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Identification and characterization of a selenium-dependent glutathione peroxidase in *Setaria cervi*

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Abstract

Setaria cervi a bovine filarial parasite secretes selenium glutathione peroxidase during in vitro cultivation. A significant amount of enzyme activity was detected in the somatic extract of different developmental stages of the parasite. Among different stages, microfilariae showed a higher level of selenium glutathione peroxidase activity followed by males then females. However, when the activity was compared in excretory secretory products of these stages males showed higher activity than microfilariae and female worms. The enzyme was purified from female somatic extract using a combination of glutathione agarose and gel filtration chromatography, which migrated as a single band of molecular mass ≈20 kDa. Selenium content of purified enzyme was estimated by atomic absorption spectroscopy and found to be 3.5 ng selenium/μg of protein. Further, inhibition of enzyme activity by potassium cyanide suggested the presence of selenium at the active site of enzyme. This is the first report of identification of selenium glutathione peroxidase from any filarial parasite.

Keywords: Selenium; Glutathione peroxidase; Bovine parasite; *Setaria cervi*

Human filariasis is a chronic debilitating disease, which causes extensive morbidity and little mortality. Three species of filarial worms, *Wuchereria bancrofti*, *Brugia malayi*, and *Brugia timori*, are the causative agents of lymphatic filariasis in humans, defined by the characteristic tropism of adult worms of each species for the afferent lymphatic. Reproductive activity leads to the release of a large number of microfilariae, which circulate in vascular system and upon ingestion by an appropriate mosquito vector develop to infective third stage larvae (L_3) within 10–15 days. After a subsequent blood meal, the infective larvae enter the definitive host via the wound and mature to adult stage over several months involving two moults, during which the entire nematode exoskeleton is replaced. These filarial parasites are long lived and by still unknown mechanisms they suppress the host's immune response.

The major immune effector mechanisms involve various types of cells (macrophages, leukocytes, and platelets) potentiated by antibodies and cytokines [1]. One mechanism that cells may employ to kill parasites is the release of toxic products, molecules, and oxygen radicals during the respiratory burst or nitric oxide release [2–4]. Toxic proteins among other things can attack the parasite lipid membrane and breach its integrity [5]. The oxygen radicals released, which include O_2^-, H_2O_2, singlet oxygen (O_2), and hydroxyl radicals ($OH^\cdot$) can damage cell membranes, unfold or inactivate proteins, degrade nucleic acid, kill cells and eventually the parasite.

To establish and maintain itself in the vasculature of the host, the parasite has evolved a number of immune evasion mechanisms [6,7] including the production of antioxidant enzymes like superoxide dismutase (SOD),

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glutathione peroxidase (GSHPx), glutathione-*S*-transferase (GST), and glutathione reductase (GR) [2,4]. The means by which filarial parasites metabolize H_2O_2 has until not been satisfactorily answered. Relatively high levels of SOD in these parasites [8,9] necessitate the presence of selenium-dependent GSHPx. However, a selenium-independent glutathione peroxidase has been cloned, expressed, and characterized in *Brugia pahangi*, *W. bancrofti, B. malayi*, and *Dirofilaria immitis* [10–12]. The enzymes from *B. pahangi* and *D. immitis* exhibited activity with a range of organic hydroperoxides including fatty acid and phospholipid hydroperoxide but differed slightly in displaying either extremely low or no significant activity towards hydrogen peroxide. This unusual substrate specificity suggests that these enzymes might not be responsible for detoxification of H_2O_2 but could act to protect cuticular membranes from oxidative damage by reducing phospholipid hydroperoxide.

In the present communication, selenium-dependent GSHPx was detected in bovine filarial parasite *Setaria cervi*. This parasite has been chosen as a model for the present study as it resembles the human parasite *W. bancrofti* in its nocturnal periodicity and antigenic pattern [13].

Materials and methods

Coomassie brilliant blue, cumene hydroperoxide, glutathione agarose beads, glutathione reduced (GSH), glutathione reductase (GR), hydrogen peroxide, 3-(4,5-dimethylthiazol-2-yl)-2,5,diphenyl tetrazolium bromide (MTT), molecular weight marker (broad range), nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), phenazine methosulfate (PMS), phenylmethylsulfonyl fluoride (PMSF), and Sephadex G-200 were purchased from Sigma St. Louis, MO, (USA). All other chemicals used were of analytical grade.

Parasites, parasite extracts, and excretory–secretory products

Adult *S. cervi* were procured from the peritoneal folds of freshly slaughtered water buffaloes (*Bubalus bubalis* Linn.) and brought to laboratory in Krebs–Ringer buffer supplemented with streptomycin, penicillin, glutamine, and 0.5% glucose. Worm extract was prepared by homogenizing in 100 mM Tris–HCl, pH 7.0, containing 1 mM PMSF, and 1 mM EDTA in REMI made homogenizer type RQ 127A at 4 °C and centrifuged first at 5000*g* followed by 12,000*g* for 30 min. The clear supernatant thus obtained was used for further studies. Detergent extractable surface antigen and interior fraction were prepared by incubating parasites in 10 volumes of Tris–HCl containing 0.1% Triton X-100 for 15 min at 37 °C followed by vortexing four times (20 s each) on ice. The worms were removed and the extract was centrifuged at 5000*g* for 15 min at 4 °C and the supernatant was saved as tegument fraction. The worm carcass was washed and resuspended in 10 volumes of the same buffer and the extract was prepared as described above.

Microfilariae (Mf) were obtained by dissecting the distal portion of the uterus of adult gravid females. Extract of Mf was prepared by sonication in 100 mM Tris–HCl, pH 7.0, containing 1 mM PMSF and 1 mM EDTA using a MSE 150 W ultrasonic disintegrator MK_2 at 20 kHz for 10 min at 2 min interval.

Excretory–secretory product (ES) of adult male, female, and Mf was collected by incubating these separately in supplemented Krebs–Ringer bicarbonate buffer for 2 h at 37 °C under sterilized conditions.

Protein estimation

Protein estimation was performed by the Bradford method [14]. Bovine serum albumin was used as the standard.

Assay of glutathione peroxidase

Glutathione peroxidase activity was assayed according to the method of Paglia and Valentine [15]. Hydrogen peroxide and cumene hydroperoxide were used as substrates. The final concentration of reagents in the assay mixture was Tris–HCl buffer (pH 7.6, 100 mM) EDTA (3 mM), NADPH (0.24 mM), GSH (6 mM), GSH reductase (0.25 mg/ml), NaN_3 (3 mM), hydrogen peroxide and cumene hydroperoxide (0.16 mM), and enzyme sample.

One unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1 μM of NADPH per milliliter per minute at 28 °C.

Activity staining of glutathione peroxidase

Specific enzyme staining was done as described by Lin et al. [16]. SDS–PAGE was performed according to the method of Laemmli [17]. After electrophoresis the gel was cut in two parts: one was fixed with 12.5% TCA for protein staining with Coomassie brilliant blue. The other part was washed with 25% isopropanol to remove SDS and incubated in 50 mM Tris–HCl buffer (pH 7.9) containing 13 mM glutathione and 0.004% hydrogen peroxide with gentle shaking for 10–20 min. The SeGSHPx activity was stained by 1.2 mM MTT and 1.6 mM PMS.

Purification of SeGSHPx from adult female S. cervi

SeGSHPx purification was optimized by affinity chromatography and gel filtration chromatography in order to get maximum purification along with a better yield of the enzyme.

Glutathione affinity chromatography. A 2 ml column of glutathione agarose beads was prepared at 4 °C and equilibrated with PBS Triton X-100 (1%) adjusting the flow rate to 12 ml/h. Soluble adult female extract containing 4.5 mg of protein was applied to the column and unbound proteins were washed extensively with PBS, pH 7.4. The matrix bound SeGSHPx was eluted using 50 mM Tris–HCl buffer, pH 7.4, and the enzyme active fractions were pooled and concentrated.

Gel filtration chromatography. The partially purified enzyme was subjected to gel filtration on Sephadex G-200 column pre-equilibrated with 50 mM Tris–HCl, pH 7.4, buffer and the enzyme was eluted in the same buffer. Homogeneity of enzyme active fractions was checked by SDS–PAGE followed by silver staining.

Selenium analysis

The selenium content of purified enzyme sample was estimated by graphite furnace atomic absorption spectrophotometrically at three different protein concentrations (1–10 μg) simultaneously with a set of standards ranging from 0 to 50 ng of selenium. Analysis of selenium in purified glutathione peroxidase was done using Varian 250 graphite furnace atomic absorption spectrophotometer and nickel nitrate (50 μg/ml) was added as chemical modifier to enhance the analyte signal.

Inhibition studies

For checking the inhibition of SeGSHPx with potassium cyanide the purified enzyme was incubated with either 10 mM KCN or 10 mM