The Activities of Antioxidant Enzymes Extracted from *Fasciola gigantica* Infecting Thin-tailed and Merino Sheep

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ABSTRAK

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Penelitian sebelumnya menunjukkan bahwa domba Ekor Tipis (ET) lebih resisten terhadap infeksi *Fasciola gigantica* dibandingkan domba Merino. Perbedaan ini kemungkinan dapat disebabkan oleh enzim antioksidan yang terdapat pada parasit. Enzim ini diketahui berperan dalam pertahanan parasit terhadap oksidan yang dihasilkan sel sistem imun inang definitif. Karena itu penelitian ini bertujuan untuk mengukur aktivitas beberapa enzim dari *Fasciola gigantica* untuk membuktikan peranannya dalam menentukan resistensi domba Ekor Tipis (ET) dan kepekaan domba Merino terhadap infeksi *F. gigantica***.** Parasit cacing diisolasi dari organ hati domba Ekor Tipis dan Merino yang diinfeksi oleh *F. gigantica* dan aktivitas enzimnya seperti superoksida dismutase (SOD), glutation S-transferase (GST) dan katalase diukur. Hasil uji aktivitas enzim SOD dan GST pada parasit dari kelompok domba Merino lebih tinggi (P<0,05) dibandingkan dengan yang diisolasi dari kelompok domba ET, sedangkan aktivitas enzim katalase tidak terdeteksi. Jumlah penghitungan sel eosinofil pada domba ET lebih banyak (P<0,05) dibandingkan domba Merino. Dari hasil penelitian ini dapat disimpulkan bahwa enzim SOD dan GST merupakan faktor determinasi yang sangat penting dalam menentukan resistensi domba ET dan kepekaan domba Merino terhadap infeksi *F. gigantica.*

Kata Kunci: *Fasciola gigantica,* Domba Ekor Tipis, Domba Merino, Superoksida Dismutase (SOD), Glutation S-Transferase (GST)

ABSTRACT

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Previous studies shown that Indonesian Thin Tailed (ITT) sheep are more resistant to *Fasciola gigantica* infection than Merino sheep. This difference could be mediated by intrinsic defense enzymes of the parasite**.** Certain enzymes are known to be crucial in parasite survival against host-derived immune responses. We measured some of them to identify if any comparative differences between the enzyme activities of the parasites from the two hosts (ITT & Merino sheep) could account for the mechanisms of parasite resistance to killing by the Merino host and susceptibility to killing by the ITT host. Parasites were extracted from the liver of infected ITT and Merino sheep and superoxide dismutase (SOD), glutathione *S*-transferase (GST) and catalase (CAT) enzyme activities were assayed. SOD and GST levels were found to be higher in parasites isolated from Merino than those of ITT sheep (P<0.05), CAT activity was not detected in any of the parasites. There was significantly higher eosinophils (P<0,05) in the ITT sheep peritoneal cells. These results suggested that SOD dan GST are important molecules in determining susceptibility in *Fasciola*-infected Merino sheep and resistance in *Fasciola*-infected ITT sheep.

Key Words: *Fasciola Gigantica,* Indonesian Thin Tailed Sheep, Merino Sheep, Superoxide Dismutase (SOD), Glutathione S-Transferase (GST)

INTRODUCTION

Anti-oxidant enzymes and the role of oxidative radicals in physiological processes have received attention in many areas of research including aging physiology and parasitology (OU *et al.,* 1995). All helminth parasites have been found to contain at least one of the major anti-oxidant enzymes: superoxide dismutase (SOD), glutathione *S*-transferase (GST) and catalase (CAT) (CALLAHAN *et al.,* 1988). The importance of these enzymes to parasites, as to other organisms, is to protect against oxidant- mediated damages. The oxidative radicals derived from the host immune response is toxic which can kill cells and even the whole organisms.

Phagocytic cells are the most important effectors in parasite killing. Phagocyte such as eosinophils have been shown to kill parasites by free radical-mediated mechanisms, usually in combination with antibody. Most phagocytes can undergo a respiratory burst releasing reactive oxygen intermediates (ROI), including hydrogen peroxide (H_2O_2) and superoxide

anions ($O_2^{-\cdot}$) which may interact to form singlet oxygen ($^1O_2$) and hydroxyl radicals (OH) through the Haber-Weiss reaction (TIZARD, 2000).

Many compounds within the intracellular and extracellular compartments are able to scavenge or quench oxidants. Some of these enzymes can accept the spare electron from free radicals and thus stabilizes the compound. Among the helminth parasites, *Trichinella spiralis* is the species on which most studies on antioxidant enzymes and *in vitro* toxicity assay have been performed (KAZURA and MESHNICK, 1984).

The GST levels are elevated in *Fasciola hepatica* parasites isolated from susceptible host (mice) and lowered in parasites from resistant hosts (rats) (MILLER *et al.,* 1992). CALLAHAN *et al.* (1988) have suggested a relationship between the level of free radical scavenger enzymes and the degree of resistance of parasites to killing by reactive oxygen intermediates (ROI).

Previous studies shown that Indonesian Thin Tailed (ITT) sheep are more resistant to *F. gigantica* infection than Merino sheep (WIEDOSARI and COPEMAN, 1990). Eosinophils isolated from ITT sheep between 2-6 weeks postinfection could effectively kill immature parasites *in vitro* by superoxide radicals produced by these cells (SPITHILL *et al*., 1999). The present study was undertaken to investigate whether the differences in susceptibility between Merino and ITT sheep to *F. gigantica* infection could be attributed to intrinsic defense enzymes of the parasite. This knowledge will important in the development of the methods to block anti-oxidant protection to improve the ways for inhibiting parasite survival.

MATERIALS AND METHODS

Experimental animals and design

Ten of each ITT and Merino sheep, approximately nine months old at the time of infection, with no detectable *F. gigantica* eggs in faeces, were used in this experiment. Animals in each breed were allocated at random into infected (n=5) and control (n=5) groups. The trial was carried out at Research Centre for Biotechnology Indonesian Institute of Sciences (LIPI), Bogor, West Java, Indonesia. The sheep were maintained in open pens on a diet consisting of freshly cut *Pennisetum purpureum* and concentrate. Metacercariae of *F. gigantica* for infections were obtained from infected *Lymnaea rubiginosa* snails collected in Surade, West Java, Indonesia. Sheep were infected with 250 viable metacercariae by loading the metacercariae contained filter paper which in turn placed inside gelatin capsules and delivered orally using a dosing gun.

Necropsy and parasite collection

Sheep were slaughtered at 10 weeks postinfection by severing the jugular vein. Livers were dissected for the recovery of *F. gigantica* parasites as previously described (WIEDOSARI and COPEMAN, 1990).

Peritoneal cell counts

Peritoneal cells from *F. gigantica*-infected sheep were collected by simple lavage using sterile PBS containing 6 mM EDTA. A small incision was made along the linea alba through which the tip of a sterile funnel was inserted into the peritoneal cavity. A volume of 2-3 litres of PBS (phosphate buffer saline) was slowly poured into the funnel, then the abdomen was gently massaged for 5 minutes. Lavage fluid was aspirated using a 50-ml syringe and placed in a 2-litre sterile flask.

The viability of the lavage cells was determined by Trypan blue exclusion test. A 5 µl sample was diluted 10 fold with PBS, 50 µl of trypan blue (0.4% w/v in PBS) was poured on a Neubauer haemocytometer and examined under a light microscope. For differential cell counts, cytospin preparations of lavage cells were made by centrifuging at 400 r.p.m. for 5 minutes at 4°C. Cytospin samples were stained using Giemsa differential stains. Either 400 or 500 cells were identified microscopically at x1000 and the relative percentage of lymphocytes, macrophages, eosinophils and neutrophils was determined.

Protein determination

Protein concentration was determined using the Bio-Rad DC colourimetric assay for protein concentration following detergent solubilization based on the method of LOWRY *et al.* (1951). Colour development occurs by a two step mechanism, of an initial reaction between protein and copper in an alkaline medium, and subsequent reduction of Folin reagent by the copper-treated protein. Protein effect a reduction of Folin reagent resulting in a characteristic blue colour. Five µl samples of diluted protein solutions were added to the protein reagents and the absorbance at 690 nm was determined using a Titertek Multiskan Spectrophotometer. Protein concentrations were determined using a standard curve generated for Ovine Serum Albumin. The protein reagents were standard preparations by Biorad.

Determination of oxidant scavenger enzymes

All parasites were homogenised in a ratio of 0.05 g worm (wet weight) to 500 µl of 0.1% Triton X-100. Approximately 10 juvenile parasites were pelleted by

centrifugation at 7.2g in a microfuge for 2 seconds in an eppendorf tube and then homogenised in a ground glass homogeniser. Crude enzyme extracts were centrifuged in a refrigerated microcentrifuge at 1892.8g for 20 seconds. The resultant supernatant was used to measure the activity of three soluble enzymes in *F. gigantica.* Reaction mixtures without parasites homogenate served as controls. All reactions were carried out at 25°C in a Shimadzu UV-160 spectrophotometer (Shimadzu Corporation, Japan) in a total reaction mixture of 1 ml, and the change in absorbance monitored continuosly for 2 minutes.

Superoxide dismutase assay

The superoxide dismutase (SOD) assay was based on the cytochrome c reduction method of FLOHE and OTTING (1984), using bovine erythrocyte SOD as a standard. Cytochrome c is reduced by the superoxide radical generated by the xanthine-xanthine oxidase enzyme system. The reduction of cytochrome c causes an increase in absorbance at a wavelength of 550 nm. In the presence of SOD, cytochrome c reduction is inhibited and, thus no increase in absorbance at 550 nm is observed. Solution A consisted of xanthine 5 µmol in 0.001 N sodium hydroxide and 2 µmol cytochrome c (horse heart type IV) admixed with 50 m*M* phosphate buffer, pH 7.8 containing 0.1 m*M* EDTA. Solution B consisted of a freshly prepared solution of 0.4 U/ml xanthine oxidase in 0.1 m*M* EDTA. Solution B was kept on ice. The baseline rate of change in absorbance of the mixture, in the absence of sample or SOD, was approximately 0.05 units.

Sample calculations for specific activity of superoxide dismutase (SOD)

One unit of superoxide dismutase activity is defined as that amount of enzyme necessary to inhibit the rate of reduction of cytochrome c by 50%. Therefore, the amount of SOD activity can be calculated in the following way:

If the control (in the absence of sample) has a change of absorbance = - 0.55 units/min then 1 unit of SOD has the activity = -0.55/2 units/min = -0.275 units/min. If the sample (40 µl) has change of absorbance = -0.06 units/ min then the activity of the sample = -0.275/-0.06 = 4.58 units in the 40 µl sample. Therefore, the activity in 1 ml= 4.58 units x 1000 µl/40 µl sample = 115 units / ml of sample. Hence, 1 ml of sample (containing 16 mg/ml of protein) = 115 units/16 mg protein = 7.16 units/mg protein.

Note: Protein concentration of *F. gigantica* = 16 mg/ml of protein. The measured SOD activity for this sample is then 7.16 units/mg of protein.

Glutathione S-Transferase assay

The glutathione S-Transferase (GST) assay was based on that of HABIG *et al.* (1974). In this assay the conversion of glutathione from the oxidised form to reduced form is monitored spectrophotometrically as an increase in absorbance at 340 nm. To each incubation, 25 µl of 8.1 mg/ml 1-chloro-2,4-dinitrobenzene (CDNB) was added to 0.97 ml of phosphate buffer (pH 7.0) containing 1 m*M* reduced glutathione and allowed to equilibrate for 1 minute before the addition of approximately 0.05 mg/ml of parasite homogenate.

Sample Calculation for Specific Activity of Glutathione S-Transferase (GST)

The specific activity of glutathione s-transferase is defined as the amount of CDNB conjugated per minute per mg of protein.

The equation used to calculate glutathione S-transferase activity was

$$C = \frac{\Delta A \times V}{D \times \Sigma \times v}$$

where:

C	=	µmoles of 1-chloro-2,4-dinitrobenzene (CDNB) conjugated / min / ml of sample
ΔA	=	change in absorbance at 340 nm
V	=	assay volume
D	=	lightpath (1 cm)
Σ	=	coefficient of extinction for NAPDH = 6.22 cm^2/µmole

Catalase assay

The catalase assay was based on the method of GANSCCHOW and SCHIMKE (1969) and measured the disproportion of hydrogen peroxide (H_2O_2) to water and oxygen as a decrease in absorbance at a wavelength of 240 nm. To each incubation, 0.1 ml of 200 m*M* H_2O_2 was added to 0.9 ml of 2.5 m*M* phosphate buffer, pH 7.0 containing 0.1% Triton X-100. Triton X-100 was added to release any tissue-bound enzyme. Once a stable baseline was obtained, an aliquot of approximately 0.05 mg/ml of parasite homogenate was added. One unit of catalase activity is defined as that amount of enzyme required to decompose 50% of 20 m*M* hydrogen peroxide in 1 minute at 25°C. The spesific activity of catalase was expressed as units of catalase per mg of protein. Bovine liver catalase was used as a standard.

Statistical analysis

Statistical analysis was performed using SAS system (1998) the ANOVA and the Duncan test were

used to evaluate the result. Differences at the P<0,05 level were considered significant.

RESULTS AND DISCUSSION

Resistance of sheep to *F. gigantica* which expressed as a percentage of the *F. gigantica* recovered from the liver is shown in Table 1. Based on that parameter, ITT sheep expressed a higher resistance to infection than Merino sheep (P<0.05). This findings were in agreement with earlier studies (WIEDOSARI and COPEMAN, 1990; SPITHILL *et al*., 1999). No flukes were recovered from the control animals. indicating that all recovered flukes from infecting animal originated from infecting metacercaria.

The activities of oxidant scavenger enzymes extracted from parasites from infecting ITT and Merino sheep are presented in Table 2. Significantly higher levels of GST and SOD were present in *F. gigantica* isolated from Merino sheep compared to adult *F. gigantica* isolated from ITT sheep. However, catalase activity was not detected in any of the parasites. This findings was an agreement with previous study on *F. hepatica* by SPITHILL *et al*. (1999). Thus, the data reveal a trend toward higher defence enzyme levels in *F. gigantica* isolated from Merino sheep relative to *F. gigantica* isolated from ITT sheep and suggest that *F.gigantica* parasites from Merino sheep have the potential to mount a more effective defence against free radical attack by host immune cells.

Both the host and parasite have developed specific defences to counteract the damage caused by free radicals during an immune response. The primary defences against free radicals and their derivatives, found in most cells and in some excretory-secretory products of parasites, are the oxidant scavenger enzymes, superoxide dismutase and catalase (CALLAHAN *et al.*, 1988; OU *et al.*, 1995). The superoxide radical ($O_2^{-\cdot}$) and the hydroxyl radical (·OH) are highly reactive species that are thought to cause the majority of biomembrane damage (CALLAHAN *et al.*, 1988; SMITH, 1992). Hydroxyl radicals combine with almost all molecules found in living cells with rate constants of 10^9-10^{10} $m^{-1}s^{-1}$. Superoxide dismutase (SOD) increases the rate of disproportion of superoxide radical to form hydrogen peroxide, H_2O_2, by 10^9 times (FRIDOVICH, 1985). This rate of disproportion, in effect, not only prevents direct damage by superoxide itself, but also inhibits the formation of the highly reactive hydroxyl radical via Fenton's reaction, of superoxide with hydrogen peroxide (FANTONE and WARD, 1982). SOD could also prevent the formation of the highly reactive hydroxyl radical from superoxide and nitric oxide (BECKMAN *et al.*, 1990). Vaccination against bacterial-specific SOD has led to an increased susceptibility of the bacteria to oxidative killing *in vivo* (BEAMAN and BEAMAN, 1990). These findings suggest that superoxide dismutases may play a central role in parasite defence against free radicals generated during an immune response by the host. Catalase and glutathione peroxidase (via the glutathione peroxidase-glutathione reductase system) catalyse the reduction of hydrogen peroxide to water. The helminth parasites examined so far contain at least one of these major oxidant scavenger enzymes and a summary of the major biochemical characteristics of these enzymes can be found in CALLAHAN *et al.* (1988).

Table 1. The average of parasite numbers (%) recovered from ITT and Merino sheep infected with *F. gigantica* (250 metacercriae)

Sheep breed	Weeks post-infection	Average of parasite number (%)
ITT Sheep (n = 5)	10	11.8
Merino Sheep (n = 5	10	24.9
Uninfected ITT (n = 5) & Uninfected Merino (n = 5) sheep	10	0

Table 2. Anti-oxidant defence enzyme levels in whole worm extracts of *F. gigantica* isolated from ITT or Merino sheep

Enzyme activity	ITT sheep	Merino sheep
	F. gigantica	*F. gigantica*
SOD (U/mg protein)	21 ± 5	52 ± 5*
GST (nmol/min/mg protein)	4214 ± 1084	5800 ± 959*
CAT	bd	bd

SOD = superoxide dismutase; GST, glutathione *S*-transferase; CAT = catalase
Values represent the mean ± Standard Deviation. bd, below detectable limit of assay
*Significant differences (P<0,05) for anti-oxidant defence enzyme activities (GST and SOD) between *F. gigantica* isolated from Merino sheep compared to *F. gigantica* isolated from ITT sheep

If these primary parasite defences are not adequate during oxidative attack, a potential array of other defences exist. Lipid peroxidation as a result of free radical damage can be detoxified by glutathione peroxidases and glutathione S-transferases. Glutathione peroxidase can convert lipid hydroperoxide into less reactive hydroxyl fatty acids (FANTONE and WARD, 1982). Glutathione S-transferases can neutralise cytotoxic carbonyls which are breakdown products of peroxides and, therefore, may provide one of the final lines of defence against the effects of oxidative stress by conjugating toxic secondary products (BROPHY and PRITCHARD, 1994).

General oxidant scavengers of free radicals such as ascorbic acid, tocopherol, carotenoids and vitamin E neutralise free radicals (HALLIWELL, 1994). Little is known of the use of general oxidant scavengers of free radicals by parasites to neutralise free radicals, but they may play a critical role in some parasites (OVINGTON and SMITH, 1992). xidised DNA is repaired by glycosylases, while oxidised proteins are degraded by proteases (EDGINGTON, 1994). Metal chelators such as lactoferrin, caeruloplasmin and transferrin prevent transition metals from catalysing free radical reactions (Fenton chemistry) (HALLIWELL, 1994). All these defensive mechanisms collectively act by blocking free radical reactions, thereby limiting the extent of cellular damage.

Parasites are not always killed by free radicals and there is a large volume of literature describing how a variety of unicellular organisms, such as bacteria and protozoa, survive attack against host-generated free radicals (HALL and JOINER, 1991). Less is known on how helminths avoid the cellular effector mechanisms of leucocytes, particularly free radicals. One important possibility, gaining more support with increased understanding, is the potential use of scavenging enzymes by helminths to render free radicals produced by leukocytes ineffective CALLAHAN *et al.* (1988). Several helminth parasites actively secrete, or in some way release, SOD into their environment (OU *et al.*, 1995). SOD activity was greater in *Nippostrongylus brasiliensis* isolated from previously primed rats as compared to naïve rats. When compared with *Nematospiroides dubius, N. brasiliensis* is several fold more susceptible to killing by free radicals. Resistance to free radicals appeared to correlate with the higher levels of activity in *N. dubius* of the enzymes, SOD and catalase. These may explain the ability of *N. dubius* to persist in the mouse intestine for several months, whereas *N. brasiliensis* is expelled within 10-12 days from the rat intestine (SMITH and BRYANT, 1986).

According to KAZURA and MESHNICK (1984), there are differences in susceptibility to granulocyte damage between newborn larvae (NBL) and adult stages of *Trichinella spiralis*. These may be a result of the differences in the enzymic defences to free radicals. In fact, adult stages of *T. spiralis* have 3-5 times higher SOD activities and at least 5 times higher GST activities than NBL. Similar to *T. spiralis,* the developmental stages of *Schistosoma mansoni* (>2 weeks old) and adult parasites are less sensitive to killing by ROI than schistosomula, and this resistance correlates with the higher enzyme activities of SOD and GST in the older parasites stages (NARE *et al.,* 1990).

There was significantly higher eosinophils ($P<0.05$) in the ITT sheep peritoneal cells than in Merino sheep (Table 3). It suggests that these cells may be able to suppress the worm enzymes more effectively than the cells from Merino sheep. HALLIWELL (1994) said that with prolonged oxidative stress, the defence mechanisms may be inadequate and extensive free radical damage to biomembranes and cells may result and parasites can be killed. This could explain why more worms survive in the Merino sheep compared to the worms in the ITT sheep which are killed. This observation is intriguing with the findings SPITHILL *et al*. (1999), that eosinophils were significantly elevated at the time of *F. gigantica* parasite killing in the ITT host relative to the *F. gigantica*–susceptible Merino host. By using a series of inhibitors, the major cytotoxic molecules mediating parasite killing, and produced by these cells, were superoxide. In addition, IFN/Interferon-γ responses were higher in the Merino sheep when compared with IFN-γ of ITT sheep (WIEDOSARI *et al.,* 2006*, in preparation*). The production of IFN-γ has been shown to inhibit superoxide production (BIELEFELDT OHMANN and BABIUK, 1984).

Table 3. Differential white blood cell counts of peritoneal lavage cells from ITT or Merino sheep infected F. gigantica

Cell type	% of total *F.gigantica*-infected ITT sheep	Leucocyte population *F. gigantica*-infected Merino sheep
Lymphocytes	11 ± 6	15 ± 5
Eosinophils	66 ± 11*	32 ± 10
Macrophages	39 ± 9	43 ± 13
Neutrophils	4 ± 1	10 ± 2

Values represent the mean ± Standard Deviation. *Significant differences (P<0,05) for eosinophils in the ITT sheep peritoneal cells compared to Merino sheep

CONCLUSION

These results suggest that the antioxidant enzymes SOD and GST are important molecules in determining susceptibility in *F. gigantica*-infected Merino sheep and resistance in *F. gigantica*-infected ITT sheep. In turn, this may leads to develop new drugs to inhibit this parasite protein.

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