46% 유사하였다. 이들 기생 연충 VAL 단백질서열에는 이 단백질의 구조적 안정화에 기여하는 히스티딘과 타이로신 잔기들이 잘 보존되어 있었다. CsVAL13 단백질 서열은 다른 기생

연충의 Group 2 VAL 단백질 서열과 분자계통분류학적으로 같은 분기에 속했다. 사람 Golgi-associated plant pathogenesis-related protein 1 (PDB ID 4aiw)을 주형으로 하여 SWISS-MODEL로 예측한 CsVAL13의 3차구조는 VAL 단백질 특유의 α-β-α sandwich를 포함하고 있었다. PCR로 증폭한 CsVAL13 cDNA를 대장균 발현 플라스미드 벡터 pET32a에 서브클로닝 하였다. 대장균에서 생산된 재조합 CsVAL13 단백질(rCsVAL13)을 Ni-NTA 흡착 크로마토그래피로 정제하였다. 재조합 CsVAL13 단백질로 BALB/c 마우스를 면역시켜 면역혈청을 얻었다. 이 면역혈청이 IgG2a 항체보다 IgG1 항체를 더 많이 갖고 있는 것으로 보아 CsVAL13은 Th1보다 Th2 면역반응에 더 영향을 주는 것으로 생각된다. 면역조직염색 결과 CsVAL13 단백질은 간흡충 성충의 표피와 자궁 내 충란에 집중적으로 분포하였다. 이상의 결과는 간흡충 성충 표피에 특이하게 분포하는 CsVAL13이 숙주-기생충 상호작용의 한 부분을 담당하며, 주변 숙주조직에 대한 면역 자극에 기여할 가능성이 있음을 의미한다.

핵심되는 말: 간흡충, venom allergen-like 단백질, SCP/TAPS 단백질, 충란, 표피