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Research Article

Partial Purification of Superoxide Dismutase and Antigenicity Nature in Setaria digitata, A Filarial Parasite

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Abstract

Setaria digitata is a filarial worm of the cattle used as a model system for anti- filarial drug screening, due to its similarity to the human filarial parasites *Wuchereria bancrofti* and *Brugia malayi*. Since filarial Superoxide dismutase (SOD) is a good biochemical target for anti filarial drug development, a study has been undertaken for the biochemical characterization of SOD from *S. digitata.* Superoxide dismutase activity was measured in different stages of growth of filarial parasites (human and cattle). The activity was almost undetected or very low in micro filarial stage but in adult worms, the enzyme activity was high. The enzyme was characterized to be a Cu/Zn superoxide dismutase. Most of the enzyme activity was associated with a detergent extractable fraction of adult (*Setaria*) parasite. The enzyme was also detected in the *in vitro* released products of adult worms. The superoxide dismutase activity was completely inhibited with IgG antibody from chronic filarial patients in contrast to IgG from normal people. Filarial patients particularly have high IgG antibody levels to purified enzyme. However, individuals from non-filarial regions of Trivandrum, Kerala, seronegative for superoxide dismutase antibodies. Antibody response to superoxide dismutase could thus be used for filarial diagnosis.

Keywords: Filariasis; Superoxide Dismutase; *Wuchereria bancrofti; Setaria digitata*

Introduction

Lymphatic filariasis, commonly known as elephantiasis, is a neglected tropical disease. Infection occurs when filarial parasites are transmitted to humans through mosquitoes. Infection is usually acquired in childhood causing hidden damage to the lymphatic system. The painful and profoundly disfiguring visible manifestations of the disease, lymphoedema, elephantiasis and scrotal swelling occur later in life and can lead to permanent disability. These patients are not only physically disabled, but suffer mental, social and financial losses contributing to stigma and poverty.

It is caused by *Wuchereria bancrofti* and *Brugia malayi* is a major health problem in tropical countries, like India. The prevalence of these diseases in coastal districts of Kerala is extremely high. Although filarial parasites live in an environment in close contact with host humoral and cellular cytotoxic factors, they have evolved mechanisms to evade the toxic (hostile) molecules of host. Super oxide radical, one such toxic molecule, is produced by normal metabolism or released by phagocytes during inflammatory processes. The normal function of SOD (E.C 1.15.1.1.) is that it act as a prominent scavenger of super oxide ($\bullet O^{2-}$), radical to protect

the cells from oxidant mediated damage caused by (•O^{2-}). During the endoparasitic phase of infection, the cells of the innate immune system of turbot generate toxic products, including reactive oxygen species (ROS) such as superoxide (•O^{2-}), hydrogen peroxide (H_2O_2) and hydroxyl radicals (•OH). The antioxidant cellular system limits the presence of ROS, preventing damage to macromolecules by these oxygen derivatives. This process involves several intracellular enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxin (Prdx) [1-5] consequently present an important defense mechanism against superoxide radical toxicity. Cell's antioxidants could not protect the proteins against peroxyl radicals [6].

There is a positive correlation between the levels of the antioxidant enzymes and parasites survival in the host [7,8]. Numerous SODs have been characterized, in the parasites of different species such as *Trichinella spiralis* [9] *Taenia taeniaeformis* [10] *Schistosoma mansoni* and in malarial parasites [7] such as *Plasmodium berghei* and *P. falciparum* [11]. probably acquired host SOD as a part of its own defense enzyme. SODs of two filarial parasites *Dirofilaria immitis* and *Onchocerca volvulus* have been characterized [7,12]. Both are Cu/Zn type, active as a dimer having molecular weights of 32 kDa and 36 kDa. Molecular cloning of the enzyme from *Onchocerca volvulus* has been achieved [12]. A complete cDNA encoding the Cu/Zn SOD *O. volvulus* was identified and its nucleotide sequence was determined. The immune response of the filarial scavenging enzyme SOD has not been studied. The SOD activity has been detected in various life cycle stages of parasites, in secretion of adult *Setaria digitata* worms and the enzyme has been partially purified. We provide evidence that antibodies in chronic filarial patients could completely inhibit the SOD activity of both bovine (*S. digitata*) and human (*W. bancrofti*) parasites.

Materials and Methods

Collection of parasites

S. digitata located in the peritoneal cavity of cattle, *Bos indicus*, were collected in Tyrode medium [13] from the local abattoir. Tyrode medium has a composition of Sodium Chloride 0.8%, Potassium Chloride 0.02%, Calcium Chloride 0.02%, Magnesium Chloride 0.01%, Sodium bicarbonate 0.015% Sodium di hydrogen phosphate 0.05% and glucose 0.5%.

The extraneous materials sticking on the surface of the parasite were removed by thorough washing with the medium. The worms were then kept in Tyrode medium at 37^{0}C until use.

Wuchereria bancrofti mf

Blood samples were collected during night time from filarial patients and mf of *W. Bancrofti* was isolated by microfiltration of the serum.

Setaria digitata mf

S. digitata were incubated in Tyrode medium at 37°C for 2 hrs. The mf was collected by centrifuging this medium.

Intact adult worms were suspended in ice cold PBS with 0·2% nonidet-p-40, vortexed at full speed for 3 times, 30 s each time and centrifuged at 2900 gfor15 min to separate the detergent extractable fraction from the carcass [8]. The detergent soluble fraction contains surface (tegument) components. The carcass was homogenized in PBS and sonicated with Branson-sonifer 450 as noted above, centrifuged to get the supernatant (interior soluble fraction, detergent resistant) and the pellet was dissolved in 0·5% NP–40 made in Tris-HCL (0·01 M), vortexed and centrifuged as above to get the insoluble cellular fraction. The parasite extract (100μl) was treated with 50 μl of chloroform/ethanol mixture (1:2, v/v), mixed thoroughly, diluted with 100 μl of distilled water and vortexed. The mixture was incubated at 37°C for about 15 min and centrifuged to spin down the precipitate. The SOD activity was determined in the supernatant to discriminate between Mn- and Cu/Zn-SOD [14].

Preparation of excretory-secretory (ES) materials

Worms were washed repeatedly in normal saline to remove adhering host materials. Washed worms were suspended in Tyrode solution (5ml/worm/hour) in translucent amber colored wide-mouthed culture bottles and kept in a thermostatic water bath at 37°C for 4 hours. The medium was replaced with fresh medium at the end of every hour and the media pooled. Proteins secreted into the spent medium were harvested for 6 days. The spent medium was dialyzed against PBS, concentrated (centricon-10) and used for SOD determination.

Assay of enzymes

Enzyme assays were done using post nuclear fractions as enzyme sources.

Superoxide dismutase (SOD) E.C.1.15.1.1

The assay system for SOD was adapted from the method of Nishikimi., *et al.* [15] and modified as follows.

Assay mixture contained 1.2 ml sodium pyrophosphate buffer (pH 8.3, 0.052M); 0.1 ml 186 μ moles of PMS, 0.3 ml 300μ moles

of NBT, 0.2 ml of 780µ moles of NADH approximately diluted enzyme preparation and water in a volume of 3ml and the reaction was started by the addition of NADH. After incubation at 30° C for 90 seconds, unless otherwise specified the reaction was stopped by the addition of 1 ml glacial CH_3COOH. Reaction mixture was stirred vigorously and 4 ml of n-butanol was added and allowed to stand for 10 min. Centrifuged and butanol layer taken out. Color intensity of the chromogen in the butanol was measured out 560 nm. Blank contained all reagents except enzyme.

Inhibition by human antibodies

IgG fraction of chronic filarial sera and non-endemic normal sera was purified by protein A-sepharose column, bound immunoglobulin were eluted with 0·1 M glycine-HCl, pH 2·8, into tubes containing 0·1 M Tris HCl, pH 9·0. Various amounts of IgG (0–100 µg) from chronic filarial (pooled) sera or non-endemic normal sera were pre incubated with SOD preparations (20 µg) in 100 µl of assay buffer, kept at 37°C for 2h and subsequently for 16 h at 4°C before performing the SOD assay.

Human sera

Serum samples were collected from people living in villages endemic for *W.bancrofti* infection, of Trivandrum, Kerala, India. Microfilaria (MF) was detected by microscopic examination of 20 µl blood smears, obtained by finger prick between 8: 30 pm and 11: 30 pm. Sera were classified as chronic filariasis (CP), individuals exhibiting elephantiasis and/or hydrocele, asymptomatic carriers (AS), microfilaraemic carriers without any clinical symptoms, and endemic normals (EN), permanent residents of the region who are free from infection as judged clinically and parasitologically. Sera were also collected from normal people of non-filarial regions with similar socio economic backgrounds, as with those in filarial cases. These sera were used as the samples of non- endemic normal for serological comparisons.

Purification of SOD

The soluble homogenate of adult *S. digitata* was applied to a DEAE-cellulose column (1·2×10 cm) equilibrated at room temperature with 0·01 M Tris HCl, pH 8·0. The unbound materials exhibited higher SOD activity than bound materials (eluted with 0·5 M NaCl in the above buffer). The unbound fractions were pooled, concentrated and passed on a sephadex G-100 column (1 × 45cm) eluting with PBS pH 7·2. The fractions with SOD activity were collected, dialyzed against distilled water and concentrated [16].

Protein estimation and SDS-PAGE

Protein concentration was measured according to the dye binding method using bovine serum albumin as standard protein [17] SDS-PAGE was performed under non-reducing conditions [18] and protein bands were visualized by Coomassie stain by the method of Laemmli [19]. Pre-stained molecular weight markers (Sigma-Aldrich) were used for the estimation of subunit mass of purified SOD.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) plates were coated overnight at 4°C with 2 µg/ml of antigen (purified SOD) in alkaline buffer, pH 9·2. Plates were saturated with 0·4% bovine serum albumin (BSA) in the buffer for 1 h at room temperature then 100 µl of 1: 200 diluted human sera were added into the wells and kept at 37°C for 3h. After washing with PBS-tween (0·1%, 3 times) 100 µl of horse radish peroxidase conjugated anti-human IgG (1: 1000, Dakopatts) were added and incubated for further 3 h. After washing with PBS, the presence of antibodies was detected with OPD substrate (O-phenylenediamine containing H_2O_2). The enzymatic reaction was stopped by adding a drop of 4 N sulphuric acid solution and the absorbance was read at 492 nm using an ELISA reader (Bio-Rad).

Results

Activity of SOD

The SOD activities in different stages of filarial parasites are shown in table 1. Adults have higher activity as compared to MF stage. Similarly the activity was undetected in *W. bancrofti* MF.

Stage	Specific activity units/mg protein
S. digitata adult	17.18 + 4.40
S. digitata mf	5.21 + 2.20
S. digitata larvae	17.20 + 4.10
S. digitata E S	24.66 + 4.80
W. bancrofti mf	ND
W. bancrofti larvae	18.20 + 2.20

Table 1: The SOD activities in different stages of filarial parasites.
*ND, Not detected.

The protein contents of parasites in the assay ranged between 15-25 µg. ES products were collected after 24 h of *in vitro* culture.

Four to five numbers of measurements were made in each case. SOD contents (unit/mg protein) in male and female worms are 16·16 ± 5·20 and 14·4 ± 4·28 respectively.

Purification of SOD from *S. digitata*

Step	Protein con.(mg)	Recovery	Specific activity	Fold purification
Homogenate	15	100	13	1
DEAE Cellulose	5	33	30	4
Sephadex G 100	0.25	1.6	382	29

Table 2: Charaterization of SOD from *S. digitata.*

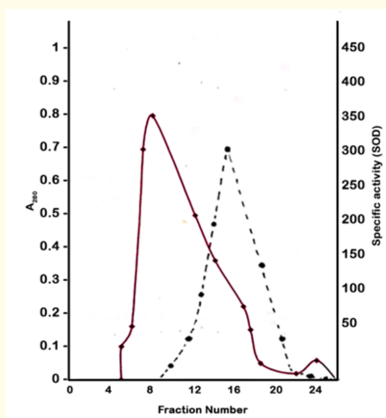

Figure 1: Sephadex G-100 chromatography of DEAE unbound materials of S.digitata adult extract A280 [♦8́ and SOD activity [●].

The distribution of SOD activity in different extractions of adult *S. digitata* worms was determined. Adult parasites were fractionated into a detergent soluble fraction, an interior fraction and insoluble fraction. From figure 2 it is evident that the detergent extractable fraction contained highest SOD activity followed by aqueous soluble fraction; aqueous insoluble fraction had lowest activity.

Extract of adult *S. digitata* was treated with organic solvent (ethanol/chloroform: 2: 1) and assayed for SOD activity. There was no loss of SOD activity following treatment with organic solvents indicating the presence of Cu/Zn SOD. Healing at 100°C for 2 min abolished 80% of enzymatic activity in *S. digitata.* SOD activity was detected in excretory-secretory (ES) products of *S. digitata* adults at different days of *in vitro* culture.

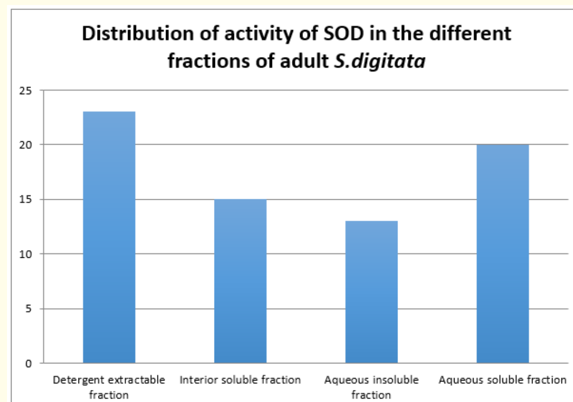

Figure 2: Distribution of activity of SOD in the different fractions of adult *S. digitata*.

Partial Purification

SOD were partially purified about 29-fold by a combination of an ion-exchange and molecular sieve chromatography. On a DEAE-cellulose column (1·2 × 10 cm; 0·01 M Tris HCl, pH 8·0), unbound materials (33% of the load) exhibited higher SOD activity than the bound materials. These fractions were pooled, and passed on a Sephadex G-100 (1×45 cm) column, eluting with PBS, pH 7·2. SOD containing fractions were pooled, dialyzed against water and concentrated. The purified SOD fractions constituted about 1·6% of total soluble proteins of adult.

S. digitata On SDS-PAGE, a prominent band was observed at 32 kDa along with two minor bands at 24 and 16 kDa (Figure 3). Activity staining using NBT (Nitro Blue Tetrazolium) of purified SOD from *S. digitata* on 10% native gel Blue-insoluble formazan appeared except in zone containing SOD (Figure 4).

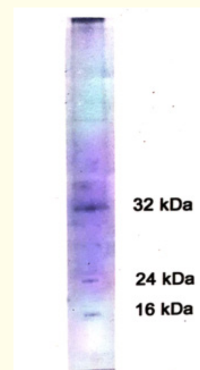

Figure 3: SDS – PAGE pattern of purified SOD in *S. digitata*.

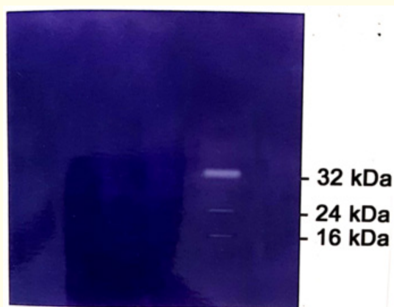

Figure 4: PAGE pattern of purified SOD (NBT-staining) in *S. digitata*.

Inhibition by filarial antibody

IgG purified by protein A sepharose column from the sera of chronic filarial patients was found to inhibit the SOD activities in *B. malayi* larvae, extracts and ES of adult *S. digitata* (Table 4).

A relatively higher extent of enzyme inhibition was observed in ES products compared to the somatic stages of the parasites. The inhibition by different amounts of filarial IgG on the SOD activity was determined using *S. digitata*. IgG from non-endemic normal sera was not able to inhibit the enzyme activity (Table 3 and 4).

Group	IgG	Positive %
	OD 492	
Endamic normals(EN)	0.10 + 0.08	33.2
Asymptamatic microfi-arimic carriers	0.22 + 0.14	76.8
Cronic filarial patients (CP)	0.28 + 0.16	100
Non endamic normals	0.06 + 0.04	0

Table 3: IgG level of levels to SOD in human filariasis sera.

Specific activity			
Paraiste	without IgG	with IgG	Inhibition%
S. digitata Adult extact	17.18 + 4.03	7.33 + 4.10	65.88
ES of adult setaria	24.88 + 5.30	5.32 + 2.86	81.94

Table 4: Inhibition of SOD activity by IgG.

Discussion

SODs has been demonstrated from various helminthes of different species such as *Schistosoma mansoni*, *Onchocerca volvulus*, *Dirofilariaimmitis*, *Brugia pahangi* and *Fasciola hepatica* [7,8]. Although the adult worms have a generally anaerobic metabolism and live in the peritoneal cavity of cattle where the oxygen tension is relatively low, oxygen is necessary for other functions such as egg generation, which generate reactive oxygen species (ROS). In addition to this normal endogenous oxidative tension, the parasite is exposed to reactive oxygen species produced by host responding cells such as macrophages, eosinophils, neutrophils and platelets. To protect themselves against oxidative stress mechanisms of hosts, parasites have developed antioxidant enzyme systems [5].

The present findings describe the content of superoxide dismutase in various life cycle stages of cattle filarial parasite, *S. digitata* and human parasites *W. bancrofti* and *B. malayi.* Enzymatic characterization, partial purification, characterization and antigenic properties of SOD were carried out using *S. digitata* because of its ready availability. The expression of SOD activity appears to be developmentally regulated. The activity was minimal in the microfilarial stage compared to infective larvae and adult stages of the worms. An increase of SOD activity in detergent extractable fraction containing surface components of parasites over the aqueous soluble fraction indicates its role in parasitic defense. Treatment with organic solvents inactivates Mn-SOD leaving Cu-Zn SOD activity unaffected [20]. The insensitivity of SOD activity of *S. digitata* to chloroform/ethanol treatment indicates the presence of Cu/Zn-SOD in *S. digitata*. The absence of high molecular weight (~ 86 K) bands in the SDS-PAGE analysis is also an evidence against the presence of Mn-SOD. In recent years considerable attention has been given to affinity chromatography for the isolation and purification of macromolecules, mainly enzymes. In the present investigation the same techniques was adapted for the isolation of SOD from *S. digitata.* Over 29 fold enrichment of the enzyme was achieved without loss in activity. Only two isozymes are found in *S. digitata* in contrast with many isozymes reported for SOD from other parasites [20,21].

The detection of SOD activity in the *in vitro* released (ES) products suggests that the parasite actively secrets the enzyme and indicates the potential importance of this enzyme in *in vivo* situations. A logical outcome of this effect would be that the secreted enzyme might be immunogenic. It is indeed interesting to note that filarial IgG could neutralize the SOD activity in *B. malayi* L3 and *S. digitata*. SOD of cattle parasite *S. digitata* possesses antigenic cross-reactivity with human parasite. Immune inhibition of SOD activity as shown here is the first report in human filariasis. As early as

1935, parasitic enzymes were proposed as targets of host immune system [22]. Antibodies capable of inhibiting enzyme (proteolytic, lipolytic) activity were detected in bovine lung worm *Dictyocalus viviparus* infection and in *Ascarissuum* infection [23,24]. However it should be mentioned that numerous instances are known where antibodies to parasite enzymes do not neutralize the activity [25].

Antibody positivity to SOD enriched fraction is detected predominantly in the infected cases (chronic filariasis and asymptomatic microfilaraemic carriers) compared to endemic normals. All the individuals in chronic filariasis are sero positive. The absence of SOD specific antibodies in individuals living in non-filarial regions Trivandrum, Kerala suggests that the antibody response that is reported here is specific for filarial infection. Many parasite enzymes have been implicated as immune diagnostic antigens [26]. Since microfilarial stage lacks SOD antibodies to the enzyme may be exploited as an immunological marker for the presence of filarial adult worms in the host. This finding might be explained by the complex protein nature of crude antigenic materials of adult *S.digitata,* as well as the persistence of the adult form, and its capability of immune evasion may provide a good chance for the production of IgG against the epitopes of this parasitic macromolecule [27, 28]. This study highlights the role of SODs as enzymes that protect the ciliate from the toxic action of $\bullet O^{2-}$ generated both in the marine environment, during its free life phase, and in the host, during the parasitic phase [29]. The extracellular forms of these enzymes, with very different amino acid sequences from the host, may yield potential diagnostic targets, as well as potential antigens, in order to produce vaccines in the future [30-32].

Conclusion

The results reported here suggest that during the course of filarial infection in humans, antibodies are produced to parasitic SOD enzyme and these antibodies have the ability to neutralize the enzymes activity. The most significant finding is the inhibition of SOD enzymes of human sera of filarial infected patients. So major antioxidant enzyme like Superoxide dismutase of the parasite could be a targeted for future drug development studies in filariasis.

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Conflict of Interest

Authors do not have any conflict of interest for the present investigation.

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