Cloning and Expression of Recombinant Glutathione Peroxidase in *Fasciola Gigantica*

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Abstract
Glutathione peroxidase (GPx) is one of the most essential antioxidant enzymes in trematode parasites that lacks catalase. GPx has been already characterized in the trematodes such as *Schistoma mansoni*, *Clonorchis sinensis*, and *Paragonimus westermani*. However, there is no reported about GPx in *Fasciola* spp. This study amplified GPx cDNA of *Fasciola gigantica* (*Fg*GPx) from cDNA library. Its sequence contained an in-frame TGA codon for selenocysteine (Sec) which is a stop codon in prokaryote. The Sec (TGA) codon was mutated into a sense codon for cysteine (TGC) using site-directed mutagenesis. The recombinant modified *Fg*GPx (rM_*Fg*GPx) protein was expressed as intracellular protein in *Escherichia coli*. Polyclonal anti-rM_*Fg*GPx was produced and immunoblotting analysis demonstrated that the anti-rM_*Fg*GPx antibodies did not cross-react with crude antigens from other trematodes including *S. mansoni*, *Opisthorchis viverrini* and *Paramphistomum* spp. The result of ELISA showed that test group had a significant increase of total immunoglobulin and IgG_{2a} when compared with control group. Moreover, there was a significant increase of total immunoglobulin in several *F. gigantica* infected animal including mice, rabbits and cows, but no cross reaction between *O. viverrini* infected hamsters sera and rM_*Fg*GPx. Therefore, rM_*Fg*GPx could be used as an immunodiagnostic tool for fascioliasis.
Keywords: *Fasciola Gigantica*, Glutathione Peroxidase, Mutagenesis, Vaccine Candidate, Immunodiagnostic Tools

Introduction
Fascioliasis is an important zoonotic helminth diseases worldwide (Cobbold, 1855). It is caused by liver flukes belonging to the genus *Fasciola*. The two main species of *Fasciola* are firstly, *Fasciola hepatica*, which is distributed in temperate regions and, secondly, *Fasciola gigantica* which has been found in tropical regions. Fascioliasis decreased productivity of domestic ruminants, especially cattle and buffaloes. In 1998, the economic losses caused by this infection had been estimated at more than 200 million dollars annually worldwide (Hammond & Sewell, 1974; Mehra, 1999). The infection also affects to humans,

approximately 2.4 million people are infected worldwide and 180 million people at risk of infection (Farag, 1998; Mas-Coma, Esteban, & Bargues, 1999).

In order to control this disease, various strategies such as eradicating the snail host, permanent destruction of snail habitats could be applied. But they are expensive and ecologically sensitive (Spithill, Smooker, & Copeman, 1999). For anthelmintics, several drugs are commonly used (El Sayad, 1997) but the limit for used and resistance to the drugs has been reported (Overend & Bowen, 1995). Prevention of infection by vaccination is considered to be the ultimate, mostly cost-effective, and sustainable strategy. Although many potentially protective candidate antigens have been reported, an effective vaccine has so far not been established (Spithill & Dalton, 1998). In order to control facioliasis effectively, a precise diagnosis for example by immunodiagnosis is important. This tool is well-suited for the detection of even the smallest amount of antigen and can be applied at an early stage of infection. A suitable antigen is needed for use as immunodiagnostic tool.

In host-parasite interaction, reactive oxygen species (ROS) are important preventive factors for the mammals (Marinho, Antunes, & Pinto, 1997; Rhee, Chang, Bae, Lee, & Kang, 2003). These factors damage parasite nucleic acids, lipids, proteins, and cell membranes, leading to death of the parasites. Nevertheless, the parasites can produce Glutathione peroxidase (GPx), an antioxidant enzyme, to cleave the ROS and to protect themselves (Flohe, 1971, 1978), GPx is thought to be an potential target for chemotherapy, as vaccine candidate and/or immunodiagnostic substance.

Glutathione peroxidase recombinant protein cannot be completely produced in prokaryote expression system because GPx contains a selenocysteine in the active site that is not coded directly in the genetic code (Roche et al., 1994; Haiping, Arvind, Julie, & Philip, 1996). Instead, the bacterial host cell is using the UGA codon as a normal stop codon and translation of selenoprotein terminate at the UGA codon, resulting in a truncated, nonfunctional enzyme. By the way, the structure of selenocysteine is identical to cysteine except that it has the element selenium where sulfur would normally reside. So, site directed mutagenesis can be performed to convert the opal codon of selenocysteine to a sense codon of cysteine (Bock et al., 1991).

The aim of this study is to express GPx in *F. gigantica* in prokaryote by using site directed mutagenesis method then evaluate the recombinant protein as a potential vaccine candidate and/or immunodiagnostic tool.

Materials and Methods

Construction of Recombinant Plasmid [pBSK$^-$_*Fg*GPx]

A cDNA containing the complete open-reading frame (ORF) of *F. gigantica* GPx was previously cloned by Dr. Supatra Chunchob. The HRG 1/3, pTripIEx2 *Fg*GPx plasmid DNA was isolated from transformant *E. coli*, XL_1Blue. The isolated *Fg*GPx DNA fragment was used as a template in a polymerase chain reaction using specific primers (HRG1-BamHI-F 5'- GGA TCC ATG TTA CAT ATG TTC G-3', HRG1-PstI-R 5'-CTG CAG CTA TTG TTT GAG CAG GTC-3'). The PCR product was subcloned into cloning vector, pBluescriptSK$^-$at *Eco*RV restriction site. The positive colony was digested with restriction endonuclease *Bam*HI and *Pst*I. The obtained DNA fragments were separated on a 1% agarose gel.

Site-Directed Mutagenesis of pBluescriptSK$^-$_*Fg*GPx

In order to mutate the GPx by changing the opal codon (TGA) to a sense codon (TGC), using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The complementary primer pairs was: MutHRG1-F 5'-GTC AAT GTG GCT TGT AAA TGC GGT TTG ACT GAT AAG-3' MutHRG1-R 5'-CTT ATC AGT CAA ACC GCA TTT ACA AGC CAC ATT GAC-3'. Colony PCR was used to verify the positive clones and the whole

plasmid mutagenesis, the used specific three primers were as follows: Muta_f1 5'-CCA TGT GGC TTG TAA ATG A-3', Muta_f2 5'-CCA TGT GGC TTG TAA ATG C-3' and Muta_f3 5'-CCA TGT GGC TTG TAA ATC C-3'. The first primer was used to verify the *Fg*GPx fragment, whereas the second primer was used for the base transition, TGA to TGC and the last primer was used to verify the previous base of point mutation.

Expression and Purification of Recombinant Modified *Fg*GPx (M_*Fg*GPx)

M_*Fg*GPx was cloned into the pQE30 expression vector following the QIAexpress System (QIAGEN) instructions at *Bam*HI and *Pst*I site before ligate and transform *E. coli*, M15 then overexpressed in bacteria. Due to adding a 6xHis-tag sequence at the N-terminus of the recombinant protein it can be purified by using Ni-NTA affinity chromatography (QIAGEN). All collected fractions were then analyzed by SDS-PAGE.

Polyclonal Anti-rM_*Fg*GPx Antisera Production

Six-week-old female BALB/c mice (obtained from National Laboratory Animal Centre, Mahidol University, Thailand) were allocated into 2 groups, 5 mice each. Mice was primed one time and boosted three times at two week intervals. In test group, animals were subcutaneously immunized with 25 µg of rM_*Fg*GPx in complete or incomplete Freund's adjuvant (Sigma, USA), respectively. The control group was immunized with PBS mixed in Freund's adjuvant (Sigma, USA). One week before immunization, pre-immune serum was collected from the tail vein of mouse by bleeding technique. The antisera was collected one week after each immunization. At the end of the experiment, the antisera was bled from the heart of the terminated mice.

Western Blot Analysis

The protein samples, rM_*Fg*GPx, *F. gigantica*'s crude whole worm (*Fg*CW), *Fg*'s tegumental antigen (*Fg*TA), *Fg*'s Excretory/secretory product (*Fg*ES), *Sm*CW, *Ov*CW and *Pa*CW (200 ng each) were sized-separated using 15% SDS-PAGE and electrotransferred to HyboundTM ECL nitrocellulose membranes (Amersham Biosciences) by a wet blot apparatus (Mini Trans- Blot Electrophoretic Transfer Cell, Bio-Rad). To block unspecific binding sites the membrane was incubated in blocking solution (5% skim milk in TBS, pH 7.5 containing 0.1% (v/v) Tween 20) for 90 min then replaced with a primary antibody solution diluted 1:200 in 1% skim milk in TBS, pH 7.5 0.1% (v/v) Tween 20 for 2 h. The membrane was washed four times with TBS, pH 7.5 for 5 min. each before incubated with diluted (1:2500) alkaline phosphate conjugated goat anti-mouse immunoglobulins (Zymed, USA) for 90 min. After repeated washes, the membrane was equilibrated two times in AP-substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$) for 5 min. each and the colorimetric detection reaction was started by addition of freshly prepared NBT/BCIP substrate solution.

Determination of Antibody Responses (ELISA)

Sera of mice was assayed for the presence of *Fg*GPx total immunoglobulin and specific IgG_1, IgG_{2a}, IgG_{2b} antibody using enzyme-linked immunosorbent assay (ELISA). The 96 wells plates were coated with the mentioned proteins in a volume of 2.5 µg/ml in 50 µl of coating buffer (0.1 M carbonate buffer, pH 9.6). Nonspecific proteins were blocked by using 100 µl of 1% BSA in 0.01 M PBS with 0.05% tween 20 (PBST), pH 7.2. After washing two times, the plate was incubated with immune sera at a suitable dilution for 2 h. then the plate was washed and blocked with the blocking solution for 10 min., following by washing, then 100 µl of secondary antibody solution horseradish peroxidase (HRP)-conjugated rabbit-anti mouse, rabbit anti mouse IgG_1, IgG_{2a} or IgG_{2b} (1:1000) was added and incubated further for 2 h. The antigen-antibody reaction was developed using OPD substrate. The reaction was stopped by adding 50 µl of 3 M H_2SO_4. The absorbance of the developed color was read at 490 nm using an automated microplate reader (model DV 990 BV4-6, GDV).

Sera of *F. gigantica* infected mice, rabbit, and cow and sera of *O. viverrini* infected hamster were used to study the sensitivity and specificity. The optimal conditions for each experiments were shown in Table 1

Table 1 ELISA conditions for *F. gigantica* infected animals and *O. viverrini* infected hamsters

infected animals	Protein antigen	1° antibody	2° antibody	substrate
***Fg*. infected mouse**	r*Fg*GPx (1µg/ml)	*Fg.* Infected mouse serum At 0, 2, 4, 6 weeks after infection (1:400)	HRP-rabbit anti mouse (1:5000)	OPD
***Fg*. infected rabbit**	r*Fg*GPx (1µg/ml)	*Fg.* Infected rabbit serum at 0, 2, 4, 6, 8, 10, 12 weeks after infection (1:600)	HRP-goat anti rabbit (1:5000)	OPD
***Fg*. infected cow**	r*Fg*GPx (1µg/ml)	*Fg.* Infected cow serum At 0, 16, 20 weeks after infection (1:600)	HRP-rabbit anti cow (1:2000)	OPD
***Ov*. infected hamster**	-r*Fg*GPx (1µg/ml), -Crude *Ov.*(1µg/ml) (positive control)	*Ov.* Infected hamster serum at 0, 2, 4, 6, 8, 10, 12 weeks after infection (1:100)	AP-goat anti hamster (1:1000)	PNPP

Research Results

Construction of Recombinant Plasmid pBluescriptSK$^-$_*Fg*GPx (pBSK$^-$_*Fg*GPx)

The specific primers were successfully used to amplify the *Fg*GPx fragment from the template HRG 1/3, pTripIEx2 *Fg*GPx. The obtained PCR product was used to transform *E. coli*, XL$_1$blue. Plasmid DNA of each selected clone was isolated before digested with *Bam*HI and *Pst*I restriction enzymes. The result showed that the positive clone contained pBSK$^-$DNA migrating at 3000 base pair (bp) and the *Fg*GPx fragment migrating at 600 bp. (Figure 1)

Site-Directed Mutagenesis of pBSK$^-$_*Fg*GPx

The changed sequence of M_*Fg*GPx was confirmed by PCR with three specific primers. The obtained 390 bp fragment confirmed that *Fg*GPx had been successfully mutated (Figure 2)

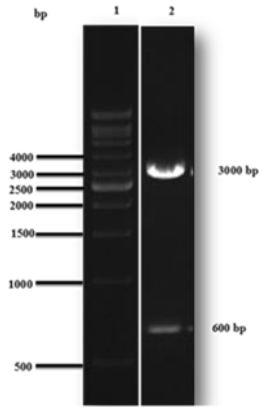

Figure 1 Ethidium bromide stained gel of 600 bp *Fg*GPx fragment after Restriction endonuclease (*Bam*HI and *Pst*I) digestion from pBSK$^-$vector. on 1% agarose gel. Lane 1: DNA marker, Lane 2: 600 bp *Fg*GPx fragment and 3000 bp pBSK$^-$plasmid DNA fragment

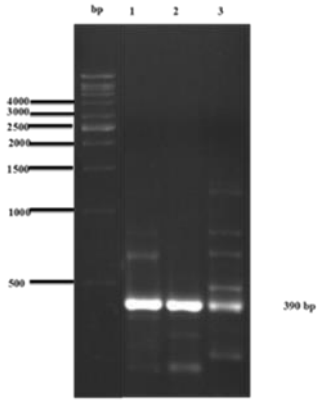

Figure 2 Ethidium bromide stained gel of Colony PCR analysis to confirm mutated *Fg*GPx. Lane 1: DNA marker, Lane 2-4: PCR product for 1st, 2nd and 3rd primer pair, respectively

Expression and Purification of Recombinant M_*Fg*GPx

M_*Fg*GPx was isolated from pBSK$^-$_M_*Fg*GPx then inserted into pQE30 by ligation. Plasmid DNA of positive clone was used to transform the expression host *E. coli*, M15. The expression of rM_*Fg*GPx in *E. coli* was analyzed to determine the optimal incubation time. The expected 19 kDa rM_*Fg*GPx was already observed 1 h after induction and gradually increased over the 3 h incubation time (Figure 3). Consequently, rM_*Fg*GPx was purified by Ni-NTA affinity chromatography under denaturing conditions. The purified 19 kDa rM_*Fg*GPx was obtained in the elution fractions. The highest yield found in the second elution fraction (Figure 4)

Western Blot Analysis

Anti-FgGPx antiserum reacted stronger with the 17 kDa native antigen in CW extract than in the TA extract and no reactivity was observed to the ES product (Figure 5). CW extracts of adult S. mansoni (Sm), O. viverrini (Ov) and Paramphistomum spp. (Pa) showed no cross reaction with the anti-rM_FgGPx antisera (Figure 6)

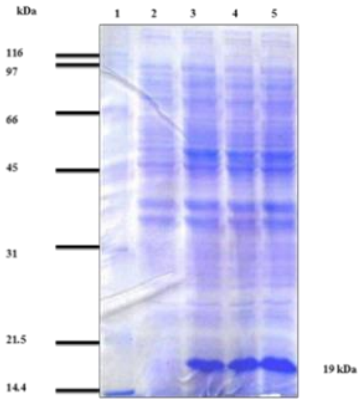

Figure 3 12% SDS-PAGE analysis and Coomassie Blue staining of total bacterial (*E. coli*, M15) protein. Lane 1: Protein standard marker, Lane 2-5: total bacterial protein at 0, 1, 2, and 3 h. after IPTG induction, respectively

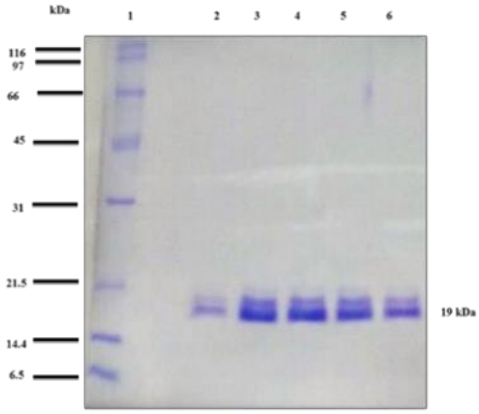

Figure 4 12% SDS-PAGE and Coomassie Blue staining of purified rM_*Fg*GPx. Lane 1: Protein standard marker, Lane 2-6: eluted protein at 1, 2, 3, 4, and 5 times with elution buffer

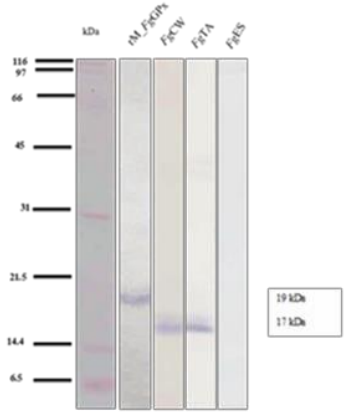

Figure 5 Western blot of purified rM_*Fg*GPx, *Fg*CW extract, *Fg*TA extract, and *Fg*ES product reacted with Anti-*Fg*GPx antiserum. Lane 1: Protein standard marker, Lane 2: *Fg*CW extract, Lane 3: *Fg*TA extract, Lane 4: *Fg*ES product

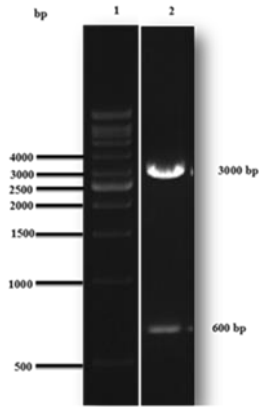

Figure 6 Western blot of CW extract from *S. mansoni*, *O. viverrini*, and *Paramphistomum* spp. reacted with mouse anti-rM_*Fg*GPx antisera. Protein standard marker are show at the left

Determination of Antibody Responses (ELISA)

rM_*Fg*GPx induced a significant antibody response (total IgG) after the second immunization (first boost) in all test groups (Figure 7). To results of isotype-specific secondary antibodies (IgG_1, IgG_{2a}, IgG_{2b}) showed a significantly increased IgG_{2a} response in all tested mice after the third immunization (Figure 8). However, significant immune responses were not observed for IgG_1, IgG_{2b} when compared with the control mice (Figure 9 and 10)

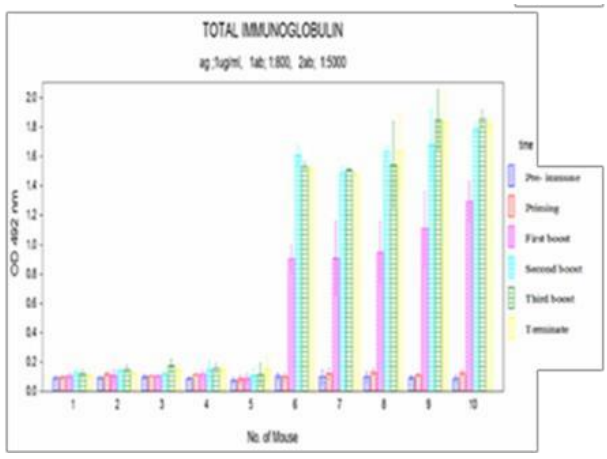

Figure 7 ELISA absorbance values for total IgG of immunized mice. The wells were coated with rM_*Fg*GPx. Columns 1-5 are values from control mice immunized with PBS, columns 6-10 are values from mice immunized with rM_*Fg*GPx

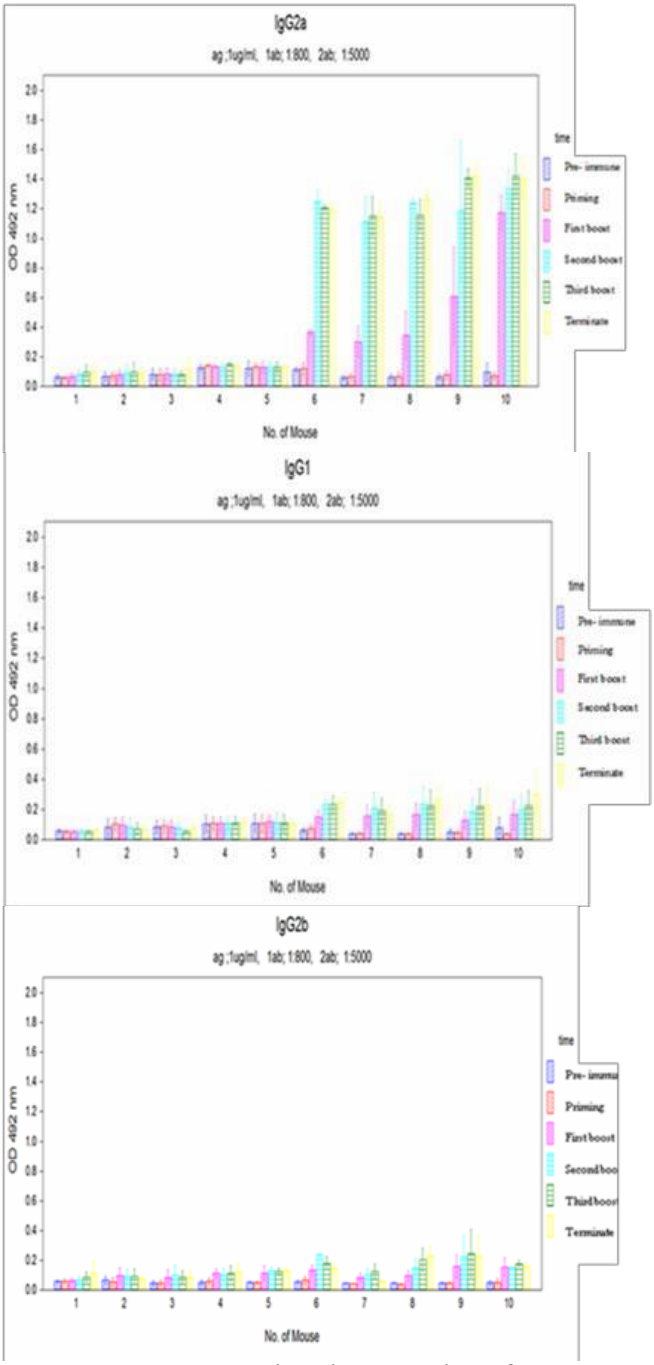

Figure 8-10 ELISA absorbance values for IgG_1, IgG_{2a} and IgG_{2b} isotype of immunized mice, respectively. The wells were coated with rM_*Fg*GPx. Columns 1-5 are values from control mice immunized with PBS, columns 6-10 are values from mice immunized with rM_*Fg*GPx.

rM_*Fg*GPx was initially reacted with the immune sera collected from *F. gigantica* infected mouse from 4 week post-infection (Figure 11). Rabbits and cows showed a response 4 and 16 weeks post-infection, respectively (Figure 12, 13) and total IgG of *O. viverrini* infected hamsters showed no reactivity to rM_*Fg*GPx (Figure 14)

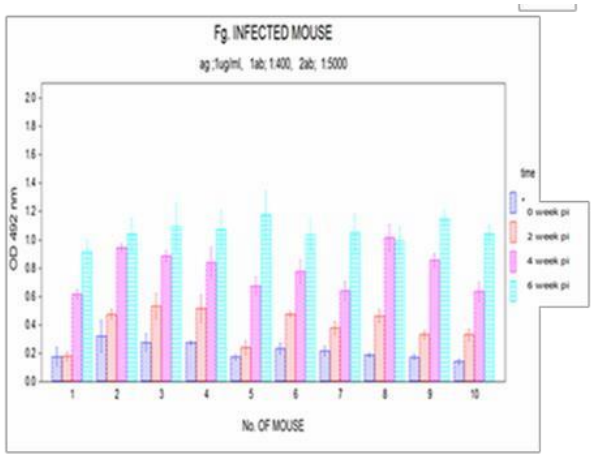

Figure 11 ELISA absorbance values of sera at 0, 2, 4, and 6 weeks post-infection from 10 *F. gigantica* infected mice probed against rM_*Fg*GPx

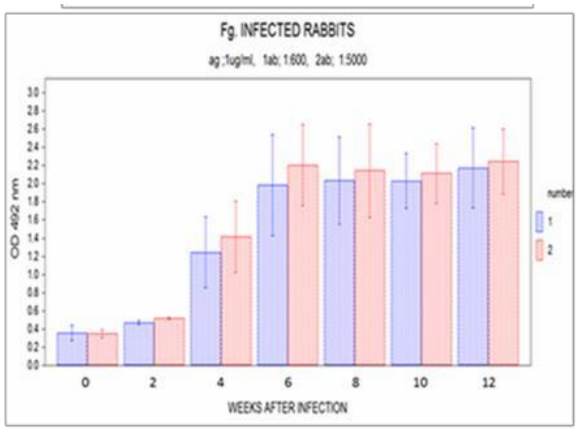

Figure 12 ELISA absorbance values of sera at 0, 2, 4, 6, 8, 10, and 12 weeks post-infection from 2 *F. gigantica* infected rabbits probed against rM_*Fg*GPx

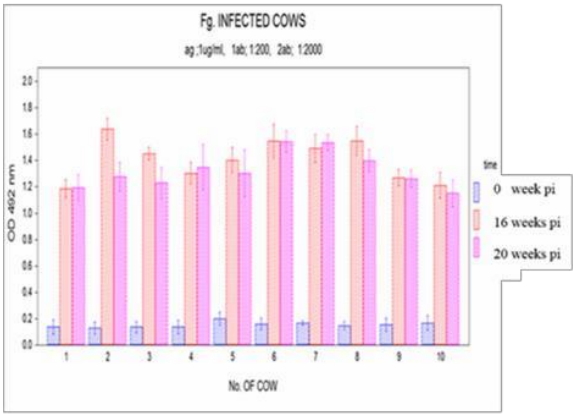

Figure 13 ELISA absorbance values of sera at 0, 16, and 20 weeks post-infection from 10 *F. gigantica* infected cattles probed against rM_*Fg*GPx

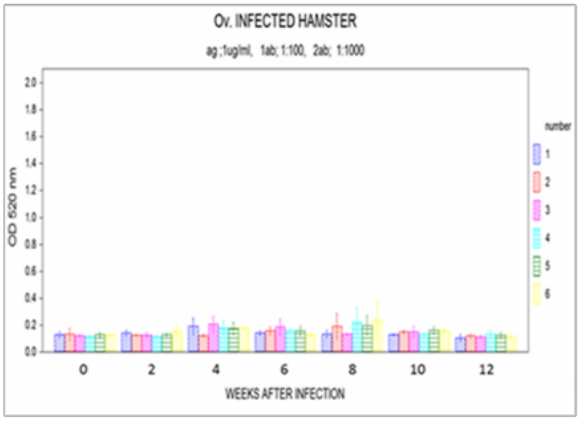

Figure 14 ELISA absorbance values of sera at 0, 2, 4, 6, 8, 10, and 12 weeks post-infection from 6 *O. viverrini* infected hamsters probed against rM_*Fg*GPx

Discussion

GPx is one of the most important enzymatic antioxidants for parasites. This enzyme regulates the intracellular hydrogen peroxide concentration. GPx has been characterized in several trematodes but not in *Fasciola* spp. In this study, the amplified 600 bp CDS of *Fg*GPx was first cloned into the bacterial cloning vector pBluescriptSK$^-$. Restriction sites for *Bam*HI and *Pst*I were introduced by PCR to facilitate insertion into the multiple cloning site of the bacterial vector. Site-directed mutagenesis was performed to convert the opal codon, TGA for selenocysteine into a sense codon, TGC for cysteine by a pair of complementary mutagenic primers. Colony PCR was used to test the positive clones with three specific primer pairs, (1) to check the *Fg*GPx fragment size, (2) to check for the introduced base transition, and (3) to check for the original base. The positive clone was isolated then subcloned into the pQE30 bacterial expression vector. An identified transformant *E. coli* was found to highly express rM_*Fg*GPx form 3 h after induction with IPTG. The recombinant protein migrated at a molecular mass of approximately 19 kDa in SDS-PAGE. rM_*Fg*GPx was purify by affinity chromatography. The highest yield was found in the second elution fraction. The elution fractions were pooled, dialyzed, concentrated and the purified protein was then used to immunize mice to produce a specific polyclonal antibody.

The antigenicity of rM_*Fg*GPx was assessed by indirect ELISA. Serum samples collected during the immunization of mice showed the level of polyclonal anti rM_*Fg*GPx IgG antibody was significantly increased after the first boost and still increasing after the second boost compared with the unimmunized group. This shows that rM_*Fg*GPx can stimulate an IgG immune response in mouse. The IgG subclass analysis showed a significant increase of IgG_{2a} whereas IgG_1 and IgG_{2b} showed no difference when compared with the control group. IgG_1 immunoglobulin isotype is considered to be controlled by Th_2 cytokines whereas IgG_2 is known to be controlled by Th_1 cytokines (Braciale et al., 1987). Th_2 secret IL-4, IL-5, IL10, and IL-13 enhance humoral immunity while suppressing cell mediated immunity. Th1 secrete cytokines IL-1, IFN-γ, lymphotoxin and are favored responses for immunity against virus and intracellular pathogens. The difference between IgG_{2a} and IgG_{2b} is IgG_{2a} fix complement by the alternative partway, part of pathogen spontaneous stimulate response of complement but IgG_{2b} fix complement by the classical partway, antigen-antibody complex (Mosmann & Sad, 1996). This suggests that *Fg*GPx stimulates a host Th_2 immune response and then stimulates complex by alternative partway. IgG_{2a} enhanced 1° antibody responses, development of germinal receptors, and immunological memory. Mechanism behind IgG_{2a}-mediated up-

regulation of antibody responses involves increased antigen presentation to CD4+ T cells by FcγR+ APCs.

Sera of *F. gigantica* infected mouse, rabbit, and cow showed a significant increase of the total IgG level to rM_*Fg*GPx since early infection compared with normal animals. Mouse showed a response 4 weeks post-infection, rabbits and cows showed a response 4 and 16 weeks post-infection, respectively. In addition, total IgG of *O. viverrini* infected hamsters showed no reactivity to rM_*Fg*GPx. Immunodetection of membrane-bound parasite antigens with polyclonal anti-rM_*Fg*GPx antibody demonstrated the presence of *Fg*GPx in *F. gigantica*'s crude worm extract (*Fg*CW) and tegument extract (*Fg*TA) while it was not found in the excretory-secretory product (*Fg*ES). The molecular weight of native *Fg*GPx in *Fg*CW and *Fg*TA is lower than that of rM_*Fg*GPx because of the added His-tag used in the purification process. Furthermore, the polyclonal anti-rM_*Fg*GPx antibody showed no reactivity to crude worm extracts of the trematodes *S. mansoni*, *O. viverrini*, and *Paramphistomum* spp. in this Western analysis. The results indicate that the prepared antibody is specific to *F. gigantica* GPx and can be further analyzed as vaccine candidate and/or immunodiagnostic tool

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