รายงานวิจัยฉบับสมบูรณ์

โครงการ การสังเคราะห์และศึกษาการแสดงออกของยีนที่เก็บรหัสแอนติเจนที่จำเพาะจากผิวพยาธิใบไม้ในตับ *Fasciola gigantica*

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กรกฎาคม 2551

สัญญาเลขที่MRG4980113

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัยสกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ: MRG4980113

ชื่อโครงการ: การสังเคราะห์และศึกษาการแสดงออกของยีนที่เก็บรหัสแอนติเจนที่จำเพาะจากผิวพยาธิใบไม้ในตับ *Fasciola gigantica*

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ระยะเวลาโครงการ: 2 ปี

ในพยาธิใบไม้ในตับ Fasciola จะใช้ Peroxiredoxin เป็น antioxidant เอมไซม์ที่มีคุณสมบัติใช้กำจัด H_20_2 ตรงตำแหน่ง active site ที่มี cysteine อยู่ เนื่องจากพยาธิชนิดนี้ไม่มี catalase และพบว่าปริมาณการ แสดงออกของ glutathione peroxidase มีอยู่น้อยมาก ในงานวิจัยนี้จึงได้ศึกษาว่าสามารถพบ Peroxiredoxins (Prxs) ในพยาธิใบไม้ในตับ *F. gigantica* ได้หรือไม่ รวมทั้งยังศึกษาการทำงานที่เกี่ยวข้องกับการต้านสารอนุมูลอิสระของออกซิเจนด้วย จึงได้โคลนยีนที่มีคุณสมบัติเป็นสาร antioxidant นั่นคือ 2-Cys Peroxiredoxin โดยวิธีการทำ immunoscreening ต่อ cDNA library ของพยาธิใบไม้ในตับ *F. gigantica* และทำปฏิกิริยากับโพลีโคลนัลแอนติบอดีต่อชั้นผิวของพยาธิและ พบยีนจำนวน 2 isoform คือ FgPrx1 และ FgPrx2 ที่แสดงลำดับที่สมบูรณ์ของกรดอะมิโน 218 ตัว โดย FgPrx1 มีน้ำหนักโมเลกุล 24.57 kDa ส่วน FgPrx2 มีน้ำหนักโมเลกุล 24.63 kDa ซึ่งทั้ง 2 isoforms นี้มีความเหมือนของ amino acids ประมาณ 98.6% และกับสัตว์เลี้ยงลูกด้วยนมประมาณ 50% identity เมื่อวิเคราะห์ phylogenic tree พบว่ายีน FgPrx1 และ FgPrx2 อยู่ในกลุ่มเดียวกับ peroxiredoxin ของ *F. hepatica* ผลของการศึกษาการแสดงออกของ mRNA ของยีน FgPrx มีการแสดงออกอย่างต่อเนื่องของการเจริญเติบโตในพยาธิตัวเต็มวัยจะพบ FgPrx ในเนื้อเยื่อ caecal epithelium เซลล์ชั้นผิว (tegument) ที่หุ้มผิวนอกของพยาธิและบุผิวในส่วน ventral sucker และในระบบสืบพันธุ์ของพยาธิเพศผู้และเมียซึ่งได้แก่ เซลล์ vitelline และไข่ และเซลล์ใน testis กับ prostrate gland ส่วนของพยาธิตัวอ่อนอายุ 4 สัปดาห์ ตัวอ่อนระยะ newly excysted juvenile และ metacercaria การแสดงออกของยีน Prx มีลักษณะคล้ายกับที่พบในพยาธิตัวเต็ม วัยคือมีการติดสีที่ caecum อย่างเด่นชัดและเซลล์ชั้นผิว หลังจากที่ได้ผลิต recombinant โปรตีนของยีนชนิดนี้ แล้วและศึกษาถึงการกระจายของ native FgPrx โปรตีนพบว่าการแสดงออกของโปรตีนคล้ายกับการแสดงออกของยีนอย่างเด่นชัด ยกเว้นในระบบสืบพันธุ์ของพยาธิเพศผู้ที่มีน้อยมาก นอกจากนี้ antiserum ต่อ recombinant FgPrx สามารถ ทำ

ปฏิกิริยากับแอนติเจนจากพยาธิใบไม้ในตับ *F. gigantica* ได้และไม่ทำปฏิกิริยากับแอนติเจนของพยาธิตัวแบนชนิดอื่นๆจากการศึกษาเหล่านี้พบว่าโปรตีน FgPrx มีการแสดงออกอย่างต่อเนื่องในแต่ละช่วงการเจริญเติบโตและเฉพาะในเนื้อเยื่อ จากการศึกษานี้ผู้วิจัยจึงสนใจที่จะศึกษาการทำงานของยีนนี้เพื่อนำไปใช้ประโยชน์ในการพัฒนาการตรวจสอบการติดเชื้อและวัคซีน

Abstract

Project Code : MRG4980113

Project Title : Molecular cloning and expression of genes encoding tegumental antigens in *Fasciola gigantica*

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Project Period : 2 ปี

Lacking of catalase and minimal expression of glutathione peroxidases in Fasciola, peroxiredoxin could be the major hydrogen peroxide-removing antioxidant in worm using an active-site cysteine residue. To determine whether peroxiredoxins (Prxs) are present within *Fasciola gigantica* and participate in the antioxidant role *in vitro*. We have cloned and characterized an antioxidant gene belonging to the 2-Cys Prxs by immunoscreening of an adult-stage *F. gigantica* cDNA library using a rabbit antiserum against tegumental antigens. Both FgPrx1 and FgPrx2 consist of 218 amino acids each with predicted molecular weights, respectively of FgPrx1, 24.57 kDa; FgPrx2, 24.63 kDa. The two predicted *F. gigantica* Prx proteins are 98.6% identical to each other and to Prx from mammalian (50% identity). A phylogenetic analysis reveals that FgPrx1 and FgPrx2 appear to be more closely related to those of *F. hepatica*. The profiling of FgPrx mRNA expression is constitutively presented in various developmental stages. In the adult parasite, FgPrx transcripts are located to the cells of gut epithelium, tegument, and reproductive organs, including prostate gland, vitelline glands, testis and eggs. In 4-week old juveniles, a similar distribution pattern is observed. Metacercaria and newly excysted juveniles positive signal is strongest in the gut epithelium and moderately in the tegumental cells. After the production and purification of recombinant FgPrx protein, the distributions of native FgPrx1 protein is remarkably similar to that of FgPrx transcription,

except less in male reproductive organs. An antiserum against recombinant FgPrx1 detects an intense protein in whole worm extract of *F. gigantica* with no cross-reactivity with Prx from the extraction of other trematode. Regarding to these analysis, FgPrx proteins constitutively express in the stages- and tissue- specificities. Taken together with the recent finding, we are further characterizing these functional proteins used for a potential candidate for immunodiagnosis and vaccine.

หน้าสรุปโครงการ (Executive Summary)

Tropical liver fluke, *Fasciola gigantica,* and tempearate liver fluke, *Fasciola hepatica,* cause serious infection in domestic animals, especially ruminants, and result in an estimated economic loss of around US$ 3 billion annually to the global animal husbandry (Spithill *et al*., 1999a). And this disease could also accidentally infect human, with an estimated 2.4 million people being infected while up to 180 million remain at risk worldwide (Over, 1982, Rim *et al*., 1994, Mas-Coma *et al*.,1999). Therefore, fasciolosis is an economically and socially important disease often resulting in huge loss in term of animal and human health and economy. In Thailand fasciolosis by *F. gigantica* causes economic loss about 400 million baths per year and the northeast being the most endemic region. To cure the infected animals, anti-parasitic drugs such as salicylanilides and triclabendazole have been used regularly. However, following prolonged drug usages the parasites could develop resistance against the drugs (Overend and Bowen, 1995; Moll *et al*., 2000; Gaasenbeek *et al*., 2001). Furthermore, chemical residues from drug treatment may remain in the meat, milk and cause side effects on consumers. Vaccine is an alternative measure against the parasite infection, as it can provide sustainable protection, and do not leave traces of chemical residues.

Vaccination with either a single defined parasites' protein or combination of proteins (cocktail) could achieve variable levels of protection after the challenge infection (Spithill and Dalton, 1998). There have been several vaccine candidates suggested for *F. hepatica*, and fewer for *F. gigantica*. Among these are the enzyme glutathione-S-transferase (GST), fatty acid binding protein (FABP), Cathepsin L (Cat-L), leucine aminopeptidase and hemoglobin (Spithill *et al*., 1999b; Mulcahy *et al*., 1999; Piacenza *et al*., 1999; Muro 1997; Dalton *et al*., 1996; Wijffels *et al*., 1994; Brophy and Barrett

1990). However, there has not yet been a sufficiently reliable vaccine that could be exploited at the field level. Furthermore, several attempts have been made to discover the parasite's other novel proteins which may have better vaccine potentail.

In addition to the development of vaccine candidates, the early diagnosis is one of the important measure that could be used to monitor the state of infection, the treatment of infection, as well as the result of vaccination. At present, the definitive method for diagnosing fasciolosis is by the stool egg count. This has disadvantages, including the difficulty in the identification and differentiation of the fasciola eggs from those of other species, and the prolonged waiting time up to 8-12 weeks for detection of the eggs which means the early detection of infection is not possible. More reliable diagnostic method than the present one is needed to detect the early as well as late phase of faciolosis. For accurate, species-specific antigen is needed.

Recent studies demonstrated that tegumental antigens are more species-specific than metabolic antigens (enzymes, FABPs) as described previously, since the surface components of parasites impart the unique features for each species. They could also be detected in the circulation of both experimentally and naturally infected cattle, and could be used for a rapid immunodetection even at the early stage of infection (Maisonnave, 1999; Viyananat *et al*., 1997; Krailas *et al*., 1999; Chaithirayanon *et al*., 2002). Apart from immunodiagnosis, they could also be used for vaccine (against schistosomiasis) as their target is to damage the tegument (Mohamed *et al*., 1998). Thus they are expected to provide a higher degree of specificity for diagnosis and protection.

In *Fasciola* spp., the studies and identification of tegumental proteins have not been extensive as in *Schistosoma* spp. Few studies reported several key antigens present in the tegumental syncytium of *F. gigantica* (28.5, 54, 58, 66 kD) that have high specificities as proven by immunoblotting and immunolocalization studies using monoclonal antibodies (Viyanant *et al*., 1997; Krailas *et al*., 1999; Sobhon *et al*., 2000; Chaithirayanon *et al*., 2002). In *F. hepatica*, Trudgett *et al*., (2000) have produced a recombinant *F. hepatica* protein from the short repetitive sequences that appears specifically in the tegument and tegumental cells of juvenile flukes, and the reproductive system of adults, and showed its potential in protecting animals against *F. hepatica* infection. Nevertheless, little is known about the genes encoding these antigens. Up to

now there have been no study on the genes encoding the tegument antigens of *F. gigantica* as previously described. Thus, to obtain tegument antigens in bulk for diagnosis and vaccine studies, the purpose of this study is to attempt the cloning and characterization of the gene encoding tegumental proteins.

1. ความสำคัญของปัญหาและที่มาของปัญหา

Introduction

Fasciolosis caused by *Fasciola gigantica* (tropical liver fluke), and *Fasciola hepatica* (tempearate liver fluke), is a debilitating parasitic disease for domestic animals resulting in an estimated economic loss of around US$ 3 billion annually to the global animal husbandry (Spithill *et al*., 1999a). Whereas this infection is accidentally in human, with an estimated 2.4 million people being infected while up to 180 million remain at risk worldwide (Over, 1982, Rim *et al*., 1994, Mas-Coma *et al*.,1999). Therefore, fasciolosis is an economically and socially important disease often resulting in huge loss in term of animal and human health and economy. For controlling the parasites by anti-parasitic drugs, there is not widespread usage due to the drugs resistance in the definitive host and many attempts are still finding potential antigen to approach the vaccine development.

Reactive oxygen species (ROS) such as superoxide anions ($O_2^{\bullet -}$), hydrogen peroxides (H_2O_2), and hydroxyl radicals ($^{\bullet}OH$) or the RNS include nitric oxide and peroxynitrite ($ONOO^-$) are by products of cellular metabolism and phagocytic cells during the respiratory burst. They can induce the oxidation protein and sugars, the lipid peroxidation and base modification of nucleic acid, leading to parasite death (Holmgren 1989). The first line of defense against ROS is involved with a superoxide dismutase (SOD) by rapidly reducing superoxide to hydrogen peroxide. Then hydrogen peroxide form is able to diffuse into the plasma membrane, causing cellular damage. Therefore, peroxiredoxins (Prxs) mainly act as a second line of defense to neutralize the H_2O_2, preventing formation of the dangerous hydroxyl radical (Netto et al., 1996). These protective enzymes play a crucial role in the parasite helminths, especially in the Fasciola and Schistosoma due to the lacking of catalase and minimal expression levels of

glutathione peroxidases (Callahan et al., 1988; McGonigle et al., 1997). Prxs, also named thioredoxin peroxidase (TPx), thiol-specific antioxidant (TSA) and alkyl-hydroperoxide-reductase-C22 proteins, are a ubiquitous antioxidant protein that reduce hydrogen peroxide and alkyl hydorperoxides to water and alcohols, respectively (Hofmann et al., 2002). The biological roles of this protein are involved in redox balance and signaling transduction processes that use hydrogen peroxide as a second messenger (Rhee et al., 2005). And they can be divided into three subclasses: the typical 2-Cys peroxiredoxin, the atypical 2-Cys peroxiredoxin and the 1-Cys peroxiredoxin (Wood et al., 2003).

In case of 2-Cys peroxoredoxins based on the number of conserved cysteine residues have been reported that during the detoxification the first active site cysteine residue near the N-terminus is converted into sulfenic acid intermediate (Cys-SOH) and the second near the C-terminus is resolved to form an intermolecular disulfide bond (McGonigle et al., 1998). In *Fasciola hepatica,* 2-Cys peroxiredoxins have been isolated and studied their biochemical characterization by several authors (McGonigle et al.,1997; Sekiya et a.,2006). For comparative purposes, we have also cloned peroxiredoxin genes from *F. gigantica* to study the gene and protein expression profiles in various developmental stages for further immunodiagnosis and vaccine development.

2. วัตถุประสงค์ของโครงการ

Overall objective: The overall objective of this project is to clone and characterize genes encoding tegumental proteins of *F. gigantica,* especially those at 66, 54, and 28.5 kDa. and study theirs expression in the tissues of various stages of the parasite life cycle, so that candidate antigens could be identified for diagnosis and vaccine development.

Specific objectives: In order to achieve the overall objective 3 specific objectives will be performed in steps as follows:-

1. To screen and clone the gene encoding tegumental antigens from adult cDNA library of *F. gigantica* by using polyclonal antibody (PoAb) against tegumental antigen of adult parasite as probes.

2. To characterize the genes encoding tegumental proteins and detect their expression in the tissues of various developmental stages of *F. gigantica*.

3. To produce the corresponding recombinant tegumental proteins and study their distribution in tissues of various stages of *F. gigantica.*

3. ระเบียบวิธีวิจัย

1. To screen and clone the gene encoding tegumental antigens from adult cDNA library of F. gigantica by PoAb against tegumental antigen as probes.

1.1. Immunoscreening of the cDNA libraries

cDNA libraries of adult *F. gigantica* is provided by Meemol *et al.*, (2004), These cDNA libraries were screened using the *pico*Blue^{TM} Immunoscreening Kit.

1.1.1 Library Amplification

A single isolated colony from the primary working plate of XL1-Blue was picked and inoculated into 15 ml of LB/$MgSO_4$ maltose broth at 37^0C overnight with shaking (140 rpm) until the OD_{600} of the culture reached 2.0. After centrifugation at 5,000 rpm for 5 min, the pellet of cells was resuspended in 7.5 ml of 10 mM $MgSO_4$. The 500 μl of bacterial suspension was added in the required number of 4-ml tubes and the lysate was diluted enough to yield 6-7 x 10^4 plaques per 150-mm plate. After incubation at 37^0C in water bath for 15 min, 4.5 ml of melted LB/$MgSO_4$ soft top agar was added to each tube, mixed and the bacteria plus phage mixture poured quickly onto LB/$MgSO_4$ agar plates. To promote even distribution, the plates were swirled quickly after pouring. Thereafter, the plates were cooled at room temperature for 10 min to allow the top agar to harden. The plates were then incubated in inverted position at 37^0C for 6-18 hr, or until the plaques became confluent. After adding 12 ml of 1X lambda dilution buffer to each, the plates were stored at 4^0C overnight. The plaques were now ready to be pooled in 1X lambda dilution buffer to form an amplified library lysate. On a platform shaker (~50 rpm), the plates were incubated at room temperature for 1 hr. The amplified λ-phage lysates were pooled into a sterile beaker. To clear the phage lysate of

cell debris and lyse any remaining intact cells, the phage lysate was mixed well and poured into a 50-ml polypropylene sterile screw-cap tube. Ten millitlire chloroform was added to lysate and vortexed for 2 min. After centrifugation at 7,000 rpm (5000 x g) for 10 min, the supernatant was transferred into another sterile 50-ml tube, and this could be kept at 4^0C for up to 6 months.

1.1.2. Titering the Amplified Library

An isolated single plasmid from the working stock plate of XL1-Blue was inoculated in 20 ml of LB/$MgSO_4$/maltose broth (without antibiotics) and incubated at 37^0C overnight with shaking (140 rpm) until the OD_{600} of the culture reached 2.0. The cells were centrifuged at 5,000 rpm for 5 min and supernatant was poured off. The pellet was resuspended in 7.5 ml of 10mM MgSO4. At the same time four LB/MgSO4 agar plates were warmed and dried (90-mm size). The phage lysate library was diluted into 1 ml of 1X lambda dilution buffer to the desired dilution. Each tube was incubated in a 37^0C water bath for 15 min. And then 3 ml of melted (45^0C) LB/MgSO4 top agar was added into these tubes and mixed quickly. The contents from each tube was poured onto separate LB/MgSO4 agar plates. The plates were swirled quickly after pouring to promote an even distribution of the melted agar. The plates were cooled at room temperature for 10 min to allow the soft agar to harden. Thereafter, plates were incubated in the inverted position at 37^0C for at least 6-7 hr. The plaques were counted and the titer (pfu/ml) calculated as follows:

$$\text{pfu/ml} = \frac{\text{number of plaques} \times \text{dilution factor*} \times 10^3\ \mu\text{l/ml}}{\mu\text{l of diluted phage plated}}$$

(* the dilution factor)

A successfully amplified library have a fairly high titer (~10^{10} pfu/ml).

1.1.3. Plaque Screening

After determining the suitability and titer of the primary antibody prior to screening cDNA libraries by using the dot blot test, the amplified cDNA library was subjected to plaque screening using polyclonal rabbit serum raised against tegumental extract (TA) and monoclonal antibody against 28.5 kDa tegumental antigen. A Stratagene *pico*Blue™ immunoscreening kit was used as the indicator system. The

positive plaques reacting with the antiserum were isolated and subjected to further screening to confirm positivity and ensure clonality.

1.1.4. Plating Phage

The bacterial cells were grown overnight in an appropriate medium supplemented with 10 mM magnesium sulfate ($MgSO_4$) and 0.2% (v/v) maltose to an OD_{600} of 1.0. These cultured bacteria were spun at 2,000 rpm for 10 minutes, and the pelleted cells were then gently resuspended in half the original volume with sterile 10 mM $MgSO_4$, and diluted to a final OD_{600} of 0.5. After preincubation the agar plates for 1 hour at 37^0C, this top agar was melted and placed in 50^0C water bath. The bacterial cells were combined with the phage as determined from table 1 below and incubated for 15 minutes at 37^0C before adding the top agar to allow the phage to be absorbed to the bacterial cells. Then, the bacterial cell and phage mixture were poured onto the agar plates prewarmed to 37^0C. Thereafter, the bacterial cell, phage and top agar mixture were quickly and evenly distributed across the surface of the agar plate and the top agar allowed to solidify at room temperature. The agar plates were incubated at 42^0C until small plaques just became visible.

Following this incubation period, the nitrocellulose membrane was treated with 10 mM IPTG for 30 minutes until the membranes were completely wet prior to use. The membranes were placed on Whatman 3 MM paper to air dry.

1.1.5. Plaque Lifts

The agar plates containing the phage plaques were applied with the numbered IPTG-treated nitrocellulose membranes. After incubation on the agar plates for 3.5 hours at 37^0C, the nitrocellulose membrane was carefully removed with forceps and washed in TBST. When the top agar sticked to the nitrocellulose membrane, the agar plate was cooled at 4^0C for 4 hours or more. In addition, a duplicate IPTG-treated nitrocellulose membrane was applied after removing the first membrane and then return the agar plate to 37^0C for 4 hours or more. The nitrocellulose membranes were then immersed in TBST and any remaining top agar removed with a gloved hand or smooth glass rod. The nitrocellulose membranes were transferred in a plastic container or in a heat-sealable hybridization bag and washed three to five times for at least 15 minutes each with 10 ml of TBST. Finally, the nitrocellulose membrane was incubated in the blocking solution

and agitated gently for 1 hour at room temperature to block the remaining protein binding sites. Nitrocellulose membranes could be stored for several days in blocking solution with 0.02%(w/v) sodium azide at 4^0C in a heat-sealable hybridization bag.

1.1.6. Detection by antibody and color development reaction

Each nitrocellulose membrane was transferred into 8 ml of fresh blocking solution containing the appropriate dilution of primary antibody (PoAb) against tegumental antigen with gentle agitation for at least 1 hour at room temperature. Then, the nitrocellulose membranes were washed three to five times for 5 minutes each in 8 ml/membrane of TBST to remove any residual unbound or nonspecifically bound primary antibody. After incubation in a fresh blocking solution containing alkaline phosphatase (AP)-conjugated secondary antibody with gentle agitation for 1 hour at room temperature, the treated nitrocellulose membrane was washed three to five times for 5 minutes each in 8 ml/membrane of TBST to remove any residual unbound or nonspecifically bound conjugate. Finally, the residual Tween 20 is removed with 8 ml/membrane of TBS alone.

The NBT (nitroblue tetrazolium) in color development solution was diluted to a final concentration of 0.3 mg/ml and BCIP (5-bromo-4-chloro-3-indolyl Phosphate) was added to a final concentration of 0.15 mg/ml. If a precipitate formed, the NBT-BCIP color development solution was warmed at 37^0C and the solution filtered through a 0.2-μm filter disk. The nitrocellulose membrane was removed from the final TBS washing and excess moisture blotted off with Whatman 3MM paper. Then the treated nitrocellulose membrane was incubated in the BCIP-NBT color development solution. The color development reaction was allowed to proceed in the dark until the positive reaction was clearly visible (usually within 30 minutes). Thereafter, the membrane was removed from the color development solution by rinsing with TBS. And the color development reaction was stopped by immersing the nitrocellulose membranes in stop solution.

1.2. Converting λTripIEx2 to pTripIEx2

After obtaining positive clones, the λTripIEx2 was converted to pTripIEx2 by the *in vivo* excision and transformed into *E.coli* BM25.8. These plasmids were isolated, cut with the

restriction enzymes and run in 1% agarose gel. Then, the positive clones with larger sizes were purified for sequencing analysis.

A single isolated colony from the working stock plate of *E.coli* BM 25.8 host cells was picked up and inoculated into 10 ml of LB broth in a 50-ml test tube or Erlenmeyer flask, and incubated at 31^0C overnight with shaking (at 150 rpm) until the OD_{600} of the culture reaches 1.1-1.4. After adding 100 μl of 1 M $MgCl_2$ to the 10-ml overnight culture of BM25.8 (final concentration of 10 mM $MgCl_2$), a well-isolated positive plaque from secondary- or tertiary-screening plates was picked and placed in 250 μl of 1X lambda dilution buffer. The plaque was vortexed and incubated at 37^0C for 3-4 hour without shaking. Alternatively, the phage was allowed to elute at 4^0C overnight. In a 20-ml test tube, 200 μl of overnight cell culture was combined with 150 μl of the eluted positive plaque and incubated at 31^0C for 30 min without shaking. Then, 400 μl of LB broth was added and incubated at 31^0C for 1 hour with shaking at 225 rpm. And 1-10 μl of infected cell suspension was spreaded on an LB/ampicillin plate to obtain isolated colonies that grew at 31^0C.

1.3. Isolation the plasmid

The single plasmid-containing *E. coli* BM25.8 clone was transferred into 3 ml LB-medium and incubated overnight at 37^0C with shaking at 280 rpm. After spinning at 4000 rpm for 10 min, the supernatant was quickly poured into the sink, and supernatant removed as much as possible by turning the tube upside-down on a tissue paper. The pellet was resuspended in 200 μl solution I (10 mM EDTA, 50 mM glucose, and 25 mM Tris, pH 8.0), and the solution transferred to a 1.5 ml tube. The 400 μl solution II (0.1 N NaOH and 1% SDS) was added in order to denature DNA and protein and mixed by inverting the tube. After incubation on ice for 5 min, 300 μl solution III (2.7 M potassium acetate, pH 4.8) was added, mixed and incubated on ice for 5 min to renature plasmid. This mixture was spun at 12000 g for 5 min at room temperature and supernatant transferred to a new tube. Then 0.6 vol isopropanol (2-propanol) was added, mixed and spun at 12,000 g, 5 min, at room temperature (RT) in order to precipitate nucleic acid. The supernatant was discarded and the remaining pellet was washed with 70% ethanol, spun at 12,000 g, 2 min, RT. Finally, the pellet was dried in a desiccator

for 15-30 min and resuspended in 50 µl of TE buffer. The isolated plasmid was stored at 4^0C or -20^0C for further use.

2. To characterize the genes encoding tegumental proteins and detect their expression in the tissues of various developmental stages of F. gigantica.

2.1. Construction of RNA probe by in vitro transcription using DIG RNA labeling Kit

The encoding cDNA was inserted into an RNA expression vector (plasmid) according to the standard procedure described by Sambrook *et al*. (2001). The RNA expression vector was linearized with restriction enzymes to allow *in vitro* run-off synthesis of both sense and antisense-oriented RNA probes. The linearized plasmid DNA was purified by phenol-choloroform extraction and ethanol precipitation. To avoid RNA polymerase inhibition, the plasmid was resuspended in DEPC-treated ddH_2O. The digoxigenin-labeled RNA probes were generated in both the sense and antisense directions following by *in vitro* transcription with the DIG-RNA labeling kit. One microgram of purified, linearized plasmid DNA was incubated in 2 µl each of 10X concentrated DIG-RNA Labeling Mix, 10X concentrated Transcription buffer, suitable RNA polymerase (SP6, T7 or T3) and DEPC-treated H_2O to bring the volume up to 20 µl at 37^0C for 2 hours. The transcription reaction was stopped by using 2 µl of 0.2 M EDTA (pH 8.0). The labeled RNA transcript was precipitated by incubating in 2.5 µl of 4 M LiCl and 75 µl prechilled 100% ethanol at -70^0C for at least 30 minutes. Then, the labeled RNA was precipitated by contrifugation at 13,000 x g for 15 minutes at 4^0C and washed with 50 µl ice-cold 70% ethanol for 5 minutes, dried and resuspended in 50 µl of DEPC-treated H_2O. After purifying, the yield of labeled probes was estimated by direct blotting procedure. And then these labeled probes were aliquoted and stored at $–80^0$C.

2.2. Detection of mRNA of the encoding genes with DIG-labeled RNA probes in the tissues of *F. gigantica* parasites at various stages

2.2.1 Collection of parasites

2.2.1.1. Metacercariae

Metacercariae of *Fasciola gigantica* were obtained from cercariae shed from laboratory colonies of the snail intermediate host, *Lymnaea ollula,* infected with miracidia developed from *Fasciola gigantica* eggs collected from the gall bladders of freshly slaughtered cattle. After being shed from the snails, the cercariae were allowed to settle and encysted on 5 cm x 5 cm plastic sheets in the snail containers. Under a stereomicrocope, the metacercariae attached to the plastic sheets, were gently brushed into 0.85% NaCl solution and kept for use in subsequent experiments.

2.1.1.2 Newly excysted juveniles

To digest the outer cyst wall, 2% pepsin and 0.5% HCl in distilled water were added in the tube containing metacercariae at 37^0C for 45 min with shaking. After rinsing 3 times with distilled water, the metacercariae were then mixed with 0.02 M sodium dithionite, 0.2% Taurocholic acid, 1% sodium hydrogencarbonate, 0.8% sodium chloride and 0.5% HCl at 37^0C for 45 min with shaking. After pouring off supernatant, these metacercariae were then washed with distilled water 3 times. The treated metacercariae were transferred into 24-well-plate containing 10 μg/ml gentamycin with 10% fetal calf serum in RPMI-1640 for 2-3 hours at 37^0C. Finally, the newly excysted juveniles were collected and kept for use in other experiments.

2.1.1.3. Juvenile worms

Juvenile *F. gigantica* were obtained from female Golden Hamster peritoneally infected with 15 newly excysted juveniles per mouse. The juvenile worms were collected from the liver of mice at 4 weeks after the infection. The immature parasites were washed several times with 0.85% NaCl solution before being used in experiments.

2.1.1.4. Adult worms

Adult *F. gigantica* parasites were obtained from the bile duct of the liver and gall bladders of freshly slaughtered cattle killed at the local abattoirs. Other trematode parasites, namely *Eurythrema pancreaticum*, and *Paramphistomum spp.,* were collected from the naturally infected cattle for cross reaction studies. Adult *Schistosoma japonicum, S. mansoni,* and *S. mekongi* were obtained from mice eight weeks after infection with cercariae. All parasite samples were washed three times with Hank's balanced salt solution (HBS), to remove all traces of contaminating blood and bile. The flukes were washed several times in 0.85% NaCl solution to remove the host blood, bile

and contaminating microorganisms before being processed further for subsequent experiments.

2.2.2. Tissue preparation

Various developmental stages of *F. gigantica*, including adult *F. gigantica*, 4 week-old juvenile, newly excysted juvenile and metacercaria were fixed in 100 mM phosphate buffer containing 4% paraformaldehyde for 3-4 hours at room temperature. After fixation, the samples were washed in 100 mM phosphate buffer saline (PBS), embedded in paraffin, and cut at 7 μm thick. The sections were placed onto HistoGrip-coated glass slides. After drying slides in an oven at 40^0C overnight, the sections were dewaxed in fresh xylene twice, for 10 minutes each. The paraffin sections were rehydrated for 5 min each in the following solutions: 100%, 95%, 70% ethanol and DEPC-treated ddH_2O, respectively.

2.2.3. Prehybridization

The paraffin sections were incubated with DEPC-treated PBS, pH 7.4 and DEPC-treated PBS containing 100mM glycine twice for 5 minutes each. The treated sections were then immersed with 0.3% Triton X-100 in DEPC-treated PBS for 15 min and washed with DEPC-treated PBS twice for 5 minutes each. The sections were incubated for 30 min at 37^0C in TE buffer (100 mM Tris-HC1,50 mM EDTA, pH 8.0) containing 20 ug/ml RNase-free Proteinase K. Thereafter, the sections were post-fixed with 4% paraformaledhyde in DEPC-treated PBS for 5 minutes at 4^0C and washed with DEPC-treated PBS twice for 10 min each. The sections were acetylated by placing the slides into a container placed on a rocking platform and incubated with 0.1 M triethanolamine (TEA) buffer, pH 8.0, containing 0.25% (v/v) acetic anhydride twice for 15 min each. Finally, the sections were incubated with prehybridization buffer (4xSSC containing 50% (v/v) deionized formamide) at 37^0C for at least 10 min.

2.2.4. *In situ* hybridization

After washing with the prehybridiztion buffer composing of 40% deionized formamide, 10%dextran sulfate, 1x Denhardt' s solution, 4 x SSC, 10 mM DTT, 1 mg/ml yeast t-RNA, and 1 mg/ml denatured and sheared salmon sperm DNA, each section was overlaid with 30 μl of hybridization buffer containing 5-10 ng of digoxigenin-labeled RNA probe. Finally, the section was incubated at 42^0C overnight in a humid chamber.

2.2.5. Posthybridization

The coverslips were removed from sections by immersing in 2xSSC for 5-10 min. The sections were washed twice with 2xSSC and 1xSSC for 15 min in a shaking water bath at 37^0C, respectively. To digest any single-stranded (unbound) RNA probe, sections were incubated for 30 min at 37^0C in NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 20 μg/ml RNase A. Thereafter, the sections were rinsed with 0.1xSSC twice for 30 minutes in a shaking water bath at 37^0C

2.2.6. Immunological detection

On a shaking platform, sections were washed twice for 10 min each with buffer 1 (100 mM Tris-HCl (pH 7.5), 150 mM NaCl) and blocked with blocking solution (buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum) for 30 min. After decanting blocking solution, the sections were incubated in a humid chamber with buffer 1 containing 0.1% Triton X-100, 1% normal sheep serum, and a suitable dilution of sheep anti-DIG-alkaline phosphatase (Fab fragments) for 2 hours. Sections were rinsed twice for 10 min each with buffer 1 on a shaking platform, and incubated in buffer 2 (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$) for 10 min. Sections were covered with 200 μl color solution (NBT/BCIP), and the slides incubated in a humid chamber for 2-24 h in the dark. When color development was optimal, the color reaction was stopped by incubating the slides in buffer 3 (10 mM Tris-HCl (pH 8.1), 1 mM EDTA), and then slides were dipped briefly in distilled water. Finally, the sections were mounted by using aqueous mounting solution.

2.3. Northern blot analysis

2.3.1. Preparation the total RNA of *F.gigantica*

Total RNA of each developmental stage of *F. gigantica*, adult and 4 wk juvenile parasites were extracted and homogenized by using 1 ml of TRIzol Reagent (GIBCO BRL Life Technologies) per 50-100 mg of tissues. After centrifugation at12000 x g for 10 minutes at 4^0C, the cleared supernant which contained RNA was collected and then incubated for 5 minutes at room temperature. Proteins and nucleoproteins were separated by adding 0.2 ml chloroform in 1 ml of TRIzol Reagent, and centrifuged at 12,000 x g for

15 minutes at 4^0C. The RNA contained in the colorless upper aqueous phase was then precipitated with 0.5 ml isopropyl alcohol in 1 ml of TRIzol Reagent, and centrifuged at 12,000 x g for 10 minutes at 4^0C. Subsequently, the RNA pellet was washed with 1 ml of 75% ethanol in 1 ml of TRIzol reagent. After centrifugation at 7,500 x g for 5 minutes at 4^0C, the RNA pellet was dried and kept at -20^0C until further used.

2.3.2. Northern transfer

The 25 μg of total RNA of *F. gigantica* was heated in 50% formamide, 2.2 M formaldehyde in 1X MOPS buffer for 5 minutes at 65^0C, and then run in the 1%agaorse gel/2.2 M formaldehyde electrophoresis. The gel was then rinsed in ddH_2O four times before being transferred onto nylon membrane as previously described for southern transfer. Finally, the nylon membrane was dried and stored at 4^0C for further used.

2.3.3. Hybridization protocol and immunological detection

The blotted membrane was prewarmed in the hybridization buffer to hybridization temperature (50^0C for Southern blot and 70^0C for Northern blot) for 30 minutes. The denatured DNA probe, as constructed in section 3.3, was added and incubated at 50^0C overnight. Then the membrane was washed twice for 5 minutes with 2× SSC, 0.1% SDS at room temperature and in 0.1 SSC, 0.1%SDS at 42-70^0C for 15 minutes twice. The membrane was rinsed with the washing buffer for 5 minutes and incubated in 1x blocking solution at room temperature for 30 minutes. The appropriate dilution of anti DIG-AP in blocking solution was added and incubated at room temperature for 30 minutes. Subsequently, the membrane was washed with washing buffer for 15 minutes twice and equilibrated in detection buffer for 5 minutes. Finally, the membrane was incubated in freshly prepared color substrate solution in the dark, and finally the reaction was stopped by washing in distilled water.

3. Production of recombinant F. gigantica tegumental proteins and production of polyclonal antibody (PoAb)

3.1 Expression of recombinant protein

3.1.1. Time course expression test

After getting the correct insertion of the encoding cDNA fragment in the plasmid, a single clone of each plasmid that encoded the desired protein was inoculated into the 10 ml of LB broth containing 100 μg/ml selected ampicillin-resistance and grown at 37^0C overnight with shaking at 260 rpm. Two milliliters of cells from the overnight culture was added into fresh pre-warmed 50 ml of LB broth containing 100 μg/ml ampicillin, then incubated at 37^0C with shaking at 260 rpm until OD_{600} reached 0.5-0.7. One ml of sample was taken as the noninduced control (non-induced, NI), for SDS-PAGE analysis. The remaining plasmid was induced by IPTG at the final concentration of 1 mM. After incubation for 3.30 - 4 hours, 1 ml of second sample was collected every 30 min as an induced control. The remainder was centrifuged at 6,500g for 15 min at 4^0C, and the pellets were stored at -20^0C until further use.

3.1.2. Purification of recombinant protein

After expression by IPTG, the cell pellet was thawed for 15 minutes on ice and resuspended in 4 ml of lysis buffer (1x Binding buffer). Lysozyme was then added to 1 mg/ml and incubated on ice for 30 min. After the sonication on ice, the cell pellet was harvested by centrifugation at 16,000g for 20 min at 4^0C, then dissolved by using 20 ml of 1xBinding buffer. The cell pellet was collected by centrifugation at 16,000g for 15 min at 4^0C and redissolved with 5 ml of 1x Binding buffer with incubation in ice for 1 h. Subsequently, in order to purify the 6xHis-tagged recombinant protein, the Ni-NTA resin was packed into the column chromatography and equilibrated with the lysis buffer. The bacterial cells lysate was then slowly applied to the column and then washed with washing buffer. Finally, the protein was eluted with the elution buffer, and the recombinant protein purified and stored at –20^0C for further used.

3.2. Production of polyclonal antibodies (PoAb) against rFgPrx protein.

Rabbit were immunized subcutaneously with 200 μg of rFgPrx-6xHistagged fusion protein with complete Freund's adjuvant. After 3 weeks, the second immunization was given with 250 μg of the same recombinant protein in the incomplete Freund's adjuvant by the same route. The rabbit with the highest antibody titer was used for further studies.

4. To study the distribution of the corresponding tegumental protein in tissues of various stages of *F. gigantica*

4.1. Immunofluorescence

The distribution of peroxiredoxin in the tissues of various developmental stages of *F. gigantica* were also detected by indirect immunofluorescence using PoAb in immune serum against rFgPrx protein. Paraffin sections of each stage of the parasite, including Met, NEJ, 4 week-old juveniles, and adult were fixed in acetone at -10^{0}C for 10 min. After washing with 10 mM PBS, pH 7.4, for 5 min, the sections were incubated with 0.1% glycine in 10 mM PBS, and subsequently in 4 % BSA in 10 mM PBS to block nonspecific binding, for 15 and 30 min, respectively. Finally, the sections were incubated with PoAbs for 60 min at room temperature. The sections were rinsed 3 times with 10 mM PBS, pH 7.4, for 5 min each, and treated with Alexa488 conjugated goat anti-rabbit IgG with 0.05% Tween-PBS, for 30 min at room temperature. The treated sections were then rinsed thoroughly with PBS and counter-stained with DAPI or Topro3 iodide. Afterthat, the sections were mounted on glass slides in glycerol-PBS (9:1), and then examined under a Nikon HB 10101 AF fluorescence microscope.

4.2. Preparation of extracts from various trematode

Various trematodes, including *S. japonicum*, *S. mansoni*, *Eurytrema* spp., and *Paramphistomum* spp. were homogenized in lysis buffer (10mM Tris-HCL, pH 7.2, 150mM NaCl, 0.5% Triton X-100, 1mM EDTA and 1mM PMSF) and rotated at 4^{0}C for 1 hour. The suspensions were centrifuged at 5,000g, 4^{0}C, for 20 min and the supernatants were collected. Protein concentrations were determined by Lowry's method. These extracts were stored at -70^{0}C until use in subsequent experiments.

4.3. Western blot

To analysis of expression rFgPrx protein, two groups of protein extracts were separated in a 12.5% SDS-PAGE according to Laemmli *et al.*, (1970) and transferred onto nitrocellulose membranes for immundetection by rabbit serum containing PoAb against rFgPrx protein. The first group was proteins extracted from various stages of *F. gignatica*, including NEJ, 4, 5 and 7 weeks juvenile, and adult worm body, tegumental extract, excretory-secretory antigens, and rFgPrx protein. The second was proteins

obtained from other trematode parsites (*S. japonicum*, *S. mansoni*, *Eurythrema pancreaticum*, and *Paramphistomum* spp.). Alkaline phosphatase (AP) conjugated goat anti-mouse IgG and goat anti-rabbit IgG at dilution 1:2000 and NBT-BCIP were used for enzymatic detection.

3)

1. Isolation and sequencing the peroxiredoxin encoding tegument of *F. gigantica*

By using Immunoscrening cDNA library of adult *Fasciola gigantica* with rabbit polyclonal antibody serum against the tegumental antigen, there are many positive clones in the initial screening of lambda phage libraries. Positive clones were plaque-purified by 3 times of rescreening. Few of which was done with ex vivo excision to be pTriplEX2. Then inserted cDNA of pTriplEX2 was digested with the *Kpn*I and *Xba*I and subclones into pBluescript SK(-) (Stratagene Cloning Systems, La Jolla, Ca) for sequencing and transformed *Escherichia coli* XL-1 blue. Following the bioinformatic computer analysis of NCBI gene bank database through the BLAST program for similar sequences, these positive clones represented the highest scoring alignments with peroxiredoxin (Prx), tegumental calcium binding protein, and cathepsin L. Therefore, peroxiredoxin cDNA of adult *F. gigantica* were selected to investigate in this study because a little of this known gene used as a tool for the immunodiagnosis and vaccine candidate. The nucleotide sequence of FgPrx1 was found to be 874 base pairs (bp) long with a 118 bp 5' untranslated region (UTR), an open reading frame (ORF) of 657 bp, 69 bp 3' UTR, and a 30 bp poly(A) tail residues. And FgPrx2 cDNA consisted of 881 bp long with a 126 bp 5' untranslated region (UTR), an open reading frame (ORF) of 657 bp, 69 bp 3' UTR, and a 29 bp poly(A) tail residues. Two cDNA of FgPrx encoding genes were completely sequenced at 218 amino acids each with a predicted molecular weights and isoelectric points (p*I*), respectively of FgPrx1, 24.57 kDa and p*I* 7.65; FgPrx2, 24.63 kDa and p*I* 7.04 (Fig. 1 & 2).

```
  1 atgtgtgatcgcgatcagtgctctccggggcgccatccacttccc
    M  C  D  R  D  Q  C  S  P  G  R  H  P  L  P
 46 cactctcatccgcatttgcaaagaccgatgttgcagcctaacatg
    H  S  H  P  H  L  Q  R  P  M  L  Q  P  N  M
 91 cccgccccgaatttttctggacaggcggtagtgggcaaggagttc
    P  A  P  N  F  S  G  Q  A  V  V  G  K  E  F
136 aaaaccatcagtttatcagactacaagggcaaatgggtgattctc
    K  T  I  S  L  S  D  Y  K  G  K  W  V  I  L
181 gccttctatccacttgatttcacgttcgtgtgtccaacggaaata
    A  F  Y  P  L  D  F  T  F  V  C  P  T  E  I
226 atcgcgttcagtgatcagatggagcagttcgcacgacgtaactgt
    I  A  F  S  D  Q  M  E  Q  F  A  R  R  N  C
271 gccgtcatcttctgctctactgactcggtttattcgcatctgcaa
    A  V  I  F  C  S  T  D  S  V  Y  S  H  L  Q
316 tggaccaaaatggatcgtaaggttggcggtataggccagctgaac
    W  T  K  M  D  R  K  V  G  G  I  G  Q  L  N
361 ttcccgctgctggcagacaagaatatgtctatctctcgcgcctat
    F  P  L  L  A  D  K  N  M  S  I  S  R  A  Y
406 ggtgttctggatgaggagcagggtaatacctaccgtggcaatttc
    G  V  L  D  E  E  Q  G  N  T  Y  R  G  N  F
451 ctcatcgatcccaaggggtcctgcgccagatcacggtgaatgac
    L  I  D  P  K  G  V  L  R  Q  I  T  V  N  D
496 cgaccggtgggccgttccgttgaagaagccttgcgtctgctcgat
    R  P  V  G  R  S  V  E  E  A  L  R  L  L  D
541 gcattcatattccacgaggagcatggagaggtctgcccggcgaac
    A  F  I  F  H  E  E  H  G  E  V  C  P  A  N
586 tggaagcctaaaagcaagaccatcgtgcctactccggatggatcc
    W  K  P  K  S  K  T  I  V  P  T  P  D  G  S
631 aaagcatatttctcctcagccaactag 657
    K  A  Y  F  S  S  A  N  *
```

Fig. 1 Nucleotide sequence and deduced amino acid sequence of the Fg_Peroxiredoxin1 cDNA

```
  1 atgtgtgatcgcgaacagtgctctccggggcgccatccgcttccc
    M  C  D  R  E  Q  C  S  P  G  R  H  P  L  P
 46 cactctcatccgcacctgcaaagaccgatgttgcagcctaatatg
    H  S  H  P  H  L  Q  R  P  M  L  Q  P  N  M
 91 cccgccccgaatttttctgggcaggcggtagtgggcaaggagttc
    P  A  P  N  F  S  G  Q  A  V  V  G  K  E  F
136 aaaaccatcagtttgtcagactacaaaggcaaatgggtgattctc
    K  T  I  S  L  S  D  Y  K  G  K  W  V  I  L
181 gccttctatccacttgatttcacgttcgtgtgtccaacggaaata
    A  F  Y  P  L  D  F  T  F  V  C  P  T  E  I
226 atcgcgttcagtgatcagatggagcagttcgcgcgacgtaactgt
    I  A  F  S  D  Q  M  E  Q  F  A  R  R  N  C
271 gccgtaatcttctgctctactgattcggtttattcgcatctgcaa
    A  V  I  F  C  S  T  D  S  V  Y  S  H  L  Q
316 tggaccaaaatggatcgtaaagttggcggtataggtcagctgaac
    W  T  K  M  D  R  K  V  G  G  I  G  Q  L  N
361 ttcccgctgctggcagacaagaatatgtctatctctcgcgcctat
    F  P  L  L  A  D  K  N  M  S  I  S  R  A  Y
406 ggtgttctggatgaggaacagggtaatacctaccgtggcaatttc
    G  V  L  D  E  E  Q  G  N  T  Y  R  G  N  F
451 ctcatcgatcccaaggggtcttgcgccagatcacggtgaatgac
    L  I  D  P  K  G  V  L  R  Q  I  T  V  N  D
496 cggccggtgggccgttctgttgaagaagccttgcgtctgctcgac
    R  P  V  G  R  S  V  E  E  A  L  R  L  L  D
541 gcattcatattccacgaggagcatggagaggtctgcccggctaac
    A  F  I  F  H  E  E  H  G  E  V  C  P  A  N
586 tggaagcctaaaagcaagaccatcgtgcctgatccggatggatcc
    W  K  P  K  S  K  T  I  V  P  D  P  D  G  S
631 aaagcatatttctcctcagtcaactag 657
    K  A  Y  F  S  S  V  N  *
```

Fig. 2 Nucleotide sequence and deduced amino acid sequence of the Fg_Peroxiredoxin2 cDNA

Analysis of the deduced amino acid sequence of *Fasciola gigantica* Peroxiredoxin (FgPrx1 and FgPrx2)

The conserved amino acid sequences reveal significant homology to several other 2-Cys of peroxiredoxins family from other organisms, markedly in the regions surrounding the first cysteine (FVCP) Cys^{81} and the second cysteine (EVCP) Cys^{202} involve in the maintenance of a dimeric structure of antioxidant activity (Fig. 3). The two predicted Fasciola Prx proteins are 98.6% identical to each other and to Prx from Bos Taurus (48.6-49% identity) as well as human (50.4-50.9% identity) (Fig. 4). The deduced amino acid sequences of FgPrx1 and FgPrx2 are highly homologous to Peroxiredoxin (CAA06158) from *F. hepatica* (87.6% and 86.7% identity). The amino terminus of FgPrx1 and FgPrx2 contains a twenty four-amino acid extension that is not present in the Peroxiredoxin from *F. hepatica*. A phylogenetic analysis where is also carried out using UPGMA from the PAUP software (Sinauer Associates, Sunderland, MA), reveals that FgPrx1 appear to be more closely related to CAA6158 of *F. hepatica* and FgPrx2, respectively (Fig. 5). All deduced amino acid sequences of peroxiredoxin from eukaryotic kingdom were obtained from the Swissprot and Genebank databases via the National Center for Biotechnology Information (NCBI).

2. Characterization of the *F. gigantica* Prx RNA

In this experiment, the localization of FgPrx1 and FgPrx2 RNA was done by *in situ* hybridization to detect the hybridization signal in various developmental stages, including adult, 4 wk-juvenile, newly exycsted juvenile, and metacercaria. Both FgPrx1& 2 antisense RNA probes showed the similar results.

2.1 Distribution of FgPrx RNA in tissues of adult *F. gigantica*

By using the paraffin cross sections of adult FgPrx1 encoding gene mRNA antisense probe demonstrated the positive staining in the tegumental cells (Tc), caecal cells of the caecum (Ca), and both reproductive organ of male and female in (Fig. 6) as compared with the negative control by using FgPrx1 mRNA sense probe (data not shown). No staining in the nuclei of caecal and tegumental cells (Fig. 6B, C). Neither the tegument nor spine was positively stained (Fig. 6B). At the ventral sucker of *F. gigantica*, FgPrx1 encoding gene was expressed on the tegumental cells (Tc) scattering

inside the ventral sucker, not in the same pattern as that of the tegument (Fig. 6D). The reproductive organs of both male, including testis (Ti, Fig. 6F), prostrate gland (Pg, Fig. 6D, E) and female, including vitelline cells (Fig. 6G) and ovum in ovary (Fig. 6H) showed the hybridization signal whereas a cirrus that was the genital opening of sperm and sperm in the seminal vesicle (Se) did not found the positive staining (Fig. 6D, E).

In 4 week-old juveniles, the similar pattern of hybridization with both FgPrx1 and FgPrx2 antisense RNA probe appeared in all cell types as in the adult parasites (Fig. 7). The detection was specifically appeared in the cytoplasm of tegumental cells (Tc, Fig. 7A, B), caecal epithelial cells (Ca, Fig. 7A, B) lining the digestive tract, not in the parenchymal cells (Pc). Moreover, FgPrx1 antisense probe showed predominantly staining along the digestive tract and moderately in the tegumental cells (Tc) of newly excysted juvenile and metacercaria (fig. 7C,D). No staining was observed in the negative control sections of juveniles and metacercaria, using a FgPrx1 sense RNA probe.

2.2. Northern hybridization analysis

In a Northern blot analysis of total RNA from adult and 4 week-old juvnile *F. gigantica* hybridized with a FgPrx1 antisense RNA probe under highly stringent hybridization conditions approximately 1 kb RNA product of the FgPrx gene was detected (Fig. 8).

3. Expression and purification of recombinant *Fasciola gigantica* Peroxiredoxin protein (rFgPrx)

The entire coding sequence cDNA of FgPrx1 was amplified by PCR, then ligated into pET17b vector and finally cloned into Rosseta strain *Escherichia coli*. After isopropyl-β-D-thiogalactopyranoside (IPTG) induction, the rFgPrx1-6xHistagged fusion protein was allowed to express as a fusion protein starting from 30 min to 4 hour, and showed the intense major band at various intervals following the induction as compared with the non-inserted gene treated group (NI) by 12.5% SDS-PAGE. Molecular weight of rFgPrx1 protein was consistent with the predicted size of the 6xHistagged protein as determined by comparison with standard molecular weight protein markers, was around 26 kDa (Fig. 9A). Subsequently, the bacterial cell lysate was performed by lysozyme

incubation and sonification. This rFgPrx1-6xHistagged fusion protein was purified by His-Resin chromatography with the native condition (Fig. 9A-B). The stepwise collections, by varying the concentration of immidazole revealed rFgPrx1-6xHistagged fusion protein. Furthermore, the purified rFgPrx1-6xHistagged fusion protein was collected, pooled and used to produce polyclonal antibody in rabbit.

4. Investigation the distribution of the corresponding tegumental protein in tissues of various stages of *F. gigantica*

4.1. Localization by immunofluorescence

The dilution at 1:500 of rabbit anti-rFgPrx1-6xHistagged protein as a primary antibody, and Alexa 488 goat anti-rabbit as secondary antibody were used in the immunofluorescence detection. The green fluorescence was positively observed in the paraffin sections in all three stages of the parasites. Dapi and Topro3 were used for nuclear counterstaining.

In adult parasite, The florescence was present throughout the body of parasites as compared to the control section by pre-immune serum which did not fluoresce (Fig. 10A-B). Intense fluorescence was observed in the tegument, tegumental cells and their cytoplasmic's processes, caecal epithelial and parenchymal cells (Fig. 10B-C). Furthermore, the female reproductive organs also intensely fluoresced including ovary, Mehlis gland, vitelline cells in the vitelline gland and egg in the uterus exhibited strong green fluorescence (Fig. 10D-G). The bluish fluorescent nuclei by DAPI was also clearly exhibited in nucleus of each cell (Fig. 10C-D, G). In the uterus, all vitelline cells and ovum in the egg exhibited strong green fluorescence and bluish nuclei (Fig. 10G, 11C).
In contrast to the male reproductive organ, no green fluorescence was shown in the male gamete within a testis, and spermatozoa within a cirrus sac (Fig. 11B, D). Furthermore, only the prostrate gland showed the positive fluorescence (Fig11D).

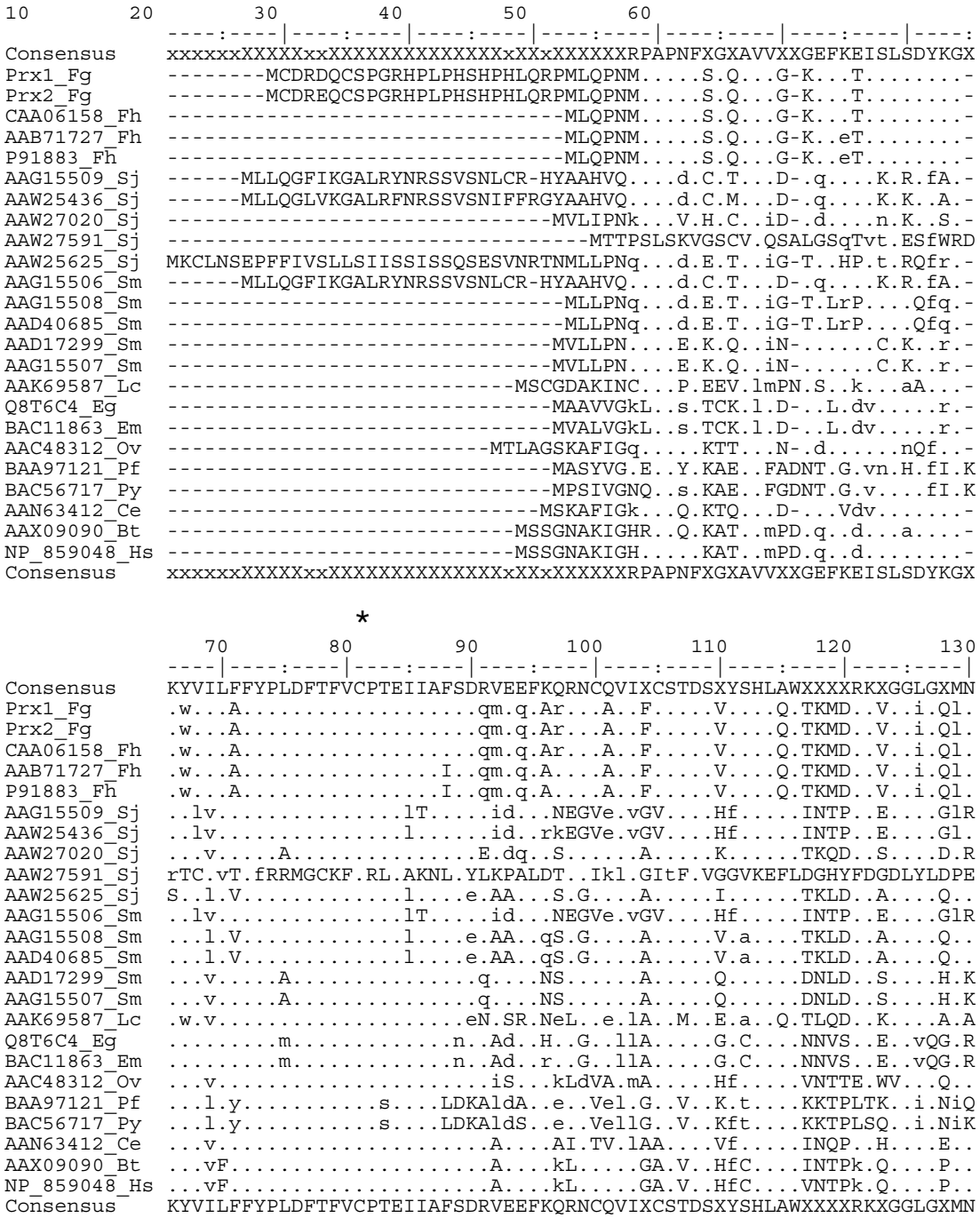
In 4 week-old juvenile parasites, the appearance of fluorescence was paralleled to that of the adult. Strong green fluorescence was observed in the tegument, tegumental cells, Mehlis gland, parenchymal and caecal epithelial cells (Fig. 12A-D). Moreover, when the tissue was counter-stained with the DAPI, the bluish fluorescent labeling was found only in the nuclei of all kinds of cells (Fig. 12B).

In newly excysted juvenile and metacercariae, the fluorescence staining was likely to those of the adult and the 4 week-old juvenile. Strong green fluorescence was clearly observed in the tegument and their cells (Fig. 12E-J). The red fluorescence due to counterstaining by Topro3 was presented in the nuclei of all cells (Fig. 12I). The autofluorescence was exhibited by the inner cyst wall of metacrcaria (Fig. 12J).

4.2. The specificity of antibody against rFgPrx by western blot

Purified rFgPrx-6xHistagged fusion protein and the various protein of parasites were separated by 12.5% SDS-PAGE and electrotransferred onto a nitrocellulose membrane, probed with rabbit anti-rFgPrx1 serum. The antibody strongly reacted with about 26 kDa of purified rFgPrx1-6xHistagged fusion protein from elutionI (EI) of bacterial lysate (Fig. 13A, lane 1). Correspondingly, the tegumental and adult worm fractions revealed the positive band approximately at 25 kDa as monomeric form and at 50 kDa as dimeric form (Fig. 13A, lane 3). In addition to the antigens of adult parasite, the protein extracts from 3, 5, and 7 week-old juveniles when reacted with anti rFgPrx-6xHistagged antibody showed two slightly lower MW at 25 kDa with the dimer at 50 kDa (Fig. 13A, lane 4-7). Whereas the newly excysted juvenile fraction could be observed the positive immunoreactivity at the dimmer FgPrx proteins similarly into the other fractions. Furthermore, no positive band was found in the excretory-secretory fraction (Fig. 13A, lane 2). When similar experiments were done with other trematode parasite extracts, such as *S. japonicum*, *S. mansoni*, *Eurytrema* spp., and *Paramphistomum* spp., no cross immunoreaction was detected against rabbit anti FgPrx1-6xHistagged protein (Fig. 13B, lane 2-5).

Figure 3. Multiple alignment of the deduced amino acid sequence of FgPrx1 & 2 with amino acid sequences of peroxiredoxin family from other species. The top and below of this alignment is shown the consensus sequence, (.) indicates identical residues, small letters indicate similar residues and capital letters indicate different residues. Star (*) indicates the two cysteine, Cys^{81} and Cys^{202} respectively. The aligned sequences were retrieved from GenBank and SwissProt.


10 20 30 40 50 60
Consensus xxxxxxXXXXXxxXXXXXXXXXXXXXXXxXXxXXXXXXRPAPNFXGXAVVXXGEFKEISLSDYKGX
Prx1_Fg --------MCDRDQCSPGRHPLPHSHPHLQRPMLQPNM.....S.Q...G-K...T........-
Prx2_Fg --------MCDREQCSPGRHPLPHSHPHLQRPMLQPNM.....S.Q...G-K...T........-
CAA06158_Fh ------------------------------MLQPNM.....S.Q...G-K...T........-
AAB71727_Fh ------------------------------MLQPNM.....S.Q...G-K..eT........-
P91883_Fh ------------------------------MLQPNM.....S.Q...G-K..eT........-
AAG15509_Sj ------MLLQGFIKGALRYNRSSVSNLCR-HYAAHVQ....d.C.T...D-.q....K.R.fA.-
AAW25436_Sj ------MLLQGLVKGALRFNRSSVSNIFFRGYAAHVQ....d.C.M...D-.q....K.K..A.-
AAW27020_Sj ------------------------------MVLIPNk...V.H.C..iD-.d....n.K..S.-
AAW27591_Sj ------------------------------MTTPSLSKVGSCV.QSALGSqTvt.ESfWRD
AAW25625_Sj MKCLNSEPFFIVSLLSIISSISSQSESVNRTNMLLPNq...d.E.T..iG-T..HP.t.RQfr.-
AAG15506_Sm ------MLLQGFIKGALRYNRSSVSNLCR-HYAAHVQ....d.C.T...D-.q....K.R.fA.-
AAG15508_Sm ------------------------------MLLPNq...d.E.T..iG-T.LrP....Qfq.-
AAD40685_Sm ------------------------------MLLPNq...d.E.T..iG-T.LrP....Qfq.-
AAD17299_Sm ------------------------------MVLLPN....E.K.Q..iN-......C.K..r.-
AAG15507_Sm ------------------------------MVLLPN....E.K.Q..iN-......C.K..r.-
AAK69587_Lc ----------------------------MSCGDAKINC...P.EEV.lmPN.S..k...aA...-
Q8T6C4_Eg ------------------------------MAAVVGkL..s.TCK.l.D-..L.dv.....r.-
BAC11863_Em ------------------------------MVALVGkL..s.TCK.l.D-..L.dv.....r.-
AAC48312_Ov ------------------------MTLAGSKAFIGq.....KTT...N-.d......nQf..-
BAA97121_Pf ------------------------------MASYVG.E..Y.KAE..FADNT.G.vn.H.fI.K
BAC56717_Py ------------------------------MPSIVGNQ..s.KAE..FGDNT.G.v....fI.K
AAN63412_Ce ----------------------------MSKAFIGk...Q.KTQ...D-...Vdv........-
AAX09090_Bt ----------------------------MSSGNAKIGHR..Q.KAT..mPD.q..d...a....-
NP_859048_Hs ----------------------------MSSGNAKIGH.....KAT..mPD.q..d........-
Consensus xxxxxxXXXXXxxXXXXXXXXXXXXXXXxXXxXXXXXXRPAPNFXGXAVVXXGEFKEISLSDYKGX
*
70 80 90 100 110 120 130
Consensus KYVILFFYPLDFTFVCPTEIIAFSDRVEEFKQRNCQVIXCSTDSXYSHLAWXXXXRKXGGLGXMN
Prx1_Fg .w...A.....qm.q.Ar...A..F.....V....Q.TKMD..V..i.Ql.
Prx2_Fg .w...A.....qm.q.Ar...A..F.....V....Q.TKMD..V..i.Ql.
CAA06158_Fh .w...A.....qm.q.Ar...A..F.....V....Q.TKMD..V..i.Ql.
AAB71727_Fh .w...A.....I..qm.q.A....A..F.....V....Q.TKMD..V..i.Ql.
P91883_Fh .w...A.....I..qm.q.A....A..F.....V....Q.TKMD..V..i.Ql.
AAG15509_Sj ..lv.....lT.....id...NEGVe.vGV....Hf.....INTP..E....GlR
AAW25436_Sj ..lv.....l.....id..rkEGVe.vGV....Hf.....INTP..E....Gl.
AAW27020_Sj ...v.....A.....E.dq..S.....A.....K.....TKQD..S....D.R
AAW27591_Sj rTC.vT.fRRMGCKF.RL.AKNL.YLKPALDT..Ikl.GItF.VGGVKEFLDGHYFDGDLYLDPE
AAW25625_Sj S..l.V.....l....e.AA...S.G....A.....I.....TKLD..A....Q..
AAG15506_Sm ..lv.....lT.....id...NEGVe.vGV....Hf.....INTP..E....GlR
AAG15508_Sm ...l.V.....l....e.AA..qS.G....A.....V.a....TKLD..A....Q..
AAD40685_Sm ...l.V.....l....e.AA..qS.G....A.....V.a....TKLD..A....Q..
AAD17299_Sm ...v.....A.....q....NS.....A.....Q.....DNLD..S....H.K
AAG15507_Sm ...v.....A.....q....NS.....A.....Q.....DNLD..S....H.K
AAK69587_Lc .w.v.....eN.SR.NeL..e.lA..M..E.a..Q.TLQD..K....A.A
Q8T6C4_Eg .....m.....n..Ad..H..G..llA.....G.C....NNVS..E..vQG.R
BAC11863_Em .....m.....n..Ad..r..G..llA.....G.C....NNVS..E..vQG.R
AAC48312_Ov ...v.....iS...kLdVA.mA.....Hf.....VNTTE.WV...Q..
BAA97121_Pf ...l.y.....s....LDKAldA..e..Vel.G..V..K.t....KKTPLTK..i.NiQ
BAC56717_Py ...l.y.....s....LDKAldS..e..VellG..V..Kft....KKTPLSQ..i.NiK
AAN63412_Ce ...v.....A....AI.TV.lAA....Vf.....INQP..H....E..
AAX09090_Bt ...vF.....A....kL.....GA.V..HfC....INTPk.Q....P..
NP_859048_Hs ...vF.....A....kL.....GA.V..HfC....VNTPk.Q....P..
Consensus KYVILFFYPLDFTFVCPTEIIAFSDRVEEFKQRNCQVIXCSTDSXYSHLAWXXXXRKXGGLGXMN

```
                  140       150       160       170       180       190
            ----:----|----:----|----:----|----:----|----:----|----:----|----:
Consensus   IPLLADXNXXISRAYGVLXEEEGXAFRGLFIIDXKGVLRQITVNDXPVGRSVDEXLRLLDAFQFX
Prx1_Fg     F.....K.MS........D..q.NTy..N.l..P...........R......e.A.......I.H
Prx2_Fg     F.....K.MS........D..q.NTy..N.l..P...........R......e.A.......I.H
CAA06158_Fh F....eK.MS........D..q.NTy..N.l..P....P......R......e.A.......I.H
AAB71727_Fh F.....K.MSv...f...D..q.NTy..N.l..P...........D......e.A.......I.H
P91883_Fh   F.....K.MSv...f...D..q.NTy..N.l..P...........D......e.A.......I.H
AAG15509_Sj Y.....YQKQvt.D....H..L.V.L......SAD.ii....i..L.A......V...vR...yT
AAW25436_Sj Y.....YQKQ.t.D..i.K.dL.V.L......nPe.iv....i..L........V...vR....T
AAW27020_Sj ......PTKS.a......D....N.........P..i........K........T.........V
AAW27591_Sj RMTYKALGYKKVSPCSGAISLFSK.A.A.NSKAKAAKiPGNLSG.GWQTGGlLVVEkGGKILYyY
AAW25625_Sj ....s.K.LK........D....H....m.l..PN..........R........Ai......I.F
AAG15506_Sm Y.....YQKQvt.D....H..L.V.L......SAD.ii....i..L.A......V...vR...yT
AAG15508_Sm ....s.K.LR.....E..D.q..H....m.l..R..i........R........Ai......I.F
AAD40685_Sm ....s.K.LR.....E..D.q..H....m.l..R..i........R........Ai......I.F
AAD17299_Sm ......RKQE..k....FD..d.N.........PN.i.....i..K........T.........V
AAG15507_Sm ......RKQE..k....FD..d.N.........PN.i.....i..K........T.........V
AAK69587_Lc ..m...KTKS.a......A.kq.V.y.......PN.mv.......M....n.e.V....e....V
Q8T6C4_Eg   ..m...T.HK...D....I.dq.I.L.......D........i..L........A.........T
BAC11863_Em ..m...T.HK...D....I.dq.I.L.......D........i..L........A.........T
AAC48312_Ov ..i...T.HA..k.....K.d..I.y.......S..i........L........T...iQ....V
BAA97121_Pf HT.is.ITKS...s.N..FG-dSVsL.AFVl..Kq..vqHlL..nLAi....e.V..ii..V.HH
BAC56717_Py HT.is.IsKS.a.s.D..FN-.SV.L.AFVl..Kq..vqHlL..nLAl......I...i..L.HH
AAN63412_Ce ..v...T.HQ...D....K.d..I.........PSQN.....i..L........T...vQ....V
AAX09090_Bt ...is.PKRT.aqD....KAd..Is........D..i.....i..L........T...vQ....T
NP_859048_Hs ...vs.PKRT.aqD....KAd..Is........D..i........L........T...vQ....T
Consensus   IPLLADXNXXISRAYGVLXEEEGXAFRGLFIIDXKGVLRQITVNDXPVGRSVDEXLRLLDAFQFX

                  *
              200       210       220       230       240       250       260
            ----|----:----|----:----|----:----|----:----|----:----|----:----|
Consensus   EKHGEVCPANWKPXSXTIKPDXKXSKXYFSXXXXxxxxxxxx
Prx1_Fg     .e..........K.K..V.TPDG..A...SANS--------
Prx2_Fg     .e..........K.K..V..PDG..A...SVNS--------
CAA06158_Fh .e..........K.K..V.TPDG..A...SAN---------
AAB71727_Fh .e..........K.K..V.TPDG..A...SAN---------
P91883_Fh   .e..........K.K..V.TPDG..A...SAN---------
AAG15509_Sj d.y......d.q.KGP.....L.KY.E..HKVN---------
AAW25436_Sj d........d.q.KGP.....L.QY.E..HKVH---------
AAW27020_Sj ..LW-------------------------------------
AAW27591_Sj .qKEV.RHPdy.KIIDVl.I.T.DVPEfAtVLSQECDDSCKM
AAW25625_Sj ..n.........K.A.....PTAaLS...SVN---------
AAG15506_Sm d.y......d.q.KGP.....L.KY.E..HKVN---------
AAG15508_Sm ............N.A.....PVA.LS...SVH---------
AAD40685_Sm ............N.A.....PVA.LS...SVH---------
AAD17299_Sm ........V...RGQHG..VnQ.------------------
AAG15507_Sm ........V...RGQHG..VnQ.------------------
AAK69587_Lc ............KGaP.m..eP.A.VEGyFSKL---------
Q8T6C4_Eg   d..........q.G.K.F..SAGDL.SfM.S-----------
BAC11863_Em d..........H.G.K.F..SAGDL.SfM.S-----------
AAC48312_Ov dN.........q.G.E....eV.E..E..GKH----------
BAA97121_Pf .q..d.......KGKVAm..SEeGVSE.L.KL----------
BAC56717_Py ..y.d......qKGKEsm..SEeGVAK.L.NL----------
AAN63412_Ce .........G.T.G.D....GV.E.qE..KKH----------
AAX09090_Bt d........G...G.D.....VqK..E...KQK---------
NP_859048_Hs d........G...G.D.....VqK..E...KQK---------
Consensus   EKHGEVCPANWKPXSXTIKPDXKXSKXYFSXXXXxxxxxxxx
```

Figure 4 Indentity of the value (%) Fasciola gigantica Peroxiredoxin amino acid sequence in pairwise comparison with homologous Prx from other species.

Sequence Identity Matrix

Seq->	Prx1_Fg	Prx2_Fg	AAB71727	P91883	CAA06158	AAW27591	AAW27020	AAW25625	AAW25436	AAG1550	AAG15508	AAD17299	AAD40685	AAG15506	AAG15507	AAX09090	NP_859048
Prx1_Fg	**ID**	0.986	0.858	0.858	0.876	0.086	0.520	0.603	0.479	0.457	0.611	0.547	0.611	0.457	0.547	0.486	0.504
Prx2_Fg	0.986	**ID**	0.849	0.849	0.867	0.091	0.520	0.612	0.488	0.466	0.621	0.547	0.621	0.466	0.547	0.490	0.509
AAB71727,Fh	0.858	0.849	**ID**	1.000	0.958	0.101	0.564	0.579	0.459	0.447	0.670	0.594	0.670	0.447	0.594	0.515	0.535
P91883,Fh	0.858	0.849	1.000	**ID**	0.958	0.101	0.564	0.579	0.459	0.447	0.670	0.594	0.670	0.447	0.594	0.515	0.535
CAA06158,Fh	0.876	0.867	0.958	0.958	**ID**	0.097	0.574	0.588	0.468	0.447	0.680	0.605	0.680	0.447	0.605	0.525	0.545
AAW27591,Sj	0.086	0.091	0.101	0.101	0.097	**ID**	0.096	0.092	0.094	0.116	0.097	0.106	0.097	0.116	0.106	0.100	0.100
AAW27020,Sj	0.520	0.520	0.564	0.564	0.574	0.096	**ID**	0.513	0.472	0.461	0.579	0.735	0.579	0.461	0.735	0.527	0.537
AAW25625,Sj	0.603	0.612	0.579	0.579	0.588	0.092	0.513	**ID**	0.495	0.500	0.769	0.553	0.769	0.500	0.553	0.488	0.497
AAW25436,Sj	0.479	0.488	0.459	0.459	0.468	0.094	0.472	0.495	**ID**	0.877	0.486	0.509	0.486	0.877	0.509	0.588	0.583
AAG15509,Sj	0.457	0.466	0.447	0.447	0.447	0.116	0.461	0.500	0.877	**ID**	0.470	0.484	0.470	1.000	0.484	0.563	0.559
AAG15508,Sm	0.611	0.621	0.670	0.670	0.680	0.097	0.579	0.769	0.486	0.470	**ID**	0.620	1.000	0.470	0.620	0.565	0.580
AAD17299,Sm	0.547	0.547	0.594	0.594	0.605	0.106	0.735	0.553	0.509	0.484	0.620	**ID**	0.620	0.484	1.000	0.572	0.572
AAD40685,Sm	0.611	0.621	0.670	0.670	0.680	0.097	0.579	0.769	0.486	0.470	1.000	0.620	**ID**	0.470	0.620	0.565	0.580
AAG15506,Sm	0.457	0.466	0.447	0.447	0.447	0.116	0.461	0.500	0.877	1.000	0.470	0.484	0.470	**ID**	0.484	0.563	0.559
AAG15507,Sm	0.547	0.547	0.594	0.594	0.605	0.106	0.735	0.553	0.509	0.484	0.620	1.000	0.620	0.484	**ID**	0.572	0.572
AAX09090,Bt	0.486	0.490	0.515	0.515	0.525	0.100	0.527	0.488	0.588	0.563	0.565	0.572	0.565	0.563	0.572	**ID**	0.969
NP_859048,Hs	0.504	0.509	0.535	0.535	0.545	0.100	0.537	0.497	0.583	0.559	0.580	0.572	0.580	0.559	0.572	0.969	**ID**

Figure 5 Phylogenetic analysis of Peroxiredoxin. A multiple sequence alignment of the listed Prx sequences was done in the Clustral W program [13]. The PAUP 4.0 program was used to create an rooted phylogenetic tree using UPGMA.

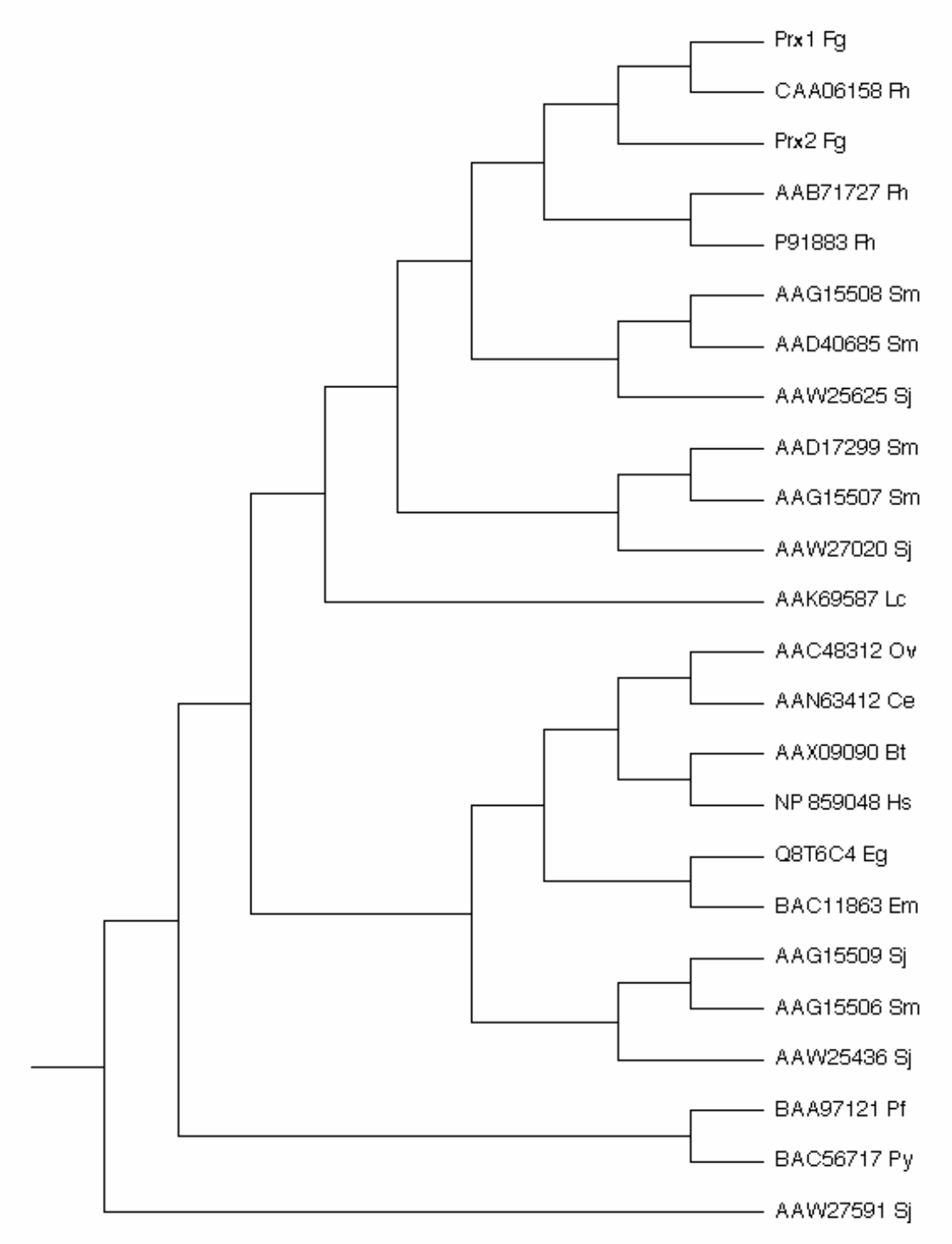

Prx1 Fg
CAA06158 Rh
Prx2 Fg
AAB71727 Rh
P91883 Rh
AAG15508 Sm
AAD40685 Sm
AAW25625 Sj
AAD17299 Sm
AAG15507 Sm
AAW27020 Sj
AAK69587 Lc
AAC48312 Ov
AAN63412 Ce
AAX09090 Bt
NP 859048 Hs
Q8T6C4 Eg
BAC11863 Em
AAG15509 Sj
AAG15506 Sm
AAW25436 Sj
BAA97121 Pf
BAC56717 Py
AAW27591 Sj

Figure 6 Expression of FgPrx gene in adult *F. gigantica* as demonstrated by in situ hybridization using a FgPrx antisense RNA probe

(A) Cross section of adult parasite, strong staining of a FgPrx by antisense RNA probe was observed in the tegumental cells (Tc), caecal epithelial cells (Ca), and vitellin cells (Vi). tegument (Te) were negatively stained.

(B) High magnification of cross section, showing positive staining in the tegumental cells (Tc), caecal epithelial cells (Ca), and vitelline cells (Vi). Whilst their nuclei, tegument, spines and were negatively stained.

(C) Highest magnification of cross section, showing positive staining in the cytoplasm of tegumental cells (Tc).

(D) At the ventral sucker (Vs), the staining was found only in the tegumental-type epithelial cells and prostrate gland (Pg).

(E) Highest magnification of prostrate gland, showing positive staining in the cytoplasm of the prostate gland cells (Pg), whilst the cirrus (Ci) and sperm in the seminal vesicle (Se) showed negatively staining.

(F) The positive staining was present in the cells of various spermatogenesis stages within the testis (Ti)

(G) High magnification of the vitelline cells (Vi), the positive staining was detected.

(H) High magnification of the ovary (Ov), the hybridization signal was found in the cytoplasm of ovary.

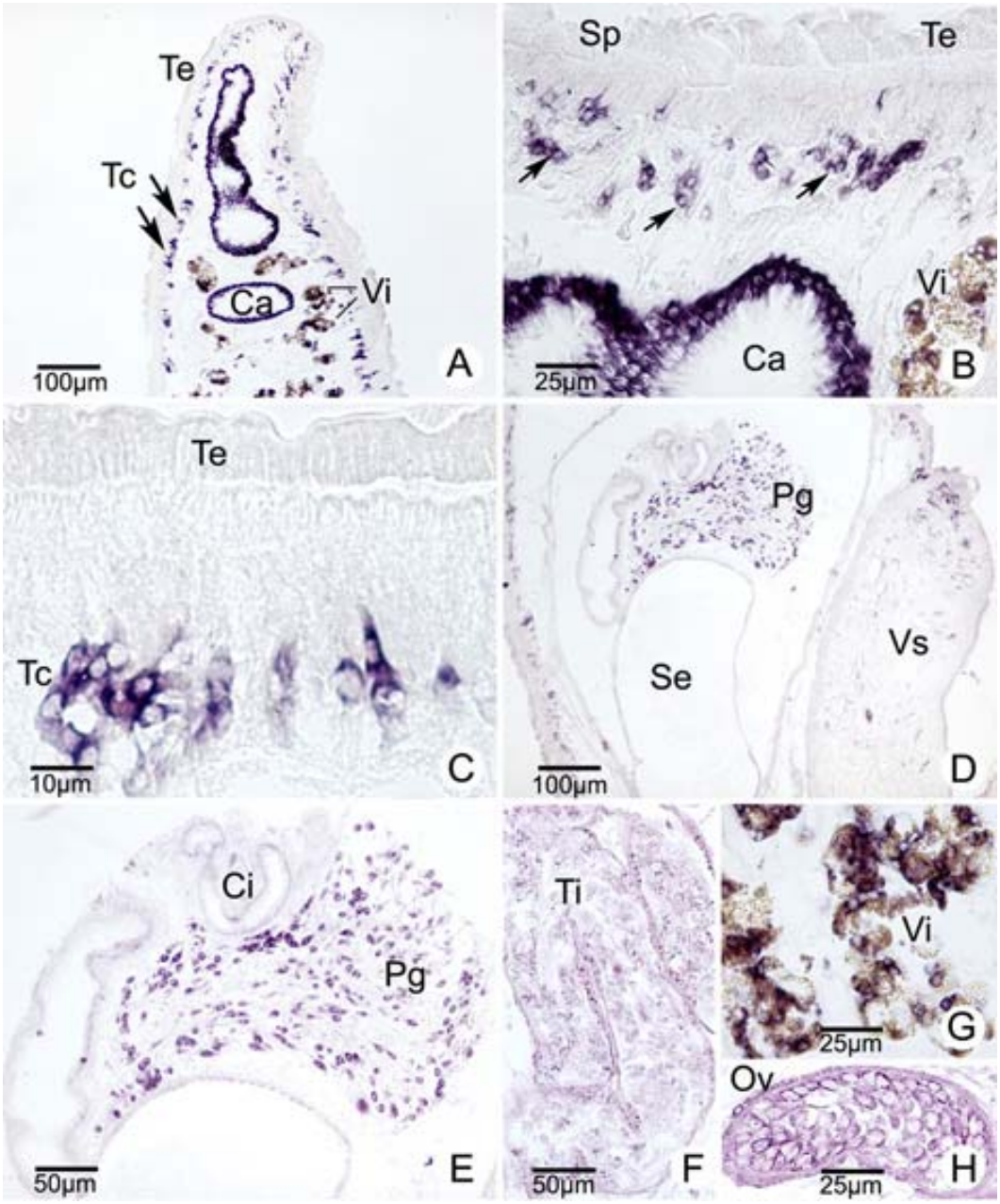

Te
Tc
Ca
Vi
100μm
A
Sp
Te
Vi
Ca
25μm
B
Te
Tc
10μm
C
Pg
Vs
Se
100μm
D
Ci
Pg
50μm
E
Ti
50μm
F
Vi
25μm
G
Ov
25μm
H

Figure 7 Expression of FgPrx gene in 4 week-old juvenile, newly excysted juvenile and metacercaria of *F. gigantica* as demonstrated by in situ hybridization using a FgPrx antisense RNA probe

(A) In 4 week-old juveniles, FgPrx antisense RNA probe showed strong staining in tegumental cells (Tc, arrows), caecal epithelial cells (Ca), but not in the tegument (Te).

(C) Higher magnification of 4 week-old juvenile, the positive staining was found in the tegumental cells (Tc) beneath the subtegument and cells of caecal epithelial cells (Ca).

(D) A metacercaria, the positive staining was predominantly present in cells along with digestive tract and moderately in the tegumental cells (Tc). Whist the inner cyst wall (Cw2) showed strong staining.

(E) Control cross-section of newly excysted juvenile, showing strong staining in two separating branches of caecum (Ca) along the digestive tract and moderate staining in the tegumental cells (Tc, arrow head)

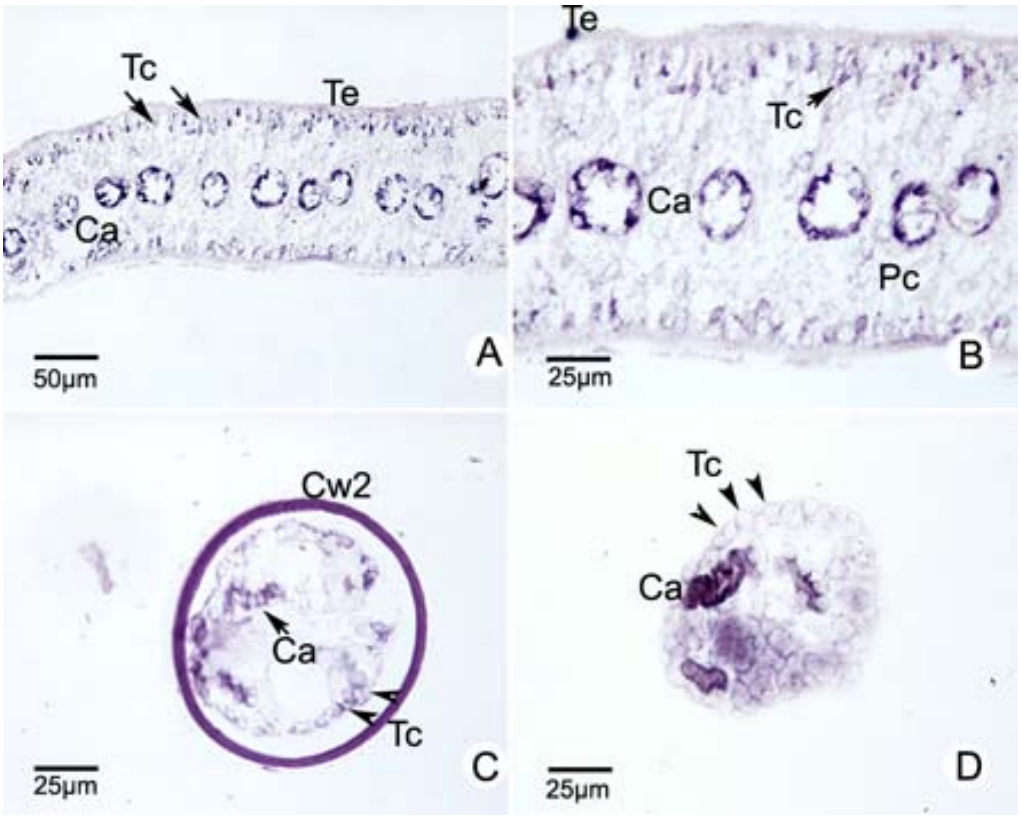

Tc
Te
Ca
50μm
A
Te
Tc
Ca
Pc
25μm
B
Cw2
Ca
Tc
25μm
C
Tc
Ca
25μm
D

Figure 8 Northern blot analysis of *F. gigantica*. Total RNA (20 μg) extracted from 4-week old juvenile (lane 1) and adult stages (lane 2) were separtated by electrophoresis in a 1.5% agarose – formaldehyde gel and probed with the coding sequence of FgPrx1. A single hybridizing transcript of approximately 1 kb was produced in both stages of parasites

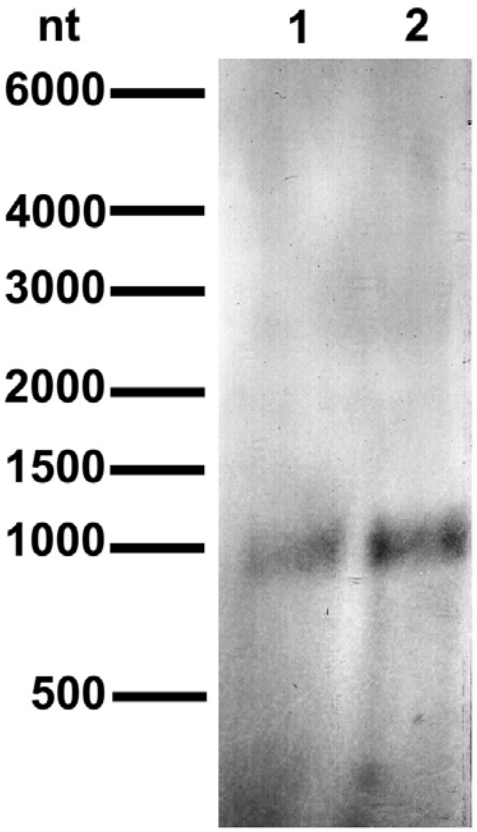
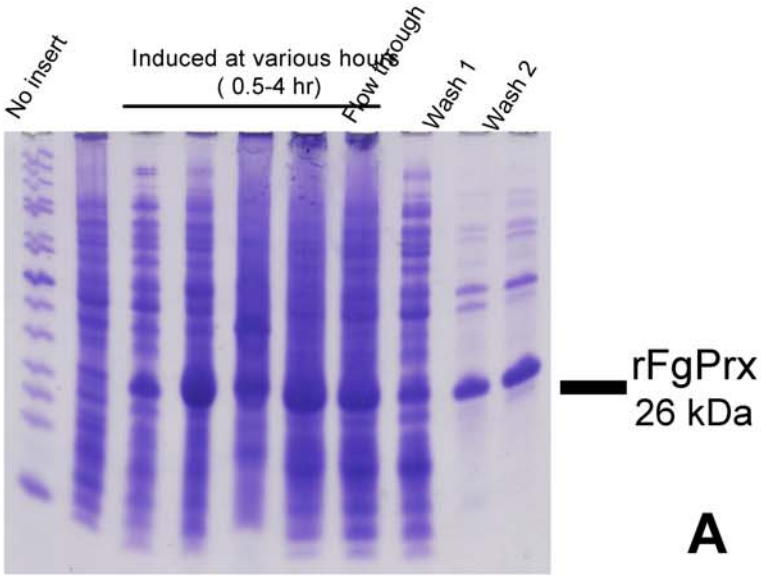

nt
1
2
6000
4000
3000
2000
1500
1000
500

Figure 9 Electrophoretic pattern of the rFgPrx1-6xHistagged fusion protein expression profiles in bacterial lysates (A) and the purification of a rFgPrx1-6xHistagged fusion protein (B) was analyzed using 12.5% SDS-PAGE stained with Coomassie Blue.

(A) A time course of expression of recombinant FgPrx-6xHistagged fusion protein. A sample of total bacterial cell transformants with non-inserted gene was collected (No insert). After induction in the presence of 1mM IPTG, the total bacterial cells were kept at various hours starting from 30 min and 1, 2, 3, and 4 hour. After induction with IPTG for 4 h, the bacterial cell lysate containing rFgPrx1-6xHistagged fusion protein was purified by His-Resin chromatography under native conditions with various immidazole, Flow-through (FT); 5mM wash (Wash I); 20 mM wash (Wash II)

(B) After purification with His-Resin chromatography, the rFgPrx1-6xHistagged fusion protein was eluted by 500 mM elution (Elute1) and 1000 mM elution (Elute 2). This rFgPrx1-6xHistagged fusion has a MW at 26 kDa.

Prx purification using Ni-NTA column



A

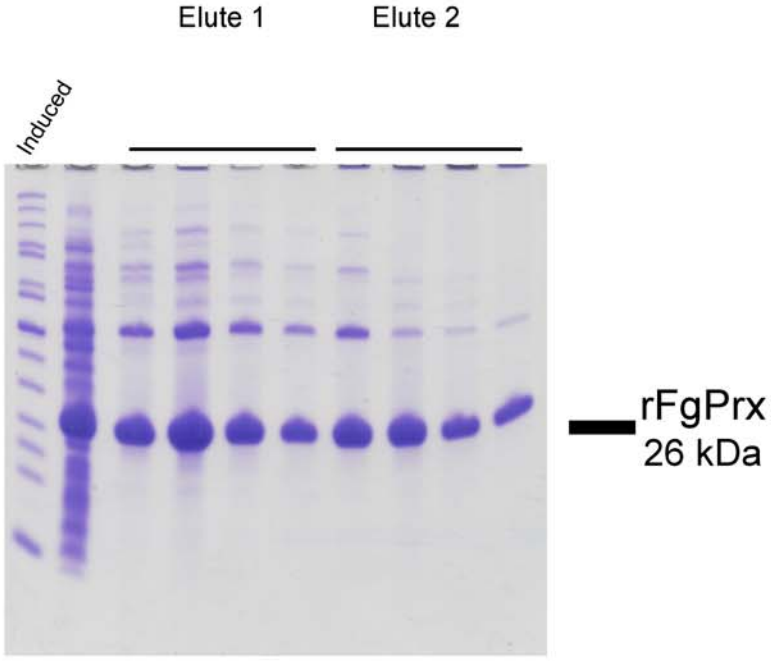


Figure 10 Localization of FgPrx1 protein in the paraffin section of adult from *F. gigantica* by immunofluorescence technique using polyclonal antibody generated from rabbit immunized with rFgPrx protein as probe.

(A) Control section stained with pre-immune sera, showed no specific fluorescence in the parasite's tissues. Ca = caecal epithelial cells, Te = tegument, Ti = Testis.

(B) Longitudinal section of an adult worm, showing the positive fluorescence throughout the body of parasite, except the spines (Sp). Os= Oral sucker, Pc= Parenchymal cells

(C) Higher magnification showing the intense fluorescence was observed in the tegument (Te) and tegumental cells (Tc) while no fluorescence was observed in the spine (Sp, arrow).

(D-G) The fluorescence was appeared particularly strong in the female reprocductive organs including ovary (Ov), Mehlis gland (Mg), vitelline cells (Vi), and egg (Eg). The bluish fluorescence showed the counterstaining of nuclei with DAPI.

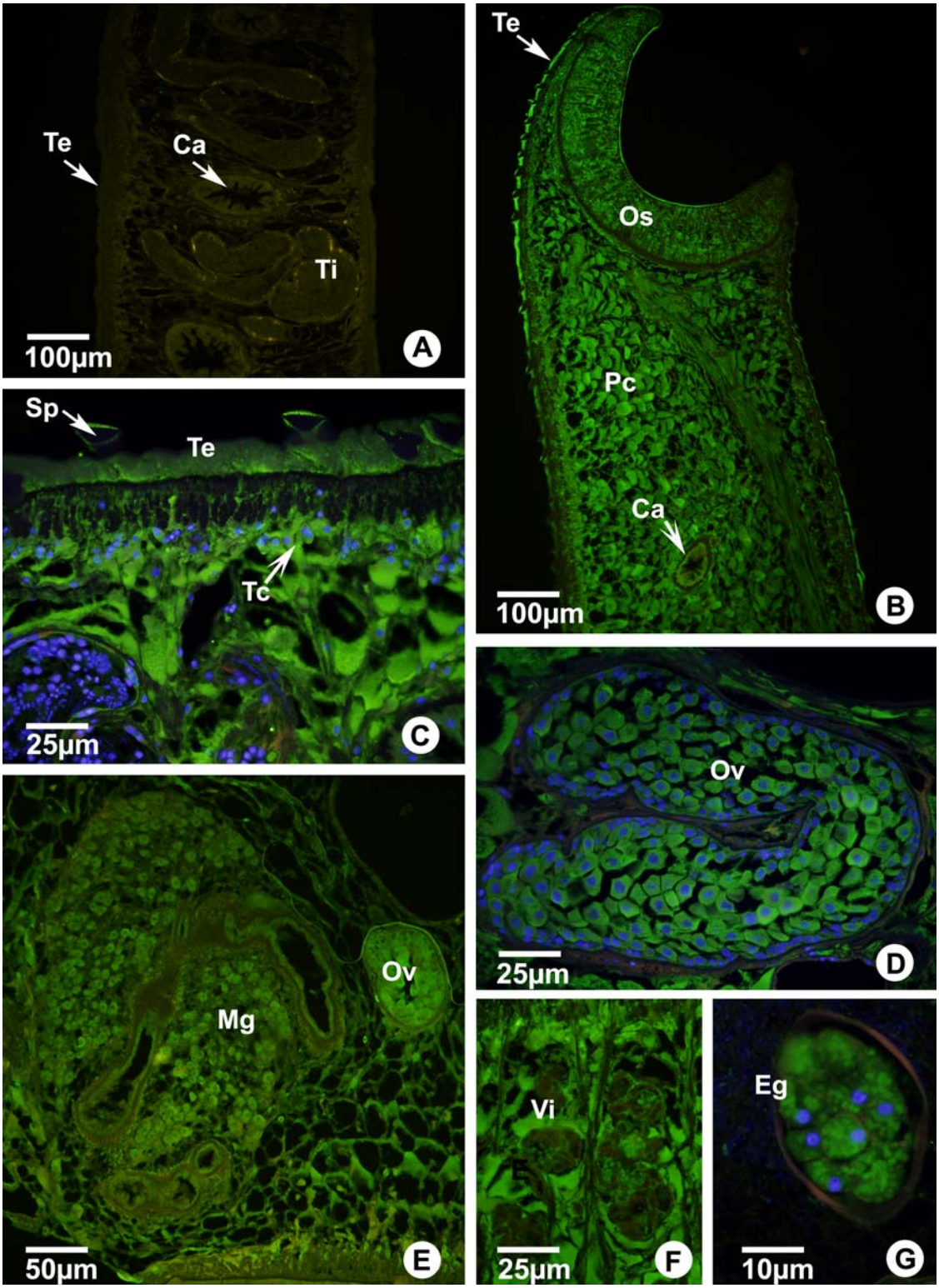

Te
Ca
Ti
100µm
A
Te
Os
Pc
Ca
100µm
B
Sp
Te
Tc
25µm
C
Ov
25µm
D
Ov
Mg
50µm
E
Vi
25µm
F
Eg
10µm
G

Figure 11 Localization of FgPrx protein in the paraffin section of male reproductive organs of an adult *F. gigantica* by immunofluorescence technique using polyclonal antibody generated from rabbit immunized with rFgPrx protein as probe.

(A) Cross section of an adult worm, showing no fluorescence in the body of parasite, particularly in the prostrate gland (Pg), prostrate duct (Pd), and egg (Eg) of male and female reproductive organs. The autofluorescence was found in the egg shell.

(B) High magnification of testis, showing no green fluorescence in spermatogenic cells in the testis (Ti). The bluish fluorescence was observed in the nucleus of various cells , spermatogonia as well as spermatid.

(C) Longitudinal section of an adult worm counter-stained with DAPI, showing green fluorescence throughout the body, especially intense in egg in the uterus (Ut). While the bluish staining showed the nucleus of various cells, especially in the mature spermatozoa.

(D) High magnifications of prostrate gland and cirrus, showing green fluorescence. The autofluorescence was found in the egg's shell within cirrus. Vs= ventral sucker

(E) High magnification of cirrus, showing the green fluorescence in the vitelline cells of egg (Eg) but not mature spermatozoa. The bluish staining counterstained the nucleus of mature spermatozoa.

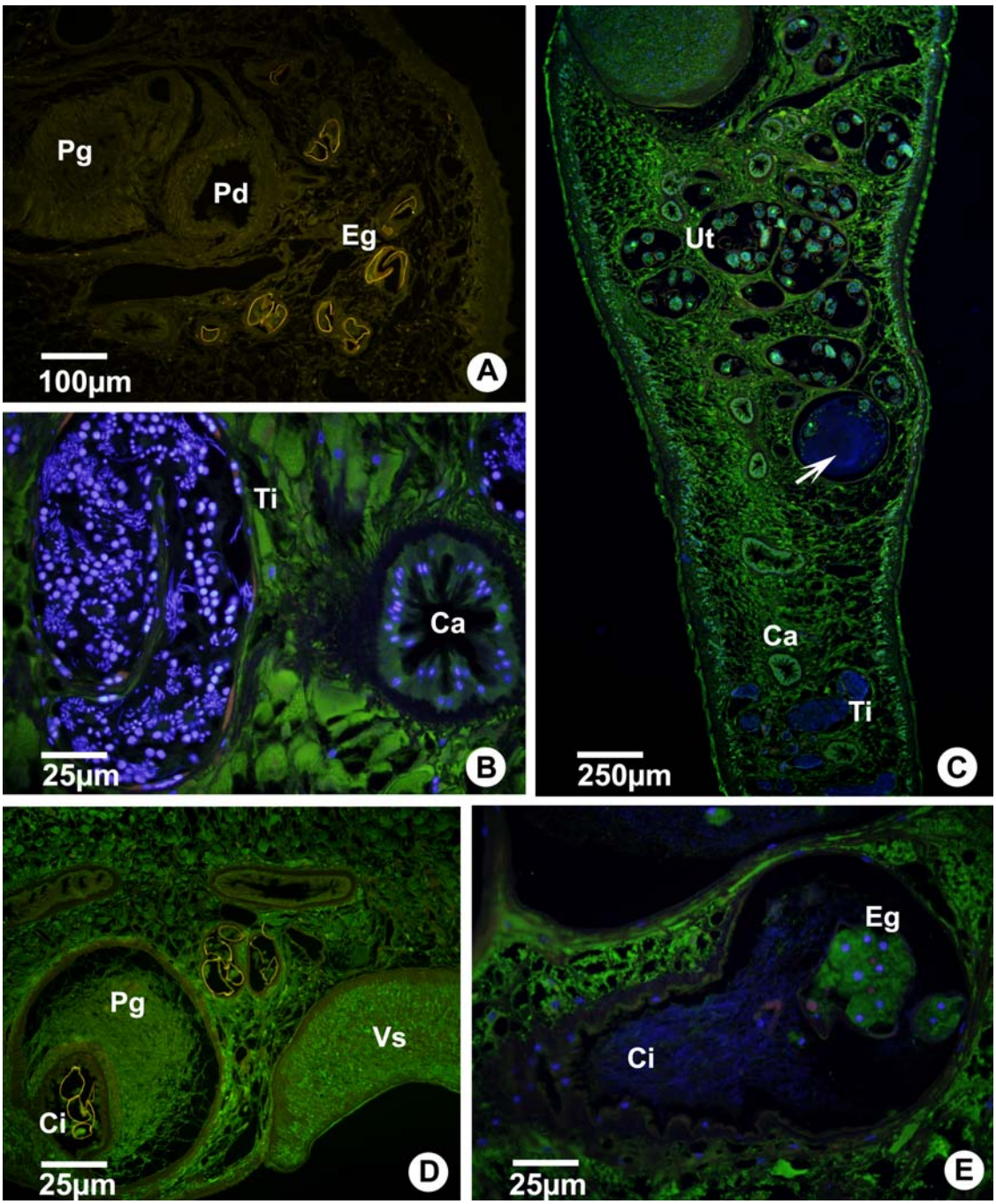

Pg
Pd
Eg
100µm
A
Ti
Ca
25µm
B
Ut
Ca
Ti
250µm
C
Pg
Vs
Ci
25µm
D
Eg
Ci
25µm
E

Figure 12 Localization of FgPrx protein in the paraffin section 4 week-juvenile, newly excysted juvenile and metacercaria *F. gigantica* by immunofluorescence technique using polyclonal antibody generated from rabbit immunized with rFgPrx protein as probe.

(A) Longitudinal section of the juvenile parasite, showed positive staining throughout the worm body, including ventral sucker (Vs), tegument (Te), parenchymal cells (Pc), caecal epithelial cells (Ca), but not the spine (Sp).

(B) Superimposed micrograph of 4 week-old juvenile parasite, counterstained the DAPI, showing the green fluorescence throughout the worm body as found in Fig. A. The bluish fluorescence is specific to the nuclei.

(C, D) High magnifications of the Mehlis gland and lower part of juvenile parasite, showing the positive staining throughout the worm, including tegument (Te), tegumental cells (Tc, arrows), parenchymal cells (Pc), caecal epithelial cells (Ca).

(E) Low magnification of newly excysted juvenile and metacercaria, showing the green fluorescence in tegument (Te).

(F) Superimposed micrograph of newly excysted juvenile and metacercaria counterstained the DAPI, showing the strong green fluorescence in the tegument as found in Fig. E.

(G-I) High magnification of newly excysted juvenile counterstained with the Topro3 (Fig. I), showing the strong green fluorescence in the cytoplasm of tegumental cells (arrows), tegument (Te), ventral sucker (Vs) and moderate staining in the parenchyma.

(J) High magnification of metacercaria, showing the positive signal throughout the body of worm with the strong fluorescence in the cytoplasm of tegumental cells (arrows). The autofluorescence was appeared in the inner cyst wall as the orange fluorescence.

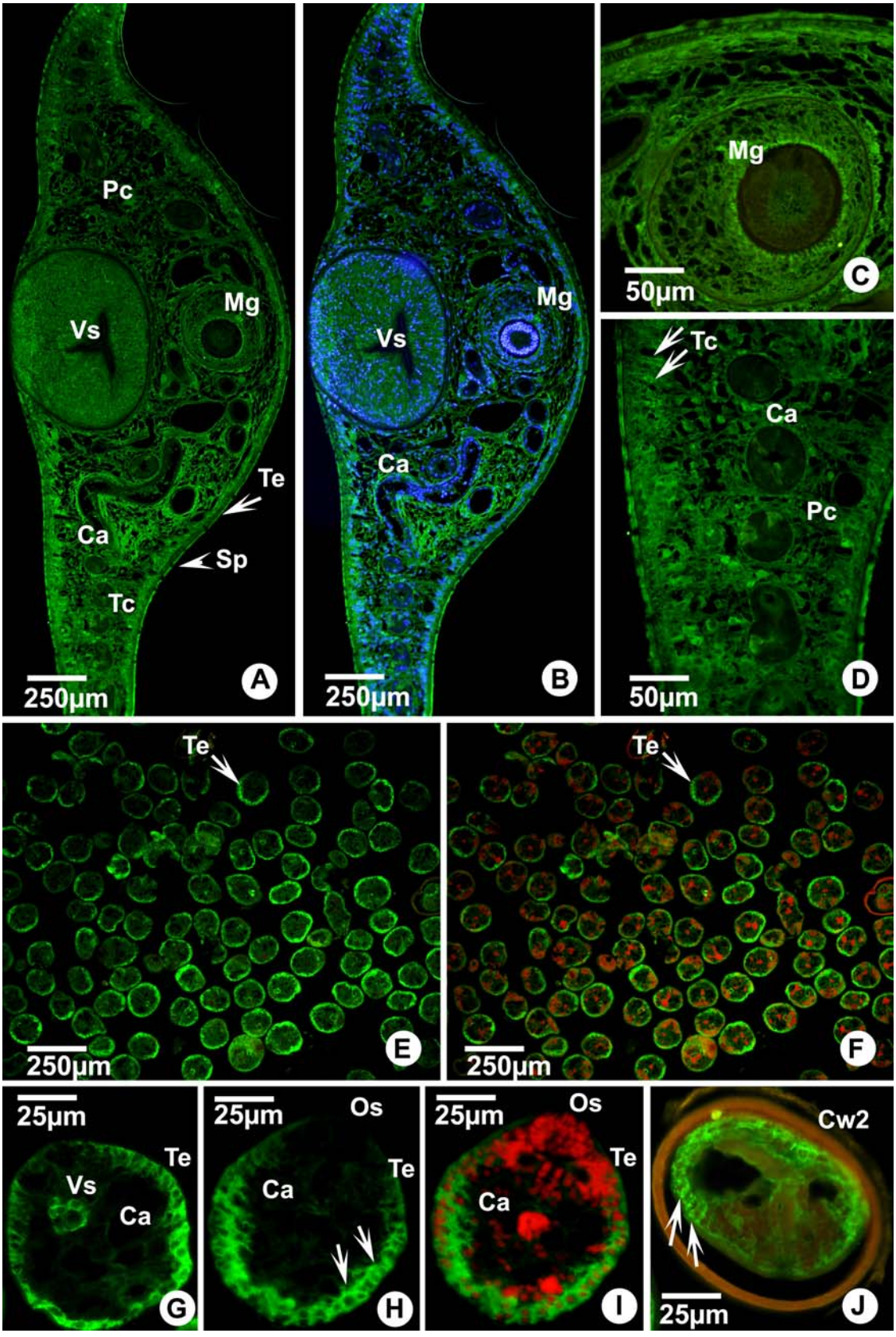
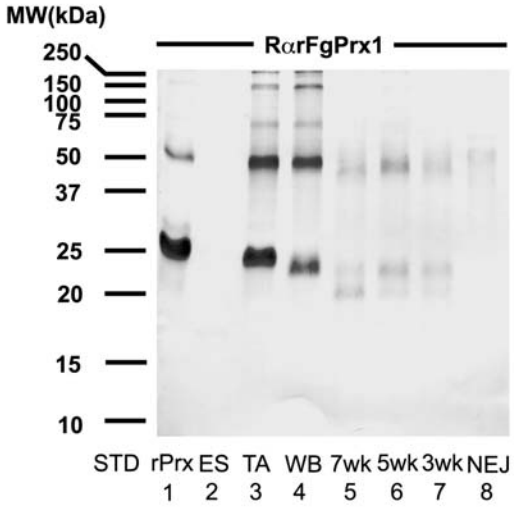

Pc
Vs
Mg
Te
Ca
Sp
Tc
250µm
A
Vs
Mg
Ca
250µm
B
Mg
50µm
C
Tc
Ca
Pc
50µm
D
Te
250µm
E
Te
250µm
F
25µm
Te
Vs
Ca
G
25µm
Os
Te
Ca
H
25µm
Os
Te
Ca
I
Cw2
25µm
J

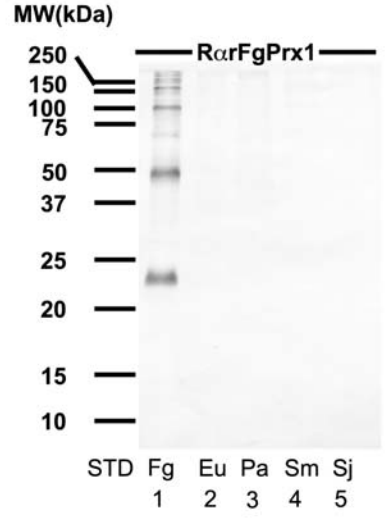
Figure 13 The specificity of antiserum to rFgPrx1 protein was determined using immunoblotting technique. STD is the lane containing standard protein molecular weight markers.

(A) The presence of approximately 25 kDa Prx protein was shown only in the tegument TA (tegumental fractions) in lane 3 as well as the slightly lower MW band with the dimeric Prx was observed in the various developmental stages of *F. gigantica*: WB (whole body of adult worm) in lane 4, 7 wk (7 week-old juvenile) in lane 5, 5 wk (5 week-old juvenile) in lane 6, 3 wk (3 week-old juvenile) in lane 7, NEJ (newly excysted juveniles) in lane 8. While the excretory-secretory fraction showed no positive band.

(B) The presence of 14-3-3 protein in various trematode parasites were shown by binding rabbit anti rFgPrx-6xHistagged fusion protein to crude worm extracts from *F. gigantica* in lane 1, Eu (*Eurytrema* spp.) in lane 2, Pa (*Paramphistomum* spp.) in lane 3, Sman (*S. mansoni*) in lane 4, Sj (*S. japonicum*) in lane 5. When compared with the immunoreactive band in *F. gigantica*, no cross-reaction was found in other trematode parasites.


MW(kDa)
RαrFgPrx1
250
150
100
75
50
37
25
20
15
10
STD rPrx ES TA WB 7wk 5wk 3wk NEJ
1 2 3 4 5 6 7 8

A


MW(kDa)
RαrFgPrx1
250
150
100
75
50
37
25
20
15
10
STD Fg Eu Pa Sm Sj
1 2 3 4 5

B

4. สรุปและวิจารณ์ผลการทดลอง

During the parasites' migration after excystment, Fasciola juvenile parasites have exposed to ROS not only from the immune effector cells but also from the hosts' catabolism and parasites' metabolism. A first line of defense oxygen toxicity is occurred by superoxide dismutase (SOD) to dismutate superoxide anion to hydrogen peroxide, which can continue to form a more dangerous molecules as hydorxyl radicals (Kim et al., 2000). Thus, hydrogen peroxide is commonly eliminated by catalase or glutathione peroxidases (GPxs) in many organisms. On the contrary, these antioxidant proteins are not found in protozoa and helminthes such as glutathione peroxidases and catalase in Leishmania (Barr and Gedamu 2001), and Schistosoma respectively (Mkoji et al., 1998). There are several reports the peroxiredoxin in *Fasciola hepatica* that can scavenge H_2O_2 without the metals or prosthetic groups (co factors) to water and alkyl hydroperoxides (Sekiay et al., 2006; McGonigle et al.,1997). By immunoscreening, we have cloned and characterized two isoforms of 2-Cys peroxiredoxin (FgPrx1 and FgPrx2) with a highly conserved amino acid sequence and catalytic motif to the mammalian. When compared with *F. hepatica*, both FgPrx1 and FgPrx2 showed the identity 87-88% and possessed an extension amino acid at N-terminal region. This extension was not a typical hydrophobic signal peptide sequence. Therefore, both FgPrx are interest of antioxidant protein to investigate the gene and protein expression profiles as well as their functions.

In Schistosoma, the localization of Prxs were observed throughout the body dependent and differential tissue dependent on the type of Prxs genes. SjPrx1 was detected in the tegument and both reproductive organs, whereas SjPrx2 was localized in the sub-tegumental layer, parenchyma, caecal epithelial cells and vitelline gland. The authors suggested that the differential expression of Prxs genes should have the varied functions. SjPrx1 could protect the ROS from the host immune cells. SjPrx2 played roles in intracellular redox signaling and/or in the reduction of ROS generated by the process of hemoglobinolytic process. In *F. gigantica*, our data performed the profiling of FgPrx1 & 2 mRNA and native FgPrx1 protein expressions, showing the stage-specificity of 2-Cys peroxiredoxin. These mRNA and protein expression patterns were quite similarly that the Prx distributed throughout the body of worm. Female reproductive organs, including egg, vitelline cells, and ovary were also positively stained correspondingly with

the Prx protein from other species. For example, *S. japonicum, S. mansoni, C. elegans* Prx protein are secreted from the eggs across the eggshell and believed its function to protect the egg from ROS of effector cells during the digestive system (Kumagai et al., 2006; Williams et al., 2001; Alger et al., 2002; Isermann et al., 2004; Kazura et al., 1985). Except, the FgPrx mRNA expression did not exit the hybridization signal in the parenchymal cells, despite the positive immunofluorescence of these cells was observed. Taken together the report of Prxs from Schistosoma, our data might be imply that these enzymes play a protective role against not only the internal oxidative damage but also the external oxidative stress.

The recombinant FgPrx1-6xHistagged protein was expressed in the bacterial system and used for the production of polyclonal anti-rFgPrx1 rabbit serum. The western blot show the immunoreactive band against the native Prx protein in the tegumental antigen at approximately 25 kDa, and 50 kDa in accordance with a predicted Prx amino acid (24.63 kDa). This finding supports FgPrx protein surrounding the tegument plays a major role of protection against ROS from the host immune cells. In *F. hepatica*, FhePrx served as the secreted protein due to the immunoreactive band at a major 26 kDa (monomeric form) and a minor 42 kDa (dimmer) of excretory-secretory extract (Donnelly et al., 2005; Jefferies et al., 2001; Salazar-Calderón et a., 2000). However, the molecular weight of the native antigen was not equal with the predicted amino acid sequence of FhPrx at 21.723 kDa and the author could not find the reasons of the extension of molecular weight (Salazar-Calderón et al., 2000). Interestingly, here two positive bands of whole *F. gigantica* by western blot were detected slightly lower than 25 kDa and one of these bands was corresponded with the native FhePrx protein. However, FgPrx was not shown in the excretory-secretory (ES) antigen from *F. gigantica*. Together, the similar observation was also found in the Prx from Brugia malayi (as a filarial parasite) (Ghosh et al., 1998).

In this summary, 2-Cys Peroxiredoxins from *F. gigantrica* show the stage-and specie-specificity, regarding to FgPrxs gene and protein expression throughout the body. These antioxidant enzymes, as an excreted protein, play a critical role in the host-parasite interface against oxidative damage during development. Consequently, the future

advance we would like to develop these enzymes as a vaccine candidate or immunodiagnosis development.

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6. Output

Veterinary parasitology **Acta Tropica** **Molecular Cloning and Characterization of Peroxiredoxin from *Fasciola gigantica***

2008

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