

A tegument-specific venom allergen-like protein of *Clonorchis sinensis*

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Abstract Venom allergen-like (VAL) proteins, members of the SCP/TAPS (sperm coating protein/Tpx-1/Ag5/PR-1/Sc7) protein superfamily, have been reported from several parasitic helminths. As little is known about their biological functions, a VAL protein of the Chinese liver fluke *Clonorchis sinensis* was cloned and characterized. A complementary DNA (cDNA) encoding a 25-kDa protein was identified from an 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 the SCP/TAPS domain of CsVAL13 shares 39–46 % sequence identity with VAL proteins from parasitic helminths. His and Tyr, which help to stabilize protein structure, were highly conserved across the VAL protein sequences. Phylogenetic analysis showed that the SCP/TAPS domain of the CsVAL13 sequence clusters together with other group 2 VAL protein sequences. In the homology-modeled structure of CsVAL13, an α - β - α sandwich and residues for a putative active site were highly conserved. Immune sera were obtained from BALB/c mice immunized with the recombinant CsVAL13 protein. 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.

Keywords *Clonorchis sinensis* · SCP/TAPS · VAL protein · Tegument

Introduction

The Chinese liver fluke *Clonorchis sinensis* resides in intrahepatic bile ducts and causes clonorchiasis, which is endemic in the Far East (Rim 2005). Humans can be infected by eating raw or undercooked freshwater fish. Human clonorchiasis is generally asymptomatic in the case of light infection. However, moderate or heavy infection can cause inflammatory injury, tissue damage, and edema (Keiser and Utzinger 2004). Chronic inflammation due to infection with adult *C. sinensis* can provoke periductal fibrosis and even cholangiocarcinoma (Bouvard et al. 2009).

The sperm coating protein/Tpx-1/Ag5/PR-1/Sc7 (SCP/TAPS) protein family is found in a wide range of eukaryotes (Cantacessi et al. 2009). The extracellular SCP/TAPS domain participates in various signaling pathways by acting as a Ca^{2+} chelator (Fernández et al. 1997). SCP/TAPS domains are composed of a distinctive α - β - α sandwich structure (Chalmers and Hoffmann 2012) that contains four residues (His51, Glu59, Glu84, and His100) as a putative active site.

In animals, SCP/TAPS proteins are involved in immune signaling, testis/sperm development, envenomation, and parasitic nematode invasion (Chalmers et al. 2008). Venom allergen-like (VAL) proteins of the parasitic helminth, members of the SCP/TAPS protein family, contain two distinct types, groups 1 and 2 (Chalmers and Hoffmann 2012). Group 1 VAL proteins contain signal peptides, three conserved disulfide bonds, and an extended first loop region.

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Group 2 VAL proteins have no signal peptide but contain highly conserved His and Tyr residues, which are thought to help stabilize SCP/TAPS helix domains by intramolecular hydrogen bond formation.

The VAL gene family of *Schistosoma mansoni* is upregulated during the infective stages of the worm life cycle, suggesting that they may be involved in the establishment of chronic host-parasite interactions (Chalmers et al. 2008). The SmVAL9 protein of *S. mansoni* is localized to parenchymal cells/vesicles and larval germinal cells (Yoshino et al. 2014). Excreted/secreted SmVAL9 is thought to be involved in tissue reorganization and extracellular matrix remodeling during the worm life cycle.

Although the existence of 14 different VAL proteins was reported in *C. sinensis* by intensive genomic search (Chalmers and Hoffmann 2012), very few studies on their biological functions have been 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 (Jeong et al. 2014). In this study, a VAL protein from *C. sinensis* was cloned. To determine its localization and provide some insight into its biological role, immunohistochemical localization was performed.

Materials and methods

Parasites and animals

Rats were infected orally with 200 *C. sinensis* metacercariae, which were obtained from freshwater fishes caught at a reservoir 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°C or used immediately.

Cloning CsVAL13 cDNA

Total RNA was isolated from adult *C. sinensis* worms using Trizol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized using reverse transcription-PCR. The open reading frame (ORF) of CsVAL13 was amplified by PCR using the following primers: CsVAL13-F, 5'-GGATCCACCGTATTAAATAACGAAGGAATT-3', and CsVAL13-R, 5'-CTCGAGCTTTTATTTCCGCCTTTC-3', with an initial denaturation at 94°C for 5 min, 30 cycles of denaturation for 1 min at 94°C , annealing for 1 min at 60°C , extension for 1 min at 72°C , and a final extension for 5 min at 72°C . The amplified products were purified and sequenced. After sequencing, the amplified product was restricted by

*Bam*HI and *Xho*I and cloned into the prokaryotic expression vector pET-32a (Novagen).

Sequence analysis and homology modeling

The sequences of various VAL proteins were obtained from GenBank. Multiple sequence alignment of VAL proteins was performed in the ClustalW 2.0 program, with minor modifications. CLC Main Workbench 6.5 (CLC bio) was used to draw phylogenetic trees. The homology model of CsVAL13 was generated in SWISS-MODEL using Golgi-associated plant pathogenesis-related protein 1 (GAPPR1) of humans (PDB ID 4aiw) as a template for modeling.

Production of recombinant CsVAL13 protein

pET32a plasmids containing CsVAL13 were introduced to *Escherichia coli* BL21 (DE3). Overexpression of recombinant CsVAL13 protein (rCsVAL13) was induced by adding 1 mM IPTG to the bacterial culture and incubating for 4 h at 37°C . rCsVAL13 was purified by Ni-NTA affinity chromatography (Qiagen) under denaturation condition. The quality of the purified rCsVAL13 was examined by 10 % SDS-PAGE.

Production of mouse immune sera

Eight-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). Two weeks later, the mice were immunized again intraperitoneally with 50 μg of rCsVAL13 mixed with Freund's incomplete adjuvant (Sigma). Two weeks later, immunization was boosted by injection of 20 μg rCsVAL13 into the tail vein. Three days later, the mice were sacrificed and immune sera against rCsVAL13 were harvested.

Immunohistochemical localization of the native CsVAL13

Adult *C. sinensis* worms were washed extensively, fixed with 10 % formalin, and embedded in paraffin blocks. Slide sections were deparaffinized, rehydrated, and mounted onto glass slides. Following inactivation of endogenous peroxidases, slides were blocked with 5 % BSA for 1 h and then incubated with rCsVAL13-immunized mouse sera or normal mouse sera at the indicated dilution at RT for 1 h. After washing three times, sections were incubated with HRP-conjugated anti-mouse IgG (Dako) at RT for 1 h. The slides were washed three times, immunoreactivity was visualized using DAB reagent, and sections were then counterstained with hematoxylin. Color development on the slides was examined by light microscopy (Nikon).

HmVAL	KEKQELLDLHNQYRREVAGGKVPNQPGSSKLDLEWSTELEASQKHADSCIFEH	DGSDDRKTAQWWWVG	121		
SmVAL7	TQNSELLALHNAYRRNIKYGNVRDQQAAMSLKLTWSHKLAEWAQCVPRRS	NMTMRKGSKWTYVGC	98		
SmVAL13	QLNHDALEHNRRLRA--LHGCPP-----LKYDRRLAREAQAWAENLA----	RLKIMKH	SICDEYGE	59	
ShGAPR1	QLNHDALEHNRRLRA--LHGCPP-----LKYDSRLAREAQAWADNLA----	RMKIMKH	SICDEYGE	59	
SjGAPR1	KLNNEAIQAHNELRA--LHGCPK-----ISYDSKLASDSQKWAHLELA----	SINCLOH	SKADGYGE	59	
EmVAL	KLNEECIRAHNKLRA--LHGCA-----LTYDAKLAKQAQKHALEYLL----	KQNKMEH	STNRDYGE	60	
CsGAPR1	ELNTQAIALHNQFRE--KHGSP-----LVYDAKLAQTAQNWAELQA----	QTKCMRH	SDMETYGE	59	
CsVAL13	VLNNEGIALHNKLRE--RHGCGP-----LSYDSSLARSAQLWAEELA----	TTKCMRH	SDMATYGE	59	
HmVAL	NIAYSSSV-----AQNVKMWFD	EYKDYNYSNYCSG--VCGH	YTLVWADTTHVGC	VSRCTFSGFNA	182
SmVAL7	SIAFVPKV-----RQAASVWFE	CHKNYNFENNTCEANKTCAD	YKQLAFADTTHIGCGYAMC	FNLTGLDK	162
SmVAL13	NLASAQSTGKAEMTGARATRNWYDE	IHYHNFNKQFQSQ---	SGHFTQLIWKNTSKAGFG	IQHVS--DG-HH	124
ShGAPR1	NLATSQSTGKAELTGARATQNWYNE	IHDHNFQKQFQSQ---	TGHFTQVIWKNTSKAGFG	IQYSN--DG-HH	124
SjGAPR1	NLAFQMSLAGSLNGREATRNWYDE	EISKHDFNGQNPQ---	TGHFTQVIWKSTNKAGFG	SAKSK--DG-MK	124
EmVAL	NIALKGGTPGFQFTGYDASQMWYSE	IRAYDFKGGDLK---	CGHFTQLVWSDTKQAGFG	VAKSA--KG-DK	125
CsGAPR1	NLAYKGAWENATITGEEATKSWYAC	GDYHDFNESFTYE---	TSYFSQLIWKSGKNVGF	GRAVSE--DG-EA	124
CsVAL13	NLAYRCIEGRPGFGADEATKSWYD	QSMHDFGEGFTYE---	TSYFSQLVWKDSKLVGF	GRATSS--DG-TA	124
HmVAL	VYVVCNYGPGGNLNR	197	34%		
SmVAL7	VFVVCNYGPGGKYAN	177	36%		
SmVAL13	VFIVGRYEPGPNVNG	139	45%		
ShGAPR1	VFVVGRYEPAGNVYG	139	45%		
SjGAPR1	VYVVGRYKPAENVIG	139	42%		
EmVAL	VIIVGQYKPPGNMYG	140	42%		
CsGAPR1	AYIVAHYFPKGNIRS	139	61%		
CsVAL13	SYIVAHYPPKGNIRD	139			

Fig. 1 Multiple sequence alignment of SCP/TAPS domains of various helminth VAL proteins. *HmVAL* *Hymenolepis microstoma* venom allergen protein (CDS32832); *SmVAL7* *S. mansoni* VAL 7 protein (AAZ04924), *SmVAL13* *S. mansoni* VAL 13 protein 13 (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

Results

Sequence alignment and phylogenetic analysis

The ORF of *CsVAL13* cDNA encodes a polypeptide that is 223 amino acids long. The estimated molecular mass and isoelectric point of the polypeptide were 25.1 kDa and 9.602, respectively. The *CsVAL13* SCP/TAPS domain sequence shares 42–61 % identity with group 2 VAL proteins in platyhelminths (Fig. 1). Sequence identities to group 1 VAL proteins were relatively low. His and Tyr residues, which contribute to structural stability, were highly conserved. Among the four amino acids known to comprise the active

site of the SCP/TAPS domain, Glu84 and His100 were substituted by Gln84 and Tyr100, respectively. A phylogenetic tree was constructed with the same sequences by the NJ method and revealed that *CsVAL13* was clustered with *CsGAPR1* (Fig. 2). These were classified as group 2 VAL proteins along with *EmVAL*, *ShGAPR1*, *SmVAL13*, and *SjGAPR1*. *SmVAL7* and *HmVAL* were classified as group 1 VAL proteins.

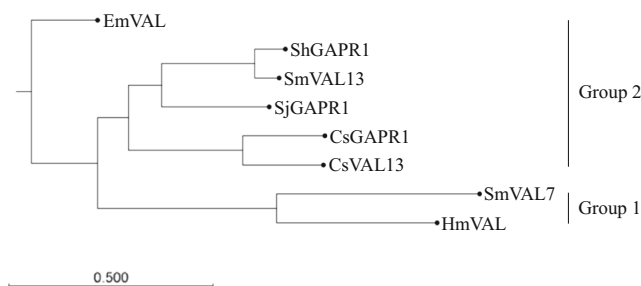


Fig. 2 Phylogenetic analysis of SCP/TAPS domain sequences of various helminth VAL proteins

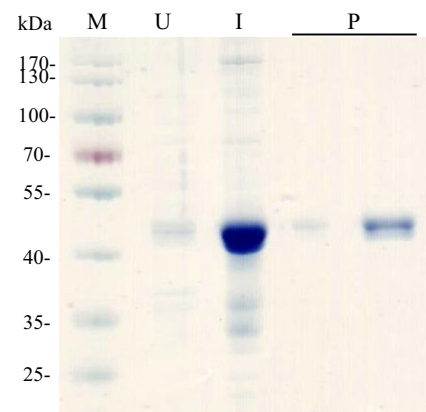


Fig. 3 Purification of recombinant *CsVAL13* protein. *M* prestained protein size marker, *U* uninduced bacterial lysate, *I* induced bacterial lysate, *P* purified recombinant protein

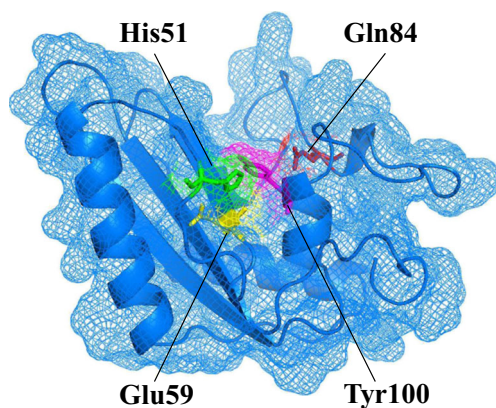
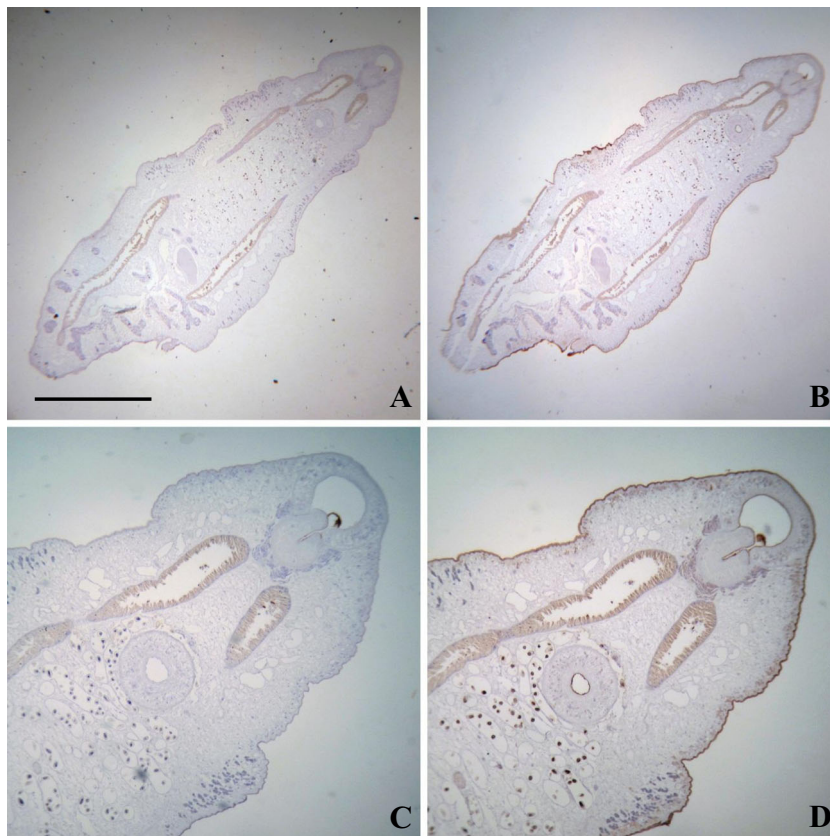


Fig. 4 Modeled structure of CsVAL13. Four residues comprising the putative SCP/TAPS domain active site are indicated

Molecular structure

The molecular structure was generated by homology modeling using the human GAPPR1 structure as a template (Fig. 3). An α - β - α sandwich structure, which is characteristic of SCP/TAPS domains, was conserved in the structure. Four residues (His51, Glu59, Gln84, and Tyr100) known to comprise the active site of the SCP/TAPS domain were closely associated and located on the surface of the main cleft.

Fig. 5 Immunohistochemical localization of CsVAL13. Adult *C. sinensis* specimens were stained and visualized under a light microscope (**a** and **b**, $\times 40$; **c** and **d**, $\times 100$). **a** and **c** specimen reacted with preimmune mouse sera; **b** and **d** specimen reacted with rCsVAL13-immunized mouse sera. Scale bar 10 mm



Immunohistochemical localization of CsVAL13

To investigate tissue localization of native CsVAL13, rCsVAL13 was generated in bacteria (Fig. 4). Immune sera obtained from mice immunized with rCsVAL13 were used for immunohistochemical localization of native CsVAL13 (Fig. 5). The immune sera stained the tegument and intrauterine eggs of adult *C. sinensis* worm. Normal mouse sera showed no positive staining.

Discussion

In this study, the CsVAL13 sequence coded for no known signal peptides and transmembrane domain. However, its location was found to be outside the cell membrane (Fig. 2), suggesting that CsVAL13 might be translocated to the outside of the cell membrane via a non-classical pathway. A *C. sinensis* protein with a non-classical translocation pathway is nitric oxide synthase-interacting protein (Bian et al. 2014). Granulin of *Opisthorchis viverrini* is also secreted without signal peptides (Smout et al. 2009; Smout et al. 2011).

The SCP/TAPS domain sequence of CsVAL13 had a conserved His and Try residue at its N- and C-terminus, respectively (Fig. 3). These conserved residues are associated with

the stabilization of SCP/TAP domains by hydrogen bond formation as referred in the SCP/TAPS domain of *S. mansoni* VAL proteins (Chalmers et al. 2008). SmVAL13 has four active site residues (His51, Glu59, Glu84, and His100) in its SCP/TAPS domain. In the CsVAL13 sequence, Glu84 and His100 are substituted by Gln84 and Tyr100, which have similar biochemical characteristics. As shown by homology modeling, the active site residues are closely associated on the surface of the large cleft in the molecule (Fig. 5), suggesting that its Ca^{2+} chelation activity (Fernández et al. 1997) is conserved.

Tegument-specific localization of native CsVAL13 protein (Fig. 5) implies that they might interact with environmental host tissues. Excretion of CsVAL13 protein with eggs from adult worms may contribute to immune stimulation. Among the VAL protein family, McCrisp1, 2, 3, and 4 of the cestode *Mesocostoides corti* (Britos et al. 2007), McCrisp2 was expressed in the apical region in tetrathyridia of *M. corti*, suggesting that cestode VALs could be involved in host-parasite relationships. VAL proteins from taxonomically different nematodes are known to be secreted during infection (Chalmers and Hoffmann 2012); they possess immunomodulatory effects (Del Valle et al. 2003; Bower et al. 2008) and can stimulate angiogenesis (Tawe et al. 2000). *Schistosoma japonicum* VAL-1 is secreted by eggs, head glands, and penetration glands of cercariae, supporting its role in host interaction with a host (Chen et al. 2010).

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.

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References

- Bian M, Li S, Wang X, Xu Y, Chen W, Zhou C, Chen X, He L, Xu J, Liang C, Wu Z, Huang Y, Li X, Yu X (2014) Identification, immunolocalization, and immunological characterization of nitric oxide synthase-interacting protein from *Clonorchis sinensis*. *Parasitol Res* 113:1749–1757
- Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Coglianov V, WHO International Agency for Research on Cancer Monograph Working Group (2009) A review of human carcinogens—part B: biological agents. *Lancet Oncol* 10:321–322
- Bower MA, Constant SL, Mendez S (2008) *Necator americanus*: the Na-ASP-2 protein secreted by the infective larvae induces neutrophil recruitment in vivo and in vitro. *Exp Parasitol* 118:569–575
- Britos L, Lalanne AI, Castillo E, Cota G, Señorale M, Marín M (2007) *Mesocostoides corti* (syn. *vogae*, cestoda): characterization of genes encoding cysteine-rich secreted proteins (CRISP). *Exp Parasitol* 116:95–102
- Cantacessi C, Campbell BE, Visser A, Geldhof P, Nolan MJ, Nisbet AJ, Matthews JB, Loukas A, Hofmann A, Otranto D, Sternberg PW, Gasser RB (2009) A portrait of the “SCP/TAPS” proteins of eukaryotes—developing a framework for fundamental research and biotechnological outcomes. *Biotechnol Adv* 27:376–388
- Chalmers IW, Hoffmann KF (2012) Platyhelminth venom allergen-like (VAL) proteins: revealing structural diversity, class-specific features and biological associations across the phylum. *Parasitology* 139: 1231–1245
- Chalmers IW, McArdle AJ, Coulson RM, Wagner MA, Schmid R, Hirai H, Hoffmann KF (2008) Developmentally regulated expression, alternative splicing and distinct sub-groupings in members of the *Schistosoma mansoni* venom allergen-like (SmVAL) gene family. *BMC Genomics* 9:89
- Chen J, Hu X, He S, Wang L, Hu D, Wang X, Zheng M, Yang Y, Liang C, Xu J, Yu X (2010) Expression and immune response analysis of *Schistosoma japonicum* VAL-1, a homologue of vespid venom allergens. *Parasitol Res* 106:1413–1418
- Del Valle A, Jones BF, Harrison LM, Chadderdon RC, Cappello M (2003) Isolation and molecular cloning of a secreted hookworm platelet inhibitor from adult *Ancylostoma caninum*. *Mol Biochem Parasitol* 129:167–177
- Fernández C, Szyperski T, Bruyère T, Ramage P, Mössinger E, Wüthrich K (1997) NMR solution structure of the pathogenesis-related protein P14a. *J Mol Biol* 266:576–593
- Jeong YI, Kim YJ, Ju JW, Hong SH, Lee MR, Cho SH, Lee WJ, Lee SE (2014) Identification of anti-allergic effect of *Clonorchis sinensis*-derived protein venom allergen-like proteins (CsVAL). *Biochem Biophys Res Commun* 445:549–555
- Keiser J, Utzinger J (2004) Chemotherapy for major food-borne trematodes: a review. *Expert Opin Pharmacother* 5:1711–1726
- Rim HJ (2005) Clonorchiasis: an update. *J Helminthol* 79:269–281
- Smout MJ, Laha T, Mulvenna J, Sripa B, Suttiaprapa S, Jones A, Brindley PJ, Loukas A (2009) A granulin-like growth factor secreted by the carcinogenic liver fluke, *Opisthorchis viverrini*, promotes proliferation of host cells. *PLoS Pathog* 5:e1000611
- Smout MJ, Mulvenna JP, Jones MK, Loukas A (2011) Expression, refolding and purification of Ov-GRN-1, a granulin-like growth factor from the carcinogenic liver fluke, that causes proliferation of mammalian host cells. *Protein Expr Purif* 79:263–270
- Tawe W, Pearlman E, Unnasch TR, Lustigman S (2000) Angiogenic activity of *Onchocerca volvulus* recombinant proteins similar to vespid venom antigen 5. *Mol Biochem Parasitol* 109:91–99
- Yoshino TP, Brown M, Wu XJ, Jackson CJ, Ocádiz-Ruiz R, Chalmers IW, Kolb M, Hokke CH, Hoffmann KF (2014) Excreted/secreted *Schistosoma mansoni* venom allergen-like 9 (SmVAL9) modulates host extracellular matrix remodelling gene expression. *Int J Parasitol* 44:551–563