

CLONING, CHARACTERIZATION, AND EXPRESSION OF VITELLINE PROTEIN BI AND ITS ENCODING GENE IN THE LIVER FLUKE, *FASCIOLA GIGANTICA*

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Abstract. A cDNA containing a 813 bp open reading frame encoding vitelline protein BI (FgVPBI) of *Fasciola gigantica* was cloned. FgVPBI has 96% sequence identity with VPBI of *Fasciola hepatica* and 84% identity with VPBII *F. hepatica*. It is far less similar to eggshell precursor proteins of other trematode species, for example, 29% identity with *C. sinensis*. Northern blot hybridization of total RNA from adult parasites demonstrated a FgVPBI transcript with a size of 1,000 nucleotides. FgVPBI mRNA is localized in the vitelline cells in both vitelline glands and intrauterine eggs. Recombinant FgVPBI was expressed as a 31.5 kDa protein in *Escherichia coli* and used for production of a polyclonal antiserum in rabbits. The FgVPBI antiserum detected immunoblotted rFgVPBI and native eggshell precursor protein at molecular weights of 31.5 kDa and 31 kDa, respectively. Immunolocalization showed strong staining in the cytoplasm of vitelline cells, in eggshell globules and the shells of eggs.

INTRODUCTION

The liver fluke *Fasciola gigantica* is the most prevalent single parasite infection of ruminants in the tropics with an incidence as high as 30-90% in Africa, 25-100% in West Asia (Iran, India), and 25-90% in Southeast Asia (Indonesia, Thailand) (Edney and Muchlis, 1962; Soesetya, 1975; Fabiyi, 1987; Sukhapesna *et al*, 1990, 1994; Spithill *et al*, 1999). The parasite can cross infect humans, and at least 2.4 million are infected with an estimated 180 million people at risk worldwide (Chitchung *et al*, 1982; Murice, 1994;

Anonymous, 1995). Infection with *F. gigantica* occurs when the host ingests the infective metacercariae, which then develop into adults within 15-16 weeks and start to release eggs into the bile ducts (Spithill *et al*, 1999). A mature liver fluke produces an average of 2,500 eggs per day (Happich and Boray, 1969). Each egg is composed of an ovum surrounded by approximately 30 vitelline cells (Stephenson, 1947; Rao, 1959). The fertilized egg is enclosed in a very strong shell, without which, the embryo would be exposed to the host's immune system and digestive enzymes, as well as the hostile environment (Irwin and Threadgold, 1972). On studying the eggshell formation in *Schistosoma* spp, Wells and Cordingley (1991) proposed that three major components, eggshell proteins, phenol oxidase tanning enzyme, and phenol oxidase activating enzyme, are

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packed together in the same membrane bound vesicle within the vitelline cells. The eggshell is formed from sclerotins or quinone-tanned proteins which results in the formation of a stable network of cross-linked proteins (Cordingley, 1987). The cross-linking reactions also change color and impart autofluorescence to the eggshell components (Kelly and von Lichtenberg, 1970). Eggshell precursor proteins are major antigens released by vitelline cells and can be identified by staining their 3, 4-dihydroxyphenylalanine (DOPA) residues with nitrite molybdate (Waite and Benedict, 1984; Waite and Rice-Ficht, 1987). Three groups of eggshell proteins have been isolated from *Schistosoma mansoni* (Johnson *et al*, 1987; Bobek *et al*, 1988; Michel *et al*, 2003; Ebersberger *et al*, 2005). The first two groups are SmP48 and SmP14 (Chen *et al*, 1992), and the third group is the SmP34 family (Ebersberger *et al*, 2005). In *F. hepatica*, eggshell precursor proteins were identified and designated as vitelline proteins (VP) A, B and C, with molecular weights of 70, 31, and 17 kDa, respectively (Waite and Rice-Ficht, 1987, 1989; Zurita *et al*, 1987, 1989; Wells and Cordingley, 1991; Rice-Ficht *et al*, 1992). These eggshell precursor proteins were localized in the vitelline cells and their synthesis by these cells has been confirmed (Koster *et al*, 1988; Kawanaka, 1991). Vitelline protein B is the major eggshell precursor protein with a molecular weight of 31 kDa and pI of 7.4. It represents about 6-7% of the total protein in mature *F. hepatica*, and it is unique in containing a rather high level of DOPA residues, which are converted from tyrosine residues during co- or post-translational processing by tyrosyl 3-hydroxylase (Waite and Rice-Ficht, 1987). Two closely related vitelline protein B isoforms exist, designated as VPBI and VPBII (Rice-Ficht *et al*, 1992). Both proteins contain 272 amino acid residues (MW 31 kDa) with a high content of glycine, tyrosine, aspartate, histidine and

lysine residues. Using RNA *in situ* hybridization, transcripts of the encoding genes were detected in cells within the adult vitelline follicles (Rice-Ficht *et al*, 1992). Equivalent vitelline proteins and their encoding genes in *F. gigantica* have not yet been identified, thus this study aims to identify the vitelline B proteins and their encoding genes, since these proteins may be antigens in the host blood and body fluid and have potential for use in immunodiagnosis.

MATERIALS AND METHODS

RT-PCR

Partial *F. gigantica* VPB encoding cDNAs were generated by reverse transcription PCR. First strand cDNA was synthesized from total RNA extracted from adult *F. gigantica* by reverse transcription using the reverse primer 5' GGA GTC CCT CGC GTA ATC ATC 3' designed from conserved sequences of *Fasciola hepatica* VPB. Double stranded 600 bp cDNAs were synthesized by PCR using the reverse transcription product as template. In detail, 10 µl RT-reaction product, 9 µl PCR buffer, 6 µl 50 mM MgCl₂, 1 µg forward primer (5' GAC TCC TAT GGC AAA TAC G 3'), 1 µl 10 mM dNTPs, 0.5 µl of Taq DNA polymerase (5 µ/µl) and autoclaved distilled water to a final volume of 100 µl, were mixed and incubated in a thermal cycler for 30 cycles at 94°C denaturing temperature, 55°C annealing temperature, and 72°C polymerization temperature, for one minute each step. The PCR products were inserted into pGEM-T easy vector (Promega, Madison, WI), and *E. coli* XL1-Blue was then transformed with the ligation product. Plasmid DNA was extracted from transformant bacterial colonies and analyzed for correctly-sized inserts by agarose gel electrophoresis. The nucleotide sequence of the inserted cDNAs was determined by the Bioservice Unit, BIOTEC (Thailand).

cDNA library screening

Plaque lifts (50,000 pfu/150 mm plate) of an adult stage *F. gigantica* cDNA library in λ ZAP II (kindly supplied by Dr Terry Spithill, McGill University, Montreal, Canada) were hybridized with a DIG-labeled RNA probe synthesized from the partial FgVBP cDNA obtained by RT-PCR (see section RT-PCR). Positive plaques were picked and the inserted cDNAs amplified by PCR using T3 and T7 primers. The PCR products were ligated into pGEM-T easy vector. After transformation of *E. coli* XL1-Blue, plasmid DNA was isolated and the inserted cDNAs sequenced as described above. Plasmids containing the complete coding sequence for vitelline protein were kept for further experiments.

Northern blot hybridization

Twenty-five μ g of the total RNA extracted from adult *F. gigantica* was resuspended in denaturing buffer containing 2.2 M formaldehyde, 50% formamide and incubated for 5 minutes at 65°C, quickly cooled on ice and separated by 1.2% agarose-formaldehyde gel electrophoresis at 90 volt for 4 hours. The RNA was then transferred to a nylon membrane and fixed by UV activated-cross linking for 3 minutes. Finally, the membrane was hybridized with a DIG-labeled FgVPB antisense RNA probe at 60°C overnight. An anti-DIG alkaline phosphatase conjugate and NBT/BCIP substrates were used for colorimetric detection of the bound probe.

In situ hybridization

Adult parasites were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and placed on glass slides coated with poly-L lysine. The sections were deparaffinized and rehydrated in DEPC-treated water. Hydrated sections were immersed in 0.1 mM DEPC treated PBS, pH 7.4, and digested with proteinase K (10 μ g/ml in TE buffer) for 15 minutes, washed with PBS

and prehybridized in 4X SSC containing 50% (v/v) deionized formamide buffer at 60°C for 30 minutes. After that, the sections were incubated in hybridization buffer containing 100 ng/ml of DIG-labeled FgVPB antisense RNA probe at 60°C for 14 hours. The sections were washed, and the presence of hybridization products in various tissues was detected as described above.

Expression and purification of recombinant *F. gigantica* vitelline protein B

The vitelline protein B encoding cDNA was cloned into the pET-30b⁽⁺⁾ expression vector (Novagen, Sandiego, CA) and the recombinant plasmid was introduced into *E. coli* BL21 (DE3) by transformation. Transformants were grown in 10 ml LB medium containing kanamycin (30 μ g/ml) at 37°C until the OD₆₀₀ reached 0.6-1.0. Then, the cells were harvested by centrifugation at 5,000g for 5 minutes at 4°C and resuspended in 200 ml fresh LB medium with kanamycin (30 μ g/ml) and incubated by shaking at 37°C for 3 hours. IPTG was added to the final concentration of 1 mM and the incubation was continued for 3 hours. After centrifugation the cell pellets were resuspended in 0.25 culture volume of cold 20 mM Tris-HCl pH 8.0 and centrifuged as before. The pellet was resuspended in 4 ml ice-cold 1X Binding buffer per 100 ml culture volume (100 mM NaH₂PO₄, 10 mM Tris-HCl, 6 M urea, pH 8) (Novagen) sonicated on ice using six 10-second bursts at high intensity with a 10-second cooling period between each burst. The lysate was centrifuged at 3,000g for 15 minutes to get rid of cellular debris. The supernatant was collected after centrifugation at 16,000g for 30 minutes at 4°C, and filtered through a 0.45 μ m membrane. The filtered supernatant was loaded into a Ni-NTA column (Novagen) and after three washes (100 mM NaH₂PO₄, 10 mM Tris-HCl, 6 M urea, pH 6.3) the recombinant protein was eluted (100 mM NaH₂PO₄, 10

mM Tris-HCl, 6 M urea, pH 4.5). Samples of collected fractions were analyzed by 12.5% SDS-PAGE and immunoblotting. The eluted recombinant protein was concentrated using Amicon ultra-15 centrifugal filter units (Millipore, Billerica, MA) and kept at -20°C until used.

Production of polyclonal antibodies (PoAb) against recombinant vitelline protein B (rFgVPB) for immunolocalization of the corresponding native protein

Female New Zealand white rabbits, eight weeks old, were subcutaneously injected with 50 µg of purified rFgVPB in 250 µl of PBS mixed with complete Freund adjuvant (Sigma, St Louis, MO). Animals were boosted twice with 50 µg of purified rFgVPB mixed with incomplete Freund adjuvant at 2 week intervals. Ten days after the third immunization; the animals were bled and checked for antibody production by ELISA.

Eggshell proteins and whole worm extract

Adult *F. gigantica* were washed several times with 0.85% NaCl solution, and vitelline glands were dissected out. The eggshell protein (ESP) was extracted using acid-urea as previously described by Waite and Rice-Ficht (1987). The whole worm proteins were extracted by placing the worms in chilled homogenization buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% Triton X-100), 10 mM phenylmethylsulfonylfluoride (PMSF) and 10 mM ethylene diamine tetraacetic acid (EDTA), homogenized, and sonicated by three 5 minutes cycles in the ice-bath. After rotation at 4°C for 2 hours, the suspension was centrifuged at 10,000g, at 4°C for 1 hour. The supernatant was collected and used as whole worm proteins. Protein concentrations were determined by Lowry's method (Bio-Rad, Hercules, CA) and the proteins were analyzed by 12.5% SDS-PAGE. Finally they were blotted onto nitrocellulose membranes and probed with

rabbit polyclonal antibody against rFgVPB.

Immunoblotting

The protein samples were separated by 12.5% SDS-PAGE, transferred electrophoretically onto nitrocellulose membrane (Hybond™-ECL™, Amersham Biosciences, Uppsala, Sweden) using a blotting apparatus (Hoefer™ TE 22, Amersham Biosciences) for 1 hour at 20 volts. Nonspecific binding to the membrane was blocked by incubation in 5% nonfat dry milk in a phosphate-buffered saline solution (PBS-T: 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, pH 7.5, 0.1% vol/vol Tween 20) for 2 hours. The blocked membrane was incubated with rabbit anti-rFgVPB antiserum diluted to 1:20,000 in PBS-T for 2 hours at room temperature. After three washes, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed Lab, South San Francisco, CA) diluted to 1:20,000 in PBS-T for 1 hour. Excess antibody was then removed by extensive washing, and bound antibody was visualized by TMB (3,3',5,5'-tetramethylbenzidine) substrate solution (Sigma-Aldrich).

Immunohistochemical detection

Adult parasites embedded in paraffin blocks were cut at 5 µm dewaxed and rehydrated. Parasite eggs collected from uterus were washed with 0.01 M PBS. Eggs and tissue sections were heated in 10 mM citric acid, pH 6.0 in a microwave oven at 700 W, 3 times, 5 minutes each. Endogenous peroxidase activity was blocked by immersing the sections in 3% H₂O₂ in absolute methanol for 30 minutes. Non-specific binding sites were blocked by incubating sections in PBS supplemented with 0.1% glycine and 4% BSA for 30 minutes each. After blocking, the samples were incubated with rabbit anti-rFgVPB antiserum diluted at 1:200 for 2 hours at room temperature. Horseradish peroxidase-conjugated goat anti-rabbit IgG

was used as secondary antibody at a dilution of 1:400 for 1 hour at room temperature. Color was developed using AEC (3-amino-9-ethylcarbazole) substrate solution (Zymed Lab). The color reaction was stopped by washing several times with tap water and the sections were observed under a light microscope.

RESULTS

Cloning and characterization of cDNAs encoding vitelline protein BI

A 600 bp FgVPB RT-PCR product was used as a probe to isolate FgVPBI (GenBank

accession no. [EF108313](#)) that contained a full-length open reading frame (813 nucleotides) from an adult stage cDNA library. Analysis of its deduced amino acid sequence (270 amino acids) showed that FgVPBI had a high content of glycine (14.1%) and tyrosine (12.6%) residues, had a molecular weight of 30.85 kDa and pI of 8.83 (EMBOSS pepstats), and was predicted to contain signal peptides (SignalP) at positions 1-18 in the amino acid sequence (Fig 1).

The orthologs of *F. gigantica* protein are highly conserved in *F. hepatica*, especially in vitelline B1 [96% amino acid (aa) sequences

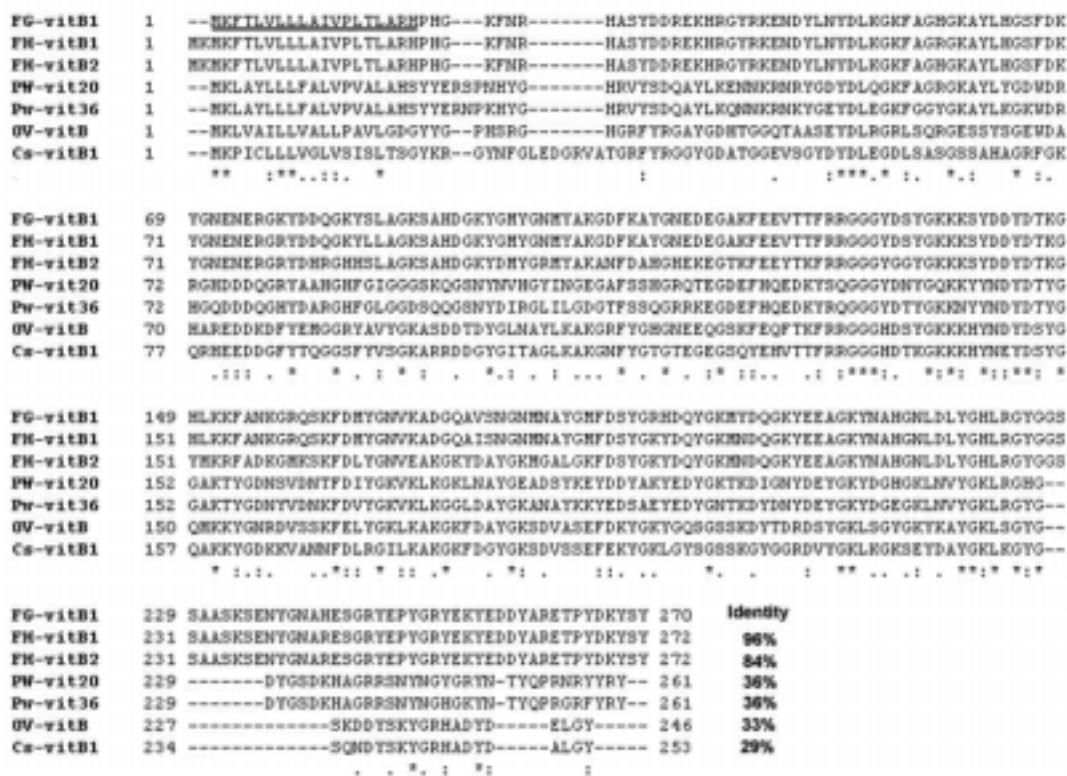


Fig 1—Alignment of deduced amino acid sequences of *Fasciola gigantica* vitelline protein BI with those of *Fasciola hepatica* (Fh-VitB1, [AAA29143](#) and Fh-VitB2, [AAA29144](#)), *Paragonimus westermani* (Pw-Vit20, [AAX32916](#) and Pw-Vit36, [AAX32917](#)), *Opisthorchis viverrini* (Ov-VitB, [AAL23712](#)), and *Clonorchis sinensis* (Cs-VitB1, [AAN64160](#)) using Clustal W program. A hyphen (-) represents gaps in the alignment. Lower asterisk (*) identifies residues that are identical in all sequences, ‘:’ and ‘.’ refer to residues that are moderately and lowly conserved, respectively. The signal peptides predicted by Signal P program are underlined. The identity values of each of eggshell protein against Fg-VitBI are shown at the end of each of polypeptide sequence.

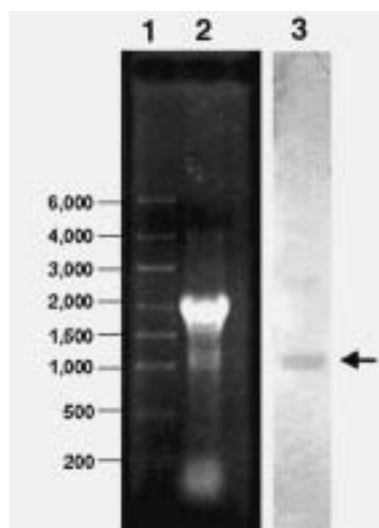


Fig 2—A photograph of 1.2% agarose gel electrophoresis of marker RNAs (lane 1) and total RNA of adult *F. gigantica* (lane 2), and Northern blot (lane 3) hybridized with vitelline BI-RNA probe which showed a single positive band at 1,000 nt.

identity with Fh-VitB1] but a search by NCBI-BLASTP for related eggshell proteins from other trematodes found only far less similar proteins in *Paragonimus westermani* (Pw-Vit20 and Vit36) (Bae *et al*, 2007), *Clonorchis sinensis* (Cs-VitB1) (Tang *et al*, 2005) and vitelline B precursor protein of *Opisthorchis viverrini* (Ov-VitB) (GenBank accession no. [AAL23712](#)) as shown in Fig 1.

Expression of vitelline protein BI encoding gene

Northern hybridization of total RNA extracted from adult *F. gigantica* with a FgVPBI antisense RNA probe showed a single transcript of 1,000 nucleotides in size (nt) (Fig 2). RNA *in situ* hybridization of parasite tissue sections using the same probe showed positive staining within the cells of vitelline follicles and eggs. In the vitelline follicles, positive staining was detected in the

developing vitelline cells from stage 1 to mature vitelline cells (stage 5) as classified by Meepool *et al* (2006). Stage 1 vitelline cells stained lightly, but this increased with increasing stages and was the most intense in mature vitelline cells (stage 5) (Fig 3A). The staining was also intense in the vitelline cells that were incorporated into eggs (Fig 3B). The negative control showed no staining when a FgVPBI sense RNA probe was used (Fig 3C, D).

Synthesis and purification of recombinant vitelline protein BI (rFgVPBI)

Recombinant vitelline protein BI of *F. gigantica* (rFgVPBI) was expressed in *E. coli* BL21 (DE3). Bacterial cell lysates prepared after induction with IPTG showed additional bands at 31.5 kDa and 31 kDa in SDS-PAGE compared with lysates of noninduced bacterial cells. The recombinant protein was contained in the insoluble protein fraction and, therefore, purified by Ni-NTA affinity chromatography under denaturing conditions. A major 31.5 kDa protein and a minor 31 kDa protein were obtained (Fig 4A).

Identification and localization of native vitelline protein BI by rabbit anti-rFgVPBI antiserum

In immunoblots, the rabbit anti-rFgVPBI antiserum reacted with purified rFgVPBI at molecular weights of 31.5 and 31 kDa and acid-urea extracted native eggshell precursor protein at a molecular weight of 31 kDa, but not with whole body extract (WB) (Fig 4B). The distribution of native vitelline protein BI antigen in adult parasite tissues was detected by immunoperoxidase staining after antigen binding with rabbit anti-rFgVPBI antiserum. Staining was intense in the vitelline cells within the vitelline glands and those incorporated in the eggs and eggshells. The vitelline follicles showed varying levels of staining intensity in the vitelline cells. Stage 1 cells stained lightly, stage 2 cells stained with mod-

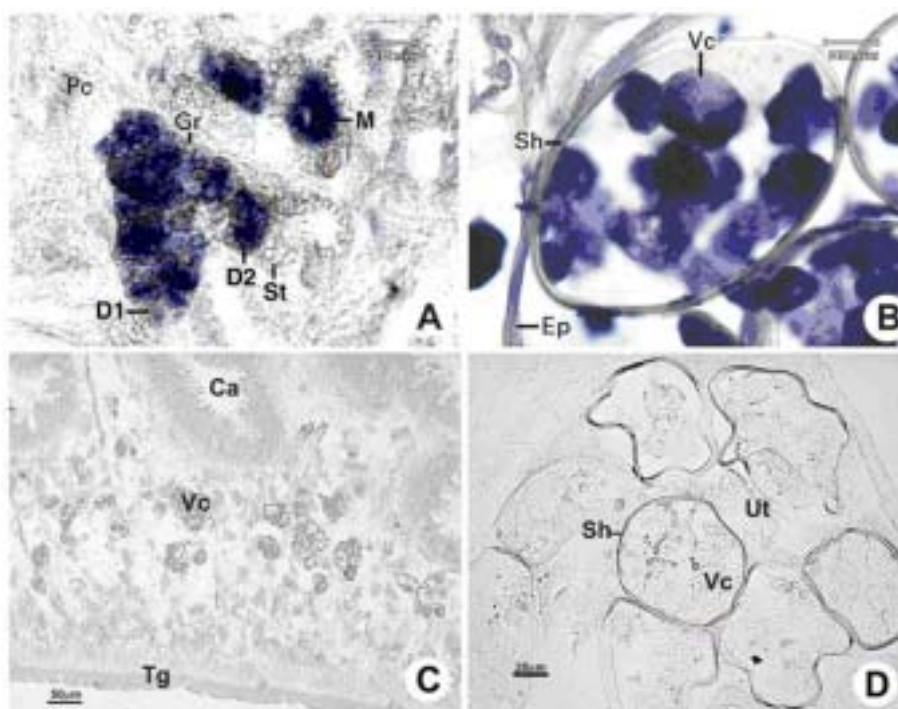


Fig 3—RNA *in situ* hybridization of adult *F. gigantica* tissue sections with a *F. gigantica* vitelline protein BI (FgVPBI) digoxigenin-labeled antisense RNA probe. (A) High magnification of vitelline follicle in vitelline glands, showing positive stain particularly, in developing vitelline cells (Vc) stage 1 (D1), stage 2 (D2) and mature stage (M), whereas the earliest stem cell (St) and parenchyma (Pc) are not stained. (B) High magnification of intrauterine eggs, showing intense staining in the vitelline cells inside the eggshell. (C) and (D) sections hybridized with FgVPBI sense RNA probe as negative control. Gr, granule; Sh, eggshell; Ep, uterine epithelium; Ut, uterus; Ca, cecum; Tg, tegument.

erate intensity, and mature cells stained with the highest intensity. Stem cells showed no staining (Fig 5B). In the uterus different levels of red stainings of AEC were observed in the vitelline cells around the ovum (Fig 5D). The outer surface of the shell of mature eggs was also stained (Fig 5F) while control sections showed no staining in eggs or vitelline cells within the vitelline gland and uterus (Fig 5A, C, E).

DICUSSION

Trematodes protect their eggs from host immune reactions and environment by a

tough, chemically resistant scleroprotein eggshell. Eggshell precursor proteins are synthesized and stockpiled in the vitellaria of these parasites (Stephenson, 1947). They have been studied in many trematode species, including *F. hepatica* (Waite and Rice-Ficht, 1987, 1989; Zurita *et al*, 1987, 1989; Rice-Ficht *et al*, 1992), *S. japonicum* (Sukiyama *et al*, 1997), *S. mansoni* (Chen *et al*, 1992), *P. westermani* (Bae *et al*, 2007), *O. viverrini* (Ruangsittichai *et al*, 2006), and *C. sinensis* (Yang *et al*, 2000; Lee *et al*, 2005; Tang *et al*, 2005). Isolated *F. gigantica* vitelline protein BI cDNA has high identity values with *F. hepatica* vitelline BI and BII cDNA sequences

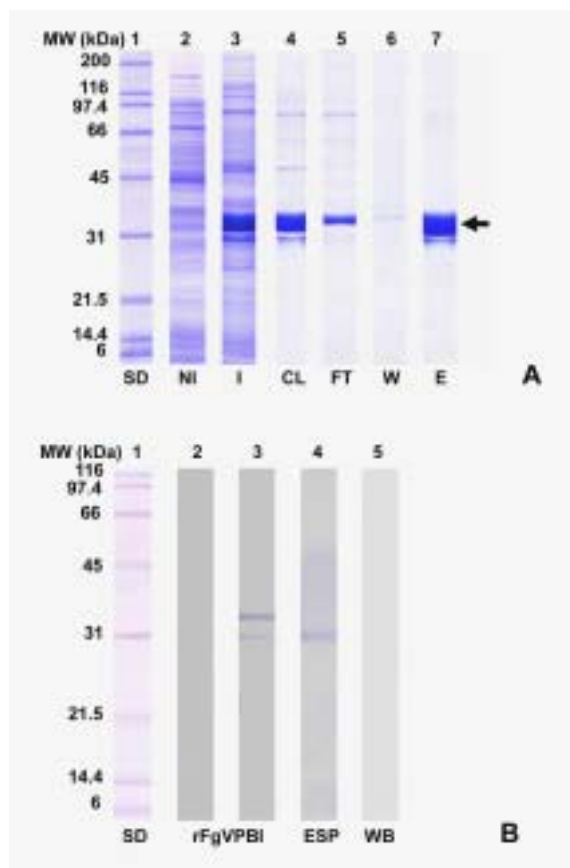


Fig 4—A) Purification of a recombinant vitelline protein BI of *F. gigantica* (rFgVPBI) by Ni-NTA affinity chromatography. Lane 1, standard protein markers (SD); lane 2, non-induced fraction (NI); lane 3, induced by IPTG fraction (I); lane 4, lysate of the expressing cells (CL); lane 5, flow-through fraction (FT); lane 6, washing fraction (W); lane 7, elution fraction (E); recombinant rFgVPBI is visible as a prominent band at 31.5 kDa (arrow). (B) Immunoblot of *F. gigantica* proteins. The PoAb against rFgVPBI reacted with rFgVPBI at the molecular weight of 31.5 and 31 kDa in lane 3 and acid-urea extracted native eggshell precursor protein (ESP) at the molecular weight 31 kDa in lane 4, but not with the whole body extract (WB) in lane 5, while no positive band appeared when the sera from a normal rabbit (NS) was used as a control in lane 2. SD (lane 1) was the lane containing standard molecular weights.

at 92% and 81%, respectively, and its translated amino sequence shows 96% and 84% identity with *F. hepatica* vitelline protein BI and BII sequences, respectively. Much lower identity values were found when compared to the eggshell protein of *P. westermani* (36%) (Bae *et al*, 2007), the vitelline B precursor protein of *O. viverrini* (33%) (GenBank accession no. [AAL23712](#)), and with the vitelline B precursor protein B1 of *C. sinensis* (29%) (Tang *et al*, 2005).

The deduced amino acid sequence of FgVPBI is shown in Fig 1 in comparison to the trematode eggshell proteins mentioned above. It is rich in glycine and tyrosine residues, similar to other eggshell proteins (Fig 1). The amino acid composition of the native vitelline protein B of *F. hepatica* showed a low content of tyrosine residues (2.1%) but a high content of DOPA (10.6%) (Rice-Ficht *et al*, 1992). The authors suggested that the tyrosine residues were converted to DOPA by tyrosyl 3-hydroxylase, and that these residues were the most important in the tanning process during eggshell formation. The pI of the native vitelline B of *F. hepatica* was 7.4 (Rice-Ficht *et al*, 1992) which was lower than the theoretical pI of 8.83 calculated for FgVPBI.

Northern hybridization using a FgVPBI antisense RNA probe revealed a single 1,000 nt transcript in adult stage total RNA. In comparison, Rice-Ficht *et al* (1992) observed transcripts between 950 nt and 1,400 nt size with a FhVPBI cDNA probe, which were interpreted as being due to differential processing. The sizes of the mRNAs of other trematode eggshell protein encoding genes varied from a 2,000 nt transcript for the pBCS44 gene in *C. sinensis* (Tang *et al*, 2005) to a 800 nt transcript for the OvESP gene in *O. viverrini*. (Ruangsittichai *et al*, 2006). By *in situ* hybridization, FgVPBI mRNA was localized in the cytoplasm of vitelline cells in both vitelline glands and in the eggs

within the uterus, whereas hybridization signals were not observed in other tissues. Depending on the developmental stages of the vitelline cells as described previously by Meepool *et al* (2006), the hybridization signal varied between low and high intensity. In the vitelline follicle, positive staining was detected in the developing vitelline cells from stage 1 to mature stage vitelline cells. In stage 1 vitelline cells, the staining was light which implied the presence of a small amount of the transcript. The staining in-

creased in stage 2 cells, which started to synthesize eggshell globules, and became most intense in mature cells whose cytoplasm was packed with eggshell globules (Meepool *et al*, 2006). The staining was also intense in vitelline cells that were incorporated in the eggs. Similar to both Pw-Vit20 and Vit36 eggshell protein in *P. westermani*, the positive signals were scattered around globule-like structures in vitelline follicles and were distributed evenly throughout the inner part of eggs when probed with Pw-Vit antisense

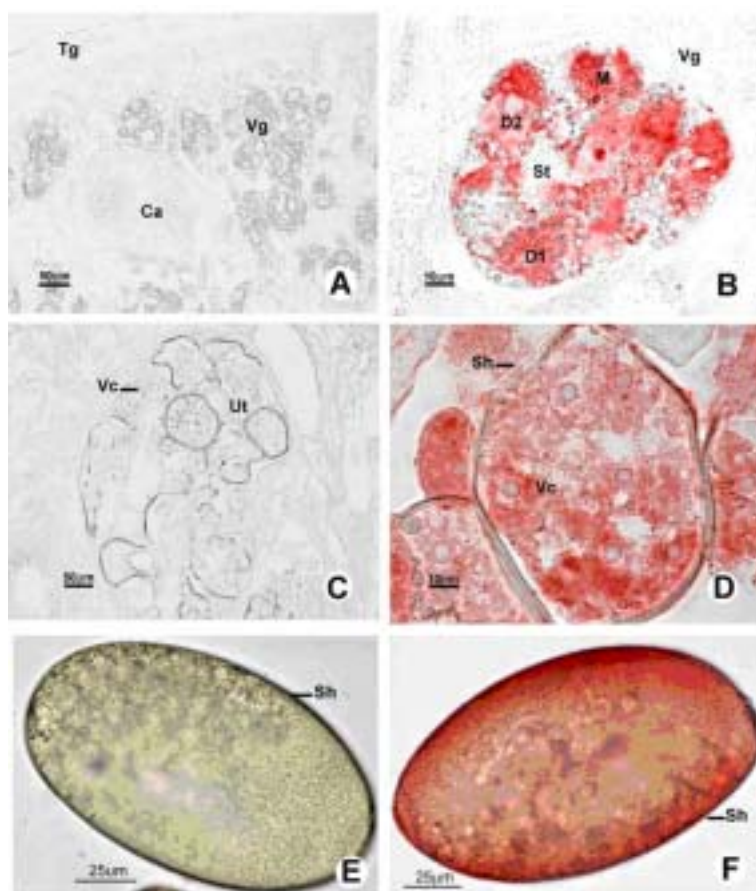


Fig 5–Immunohistochemical staining of adult *F. gigantica* tissue sections and eggs with rabbit PoAb against rFgVPBI. Positive signals shown by red staining at different levels of intensity appearing in the vitelline cells (Vc) in vitelline glands and in the egg within the uterus, the same as shown in *in situ hybridization* detection (B, D). An egg stained with antibody to FgVPBI shows intense staining of the eggshell and the area underneath the shell while the inner part of the egg is not stained (F). In the control sections (A, C, E) an egg stained with normal sera showing no staining in vitelline cells, egg (Eg) or eggshell (Sh).

riboprobe (Bae *et al*, 2007). In contrast, *F. hepatica*, a FhVPBI antisense probe could detect transcripts in mature vitelline cells within the vitelline follicle. The intensity of the hybridization signal decreased in vitelline cells in the vitelline reservoir and was absent in vitelline cells within intrauterine eggs (Rice-Ficht *et al*, 1992). In mature *S. mansoni*, the transcripts of the p48 eggshell protein gene were only detected in vitelline cells in the vitellaria (Chen *et al*, 1992), and likewise in *O. viverrini* transcripts of the OvESP gene were located in cells within the vitelline follicle (Ruangsittichai *et al*, 2006). This implies that in *F. gigantica* vitelline B transcripts were either more stable or the genes were still transcriptionally active in later stages of development. In eggshell formation, vitelline cells perform an important role by providing eggshell precursor proteins and nutrients for the developing embryo (Threadgold, 1982). Therefore, the expression of genes encoding for eggshell proteins in vitelline cells is likely to temporally coincided with egg production (Waikagul *et al*, 1986; Robinson *et al*, 2001). Recombinant FgVPBI was expressed at a molecular weight of 31.5 kDa, which was slightly larger than the 31 kDa native eggshell precursor protein due to the added His-tag and FLAG peptide. A minor 31 kDa truncated expression product was observed in addition to the full length protein. Proteolytic degradation or secondary site translation initiation are possible explanations (Preibish *et al*, 1988).

A polyclonal antiserum against rFgVPBI was produced and used for localization of the corresponding native proteins in *F. gigantica*. It reacted with the 31.5 and 31 kDa rFgVPBI and the 31 kDa native eggshell precursor protein (ESP) in immunoblots. However, this antibody could not detect eggshell protein in whole worm extracts (WB) prepared in non-denaturing lysis buffer containing 0.5% Triton-X 100. By this extraction pro-

cedure, the amount of eggshell protein from *F. gigantica* may be too low when compared with the extraction of ESP from the egg using 5% acetic acid and 4 M urea. Because the eggshell proteins were only soluble at an acidic pH, they were particularly susceptible to hydrolysis at this pH by acid protease from the gut of *F. hepatica* (Simpkin *et al*, 1980). The eggshell proteins were synthesized and packaged into secretory vesicles in the vitelline cells. These vesicles appeared to contain an emulsion of eggshell precursor material, were acidic as in other systems and this acidity stabilized the emulsion and prevented the eggshell cross-linking reactions from occurring (Wells and Cordingley, 1991).

Native *F. gigantica* vitelline B protein was detected in the adult stage using the antiserum and immunoperoxidase technique. The protein was detected in varying amounts, as reflected by the intensity of staining in the various stages of vitelline cells within the vitellaria and also in vitelline cells in the eggs within the uterus. In *P. westermani*, immunohistochemical staining of the anti-rPw-Vit20 and Vit36 antibody demonstrated immunoreactivity in the vitelline follicles and were largely restricted to the shells of eggs (Bae *et al*, 2007). The antisera raised against vitelline B of *F. hepatica* showed localization of the eggshell protein in only the vitelline globules of the vitelline cells in the vitellaria and was also detected in the same organs in *S. japonicum* when probed with antiserum raised against a 34 kDa recombinant eggshell protein, and in *C. sinensis* when probed with antiserum raised against *C. sinensis* glycine rich-C *sinensis* protein (GRCSP) and a vitelline protein B1 (CsVPB1) (Rice-Ficht *et al*, 1992; Sukiyama *et al*, 1997; Yang *et al*, 2000; Tang *et al*, 2005). A strongly positive reaction was observed in intrauterine eggs of adult *C. sinensis* using immune sera against recombinant Cs28 egg protein

(Cs28) (Lee *et al*, 2005). In the present study the immunoperoxidase pattern corresponded to the result of RNA *in situ* hybridization, as transcripts were detected in varying amounts in the cytoplasm of early and late stage vitelline cells in vitellaria and in vitelline cells of intrauterine eggs. This indicates the vitelline cells still synthesize FgVPBI after becoming detached from the follicles. To evaluate the immunologic activity of FgVPBI further studies will be carried out.

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