Experimental Parasitology 151-152 (2015) 1–7

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Full length article

Isolation and characterization of Cu/Zn–superoxide dismutase in *Fasciola gigantica*

H. Lalrinkima, O.K. Raina *, Dinesh Chandra, Siju Susan Jacob, R.K. Bauri, Subhash Chandra, H.S. Yadav, M.N. Singh, A. Rialch, A. Varghese, P.S. Banerjee, Navneet Kaur, Arvind Sharma

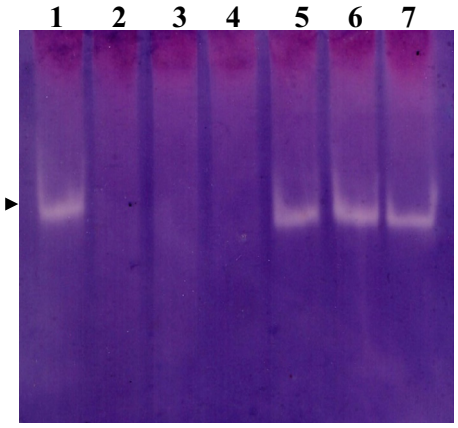
Division of Parasitology, Indian Veterinary Research Institute, Izatnagar 243122, U.P., India

HIGHLIGHTS

- A cDNA coding for *Fasciola gigantica* Cu/Zn–superoxide dismutase (SOD) was isolated.
- Differential expression of the enzyme studied in the adult, juvenile and metacercaria.
- Recombinant Cu/Zn–SOD partially characterized for enzyme activity, pH profile in NBT–PAGE.
- Cu/Zn–SODs in the somatic and excretory secretory product of the fluke were studied.

GRAPHICAL ABSTRACT

Nitro blue tetrazolium–polyacrylamide gel (NBT–PAGE) showing *Fasciola gigantica* recombinant Cu/Zn–superoxide dismutase enzyme activity (lane 1) and effect of various concentrations of hydrogen peroxide (lanes 2, 3 and 4) and sodium azide (lanes 5, 6 and 7) on the Cu/Zn–SOD enzyme activity.



ARTICLE INFO

Article history:
Received 7 October 2014
Received in revised form 23 January 2015
Accepted 27 January 2015
Available online 2 February 2015

Keywords:
Fasciola gigantica
Cu/Zn–superoxide dismutase
Recombinant SOD
Hydrogen peroxide
Sodium azide
NBT–PAGE
Western blot

ABSTRACT

A full-length complementary DNA (cDNA) encoding Cu/Zn–superoxide dismutase was isolated from *Fasciola gigantica* that on nucleotide sequencing showed a close homology (98.9%) with Cu/Zn–superoxide dismutase (SOD) of the temperate liver fluke, *F. hepatica*. Expression of the gene was found in all the three developmental stages of the parasite *viz*. adult, newly excysted juvenile and metacercaria at transcriptional level by reverse transcription-polymerase chain reaction (RT-PCR) and at the protein level by Western blotting. *F. gigantica* Cu/Zn–SOD cDNA was cloned and expressed in *Escherichia coli*. Enzyme activity of the recombinant protein was determined by nitroblue tetrazolium (NBT)–polyacrylamide gel electrophoresis (PAGE) and this activity was inactivated by hydrogen peroxide but not by sodium azide, indicating that the recombinant protein is Cu/Zn–SOD. The enzyme activity was relatively stable at a broad pH range of pH 4.0–10.0. Native Cu/Zn–superoxide dismutase protein was detected in the somatic extract and excretory–secretory products of the adult *F. gigantica* by Western blotting. NBT–PAGE showed a single Cu/Zn–SOD present in the somatic extract while three SODs are released *ex vivo* by the adult parasite. The recombinant superoxide dismutase did not react with the serum from buffaloes infected with *F. gigantica*. The role of this enzyme in defense by the parasite against the host reactive oxygen species is discussed.

* Corresponding author. Fax: +91-5812302486.
E-mail address: rainaok@rediffmail.com (O.K. Raina).

http://dx.doi.org/10.1016/j.exppara.2015.01.014
0014-4894/

1. Introduction

Fasciola gigantica (tropical liver fluke) and *F. hepatica* (temperate liver fluke), the causative agents of fasciolosis in domestic ruminants, are economically important veterinary parasites due to the substantial production and monetary losses that these parasites cause to the livestock industry. Tropical fasciolosis is a constraint on the growth and productivity of cattle, buffaloes and sheep in the tropical countries of Asia and Africa (Mehra et al., 1999; Yamasaki et al., 2002). In addition to its importance to livestock, fasciolosis is an emerging zoonosis particularly in the South American countries like Bolivia, Peru, Equador and in Egypt and Iran. These countries are considered as hyper-endemic for human fasciolosis (Mas-Coma, 2005). In the Indian sub-continent *F. gigantica* is the major cause of the disease in domestic animals. Although the adult worms have a predominantly anaerobic metabolism and inhabit in the bile duct, where the oxygen tension is relatively low, oxygen is still required for several other functions of the parasite, which produce reactive oxygen species (Tielens et al., 1982). In addition to this normal confrontation with oxidative stress, the parasite is exposed to reactive oxygen species generated by host effector cells such as macrophages, eosinophils, neutrophils and platelets (Badwey and Karnovsky, 1980; Maizels et al. 1993). Free oxygen radicals generated by these effector cells via oxidative burst are thought to contribute to the killing of parasites by the host (Nathan et al., 1979; Murray, 1981; Maizels et al. 1993). To defend themselves against oxygen-mediated killing mechanisms of hosts, parasites have evolved with antioxidant enzyme systems and the antioxidant suppression of host oxidative killing may play a protective role in the parasite life cycle (Callahan et al., 1988). Prominent among antioxidants are superoxide dismutases, which catalyze the decomposition of superoxide into hydrogen peroxide and molecular oxygen (Fridovich, 1995; Hernandez-Santoyo et al., 2011). SODs are postulated to play a role in the protection of parasites against the cellular, oxygen-mediated killing mechanisms of the host (Henkle-Duhrsen et al., 1997; Kim et al., 2000). SODs have been characterized and cloned in various helminth parasites including *F. hepatica, Schistosoma mansoni*, *Onchocerca volvulus*, *Dirofilaria immitis*, *Brugia pahangi* and *Taenia solium* (Callahan et al., 1991; Castellanos-González et al., 2002; Hernandez-Santoyo et al., 2011; Hong et al., 1992; James et al., 1994; Kim et al., 2000; Tang et al., 1994). These enzymes have been found to be cytoplasmic or secreted and play a defensive role for the parasite. The defensive role played by the SODs has been postulated in different helminths but no studies have been carried out on this enzyme in *F. gigantica*. In the present study, the cDNA coding for Cu/Zn–superoxide dismutase in *F. gigantica* was identified, functionally expressed in *Escherichia coli* (*E. coli*) and native Cu/Zn–SOD protein was detected in various developmental stages of the parasite, indicating that several SODs may be expressed in *F. gigantica*.

2. Materials and methods

2.1. Parasite collection

Fasciola gigantica adult flukes were obtained from the liver of infected buffaloes from a local abattoir and transported to the laboratory in physiological saline. These flukes were washed several times in physiological saline and incubated in RPMI-1640 medium at 37 °C for 3–4 h for harvesting excretory–secretory products (ESP) of the parasite. Live flukes were used for RNA isolation and also for the preparation of somatic extract. *F. gigantica* metacercariae were harvested from *Lymnaea auricularia* and *ex vivo* excysted to newly excysted juveniles (NEJs) (Nagar et al., 2010). These NEJs were maintained in RPMI-1640 medium, supplemented with 50 μg/ml gentamycin at 37 °C in CO_2 incubator for a few hours before isolation of RNA.

2.2. Preparation of parasite extract

F. gigantica adult flukes (n = 3) were homogenized with a sterile pestle–mortar in 4 ml phosphate buffered saline (PBS, pH 7.2) and sonicated at 5 μm amplitude × 3 cycles of 30 s each (Soniprep 150, Sanyo; UK). The homogenate was centrifuged at 20,000 *g* for 20 min at 4 °C, supernatant was aliquoted at –20 °C and used for determination of the SOD activity in the parasite. Likewise, somatic extracts of the newly excysted juveniles (n = 500) and metacercariae (n = 5000–10,000) were prepared as described for the adult fluke. The protein concentration was determined by the method of Lowry et al. 1951.

Adult fluke excretory–secretory product (ESP) was obtained by *ex vivo* culture of the flukes (50–100) in RPMI-1640 medium for 3–4 h at 37 °C. The ESP released by the flukes was concentrated (×10) by reverse dialysis with sucrose, using 10 kDa cut-off dialysis membrane (Sigma, USA). Finally, sucrose was removed from the concentrated ESP by dialyzing against phosphate buffered saline (PBS), pH 7.2 and the dialyzed ESP was used for detection of the SOD proteins in the Western blot and SOD enzyme activity by NBT–PAGE.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from three developmental stages of the parasite including adult fluke, newly excysted juveniles and metacercariae. Fifty to 100 mg tissue from live *F. gigantica* adult fluke was homogenized with a tissue homogenizer in 1 ml Trizol reagent (Invitrogen, USA) and processed for total RNA isolation following a standard protocol (Invitrogen, USA). *F. gigantica* newly excysted juveniles (300–500) were disrupted in 1 ml Trizol reagent by hard pipetting, followed by a cycle of freeze–thawing at –40 °C/37 °C. Total RNA was isolated from these juvenile parasites as described earlier for the adult flukes. RNA from 5000 to 10,000 viable metacercariae was isolated by incubating them in 1% pepsin (pepsin from porcine gastric mucosa, Sigma-Aldrich Inc., USA) with 0.4% (v/v) HCl in sterile distilled water at 37 °C for 45 min. These pepsin treated metacercariae were subsequently washed in several changes of nuclease free water to remove the outer cyst wall debris by allowing them to sediment for 5–10 min or by centrifuging them at 500 rpm for 3–5 min. A protocol of 3–4 freeze–thaw cycles at –40 °C and 37 °C or in liquid nitrogen of these pepsin treated metacercariae in 1 ml Trizol was followed by their sonication at 10 μm amplitude × 3 cycles of 10 s each. Further steps for total RNA isolation were followed as described for the two stages of the parasite discussed earlier.

Total RNA isolated from all the three developmental stages of the parasite was transcribed to cDNA using oligo-dT primer and M-MLV reverse transcriptase enzyme (MBI Fermentas, USA) following a standard protocol of cDNA synthesis. The cDNA was subjected to polymerase chain reaction (PCR) with primers specific to the temperate liver fluke *F. hepatica* SOD (GenBank accession no: AF071229). Using forward primer 5′-ATG TCG GGT TCC AGT GGC GTG-3′ and reverse primer 5′-TTA TTC CGT CAG ACC AAT TAC-3′, the full length open reading frame for SOD in *F. gigantica* was retrieved. Each PCR cycle, following an initial denaturation at 94 °C for 3 min, was carried out at 94 °C for 1 min in denaturation, 50 °C for 45 s in annealing and 72 °C for 1 min in extension. The PCR amplified product was purified from the agarose gel, ligated into TA cloning vector (pDRIVE, Qiagen, Germany) and the nucleotide sequence of the cDNA determined.

2.4. Expression and purification of recombinant protein

F. gigantica SOD protein was expressed in *E. coli* using a prokaryotic expression vector (pPROEXHT-b, Life Technologies, USA),