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Antigenicity of filarial superoxide dismutase in human bancroftian filariasis

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Abstract. 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 microfilarial 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 and IgM antibody levels to purified enzyme. However, individuals from non-filarial regions of Orissa are sero-negative for superoxide dismutase antibodies. Antibody response to superoxide dismutase could thus be used for filarial diagnosis.

Keywords. Filariasis; superoxide dismutase; *Wuchereria bancrofti; Setaria digitata.*

1. Introduction

Lymphatic filariasis: caused by *Wuchereria bancrofti* and *Brugia malayi* is a major health problem in tropical countries, like India. The prevalence of this disease in coastal districts of Orissa 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. Superoxide radical, one such toxic molecule, is produced by normal metabolism or released by phagocytes during inflammatory processes. Immune effector cells, macrophages, neutrophils and eosinophils release superoxide radical, as a host defence mechanism to kill the invading parasites (Bannister

The normal function of superoxide dismutase (SOD) (EC 1·15·1·1), a prominent scavenger of O_2^- radical, is to protect the cells from oxidant mediated damage caused by O_2^- radical. It catalyses the spontaneous dismutation of superoxide radical to hydrogen peroxide and molecular oxygen. In recent years SOD and other antioxidant enzymes (glutathione peroxidase, catalase, glutathione-S-transferase) have been implicated to protect the parasites from the host responses. There is a positive correlation between the levels of antioxidant enzymes and parasite survival in the host (Callahan *et al* 1988; Hong *et al* 1992).

Numerous SODs have been characterized, in the parasites of different species e.g., in *Trichinella spiralis* (Rhoads 1983), *Taenia taeniaeformis* (Leid and Suquet 1986) *Schistoma mansoni* (Hong *et al* 1992) and in malarial parasites. *Plasmodium*

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burgei and *P. falciparum* (Fairfield *et al* 1986, 1988) probably acquired host SOD as a part of its own defence enzyme. SODs of two filarial parasites *Dirofilaria immitis* and *Onchocerca volvulus* have been characterized (Callahan *et al* 1991; Henkle *et al* 1991). 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 (Henkle *et al* 1991). A complete cDNA encoding the Cu/Zn SOD *0. volvulus* was identified and its nucleotide sequence was determined.

The nature of human immune response to filarial SOD has not been studied. We have initiated work in this direction and the results of our study are presented here. 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, B. malayi*) parasites.

2. Materials and methods

2.1 *Parasites and preparation of parasite extract*

S. digitata adults, *W. bancrofti* infective larvae and microfilariae (MF) were obtained as described earlier (Beuria and Das 1992; Das *et al* 1992). Infective larvae of *B. malayi* and *S. digitata* were obtained from Medical Entomology Department of this centre. Adult worms were crushed in a morter and pestle for 10 min in 0·01 M phosphate, pH 7·2, 0·15M NaCl (PBS) containing protease inhibitors (1mM iodoacetamide, 1 mM phenylmethylsulphonylfluoride) and were sonicated (Branson sonifer 450, 30s each, 5 times) on ice. Soluble extract (SEA) was obtained by centrifugation at 25,7000 *g* for 20 min in the cold (Beckman J2-21). Extracts of MF and infective larvae were prepared by homogenizing initially in a glass homogenizer and then sonicated as above.

Intact adult worms were suspended in ice cold PBS with 0·2% noniodet-p-40, vortexed at full speed for 3 times, 30 s each time and centrifuged at 2900 *g*for 15 min to separate the detergent extractable fraction from the carcass (Hong *et al* 1992). 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 (100 µl) 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 (Paoletti and Macali 1990).

2.2 *In vitro maintenance of S. digitata and preparation of excretory-secretory products*

Adult *S. digitata* worms were maintained in Dulbeco's modified Eagle's medium (DME), supplemented with gentamycin (10µg/ml) penicillin (100U/ml) at 37°C

under air with 5% CO_2. Each worm was kept in 3 ml of medium which was changed daily and the spent medium was collected every day. The continued motility of worms was used as a measure of viability. Proteins secreted into the spent medium were harvested for 6 days. The spent medium was dialysed against PBS, concentrated (centricon-10), and used for SOD determination.

2.3 *Enzyme assay*

SOD activity was measured following the inhibition of NAD(P) H oxidation mediated by superoxide radical in 0·1 M triethanolamine-diethanolamine buffer, pH 7·4 (Paoletti *et al* 1986). It consists of a purely chemical reaction sequence which generates superoxide from molecular oxygen in the presence of EDTA, manganese chloride and mercaptoethanol. SOD brings about the inhibition of nucleotide oxidation. This method allows the determination of as low as 2 ng of SOD.

2.4 *Inhibition by human antibodies*

IgG fraction of chronic filarial sera and non-endemic normal sera was purified by protein A-sepharose column, bound immunoglobulins 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.

2.5 *Human sera*

Serum samples were. collected from people living in villages endemic for *W. bancrofti* infection, of Puri/Khurda districts of Orissa, India. This region has been described earlier (Das *et al* 1992). Microfilaraemia (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 (Koraput) of Orissa with similar socio economic backgrounds, as with those in filarial cases. These sera were used as the samples of non endemic normals for serological comparisons.

2.6 *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, dialysed against distilled water and concentrated.

2.7 *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 antihuman IgM or IgG (1 : 1000, Dakopatts) were added and incubated for further 3 h. After washing with PBS/T, 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, the absorbance was read at 492 nm using an ELISA reader (Biorad). The positivity was determined with non endemic samples (> mean A492 + 3SD).

3. Results

3.1 *Characterization of SOD activity*

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

Table 1. SOD activities in filarial parasites.

Stage	Specific activity (unit/mg protein)
S. digitata adult	15·18 ± 2·03
S. digitata MF	3·21 ± 1·20
S. digitata larvae	15·20 ± 3·10
S. digitata ES	22·66 ± 4·30
W. bancrofti MF	ND
B. malayi larvae	13·76 ± 1·37
W. bancrofti larvae	16·20 ± 2·20

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.
ND, Not detected.

undetected in *W. bancrofti* MF. SOD contents (unit/mg protein) in male and female worms are 16·16 ± 5·20 and 14·4 ± 4·28 respectively. The distribution of SOD activity in different extractions of adult *S. digitata* worms was determined. Adults parasites were fractionated into a detergent soluble fraction, an interior fraction and insoluble fraction. From figure 1 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

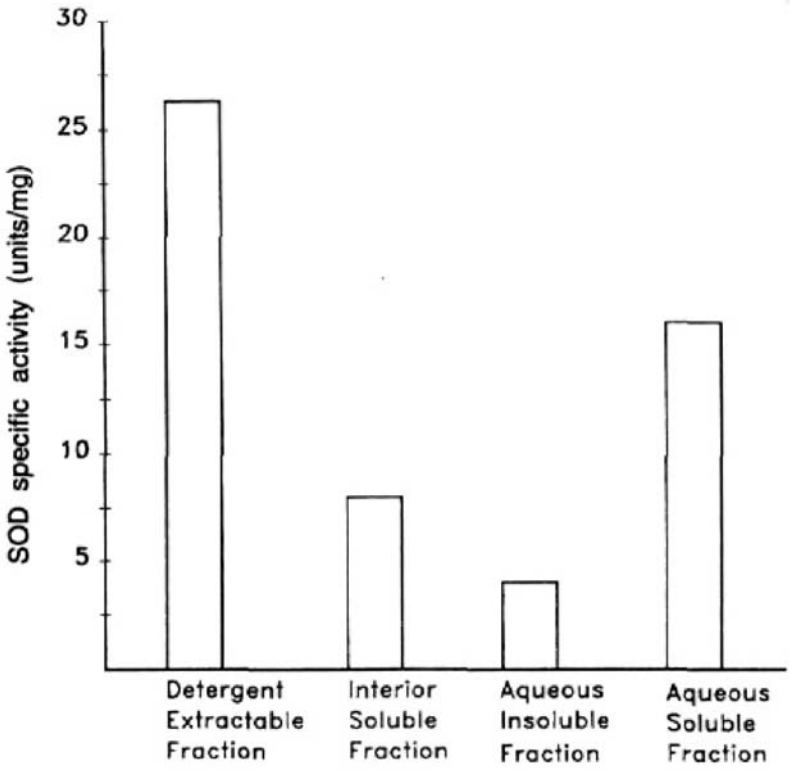

Figure 1. The distribution of SOD activity in adult *S. digitata,* Intact adult parasites were fractionated into a detergent-extractable fraction, interior (detergent-resistant caracass) soluble fraction and aqueous insoluble fraction. Activity is also shown in aqueous soluble fraction.

Cu/Zn SOD. Healing at 100°C for 2 min abolished 80% of enzymatic activity in *S. digitata.*

SOD acitivity was delected in excretory-secretory (ES) products of *S. digitata* adults at different days of *in vitro* culture. The amount of SOD released peaked (22·66 ± 4·30) at 24 h and decreased rapidly afterwards so that by 3rd day (72 h) negligible activity was noticed (figure 2).

3.2 *Partial purification*

SOD were partially purified about 29-fold by a combination of an ion-exchange and molecular seive chromatography (table 2). 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 (figure 3). SOD containing fractions were pooled, dialysed 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.

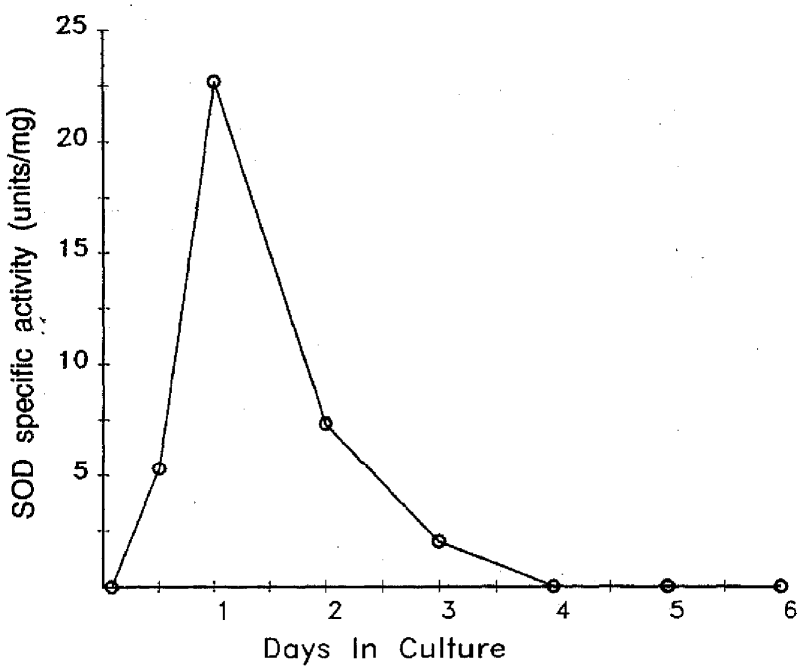

Figure 2. SOD activity in the ES products of *S. digitata* adults.

Table 2. Purification of the SOD from *S. digitata*.

Steps	Protein conc. (mg)	Recovery (%)	Specific activity (unit/mg)	Fold purification
Homogenate	15	100	13	1
DEAE-cellulose	5	33	50	4
Sephadex G-100	0·25	1·6	382	29

3.3 *Inhibition by filarial antibody*

IgG purified by protein A sepharose column from the srea of chronic filarial patients was found to inhibit the SOD activities in *B. malayi* larvae, extracts and ES of adult *S. digitata* (table 3). 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 (figure 4).

3.4 *Antibody levels to SOD in filarial sera*

The extent of IgG and IgM seropositivity to purified SOD fraction in different categories of (n=30 in each case) human filariasis was determined (table 4)

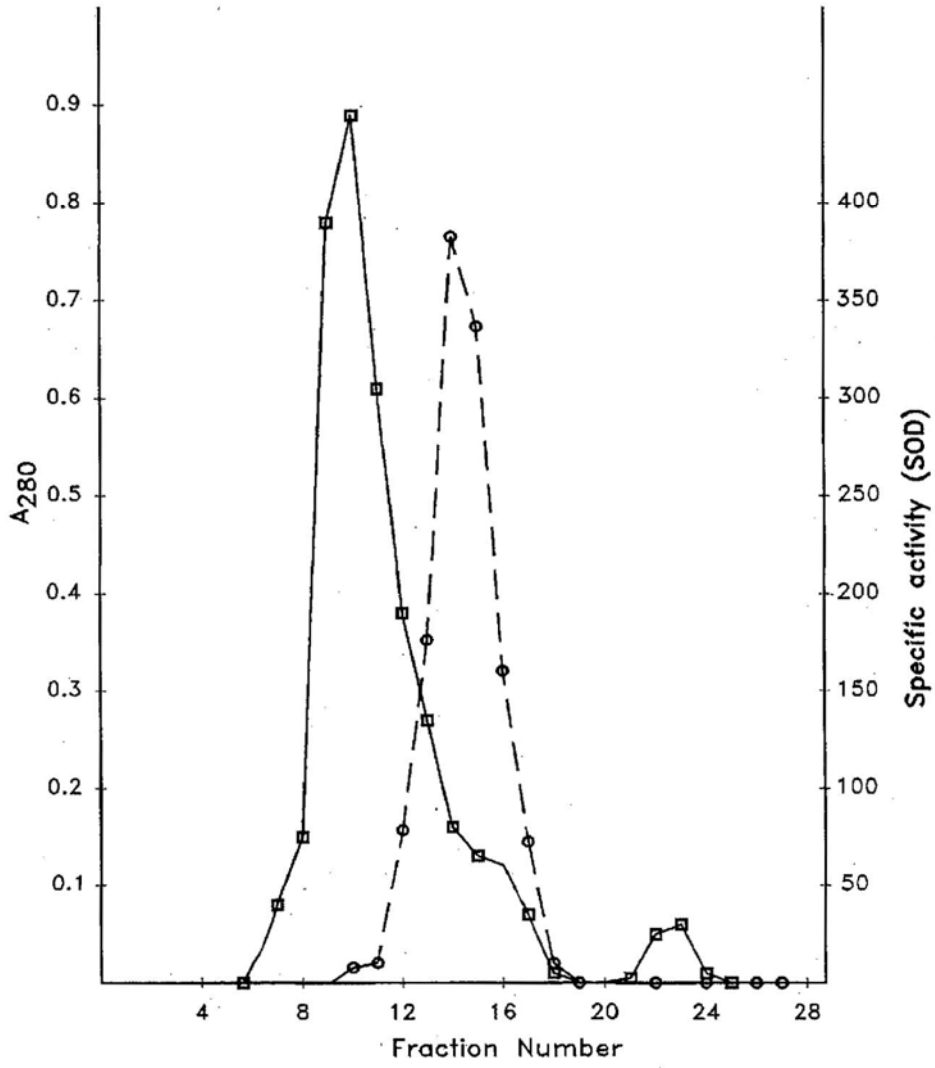

Figure 3. Sephadex G-100 chromatography of DEAE unbound materials of *S. digitata* adult extract. A_{280} (□), and SOD activity (O).

Antibody levels in AS and CP group do not differ much but are significantly higher (P<0·01) than those in EN groups. All of chronic patients (100%) showed seropositivity for both IgG and IgM antibodies. About 75% and 60% of AS group, 33% and 20% of EN are positive for IgG and IgM respectively. It is of interest to note that non-endernic normals are seronegative for both the antibodies.

4. Discussion

The present paper describes the content of superoxide dismutase in various life cycle stages of cattle filarial parasite, *S. digitata* and human parasites *W. bancrofti*

Table 3. Inhibition of SOD activity of filarial parasites (20 µg protein) by IgG (50 µg) from chronic filarial sera.

Parasite	Specific activity		Inhibition (%)
	Without IgG	With IgG	
B. malayi L_3	13·76 ± 1·37	5·47 ± 2·80	60·25
S. digitata AE (adult extract)	15·18 ± 2·03	5·33 ± 3·10	64·88
ES of adult *S. digitata*	22·66 ± 4·30	4·32 ± 2·86	80·93

The results are mean of four experiments

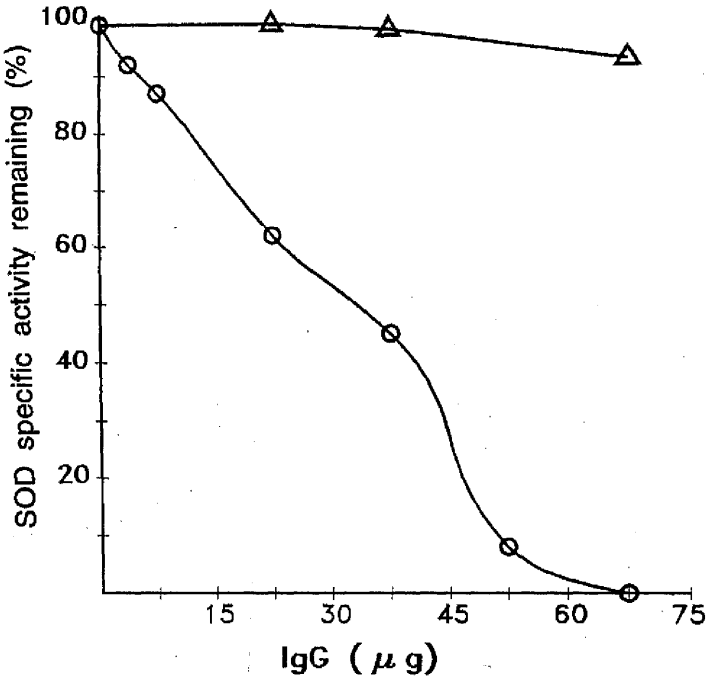

Figure 4. The effect of purified IgG from pooled sera of chronic filarial patients (O) and non endemic normals (Δ) on the SOD activity of *S. digitata.* Various quantities of IgG (0-100 µg) were incubated with purified SOD fraction at 37 C for 2h and then at 4°C overnight before performing the SOD assay.

and *B. malayi.* Enzymatic characterization, partial purification and antigenic reactivity 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 minimum in the microfilarial stage compared to infective larvae and adult stages of the worms. Similarly the SOD activity has been reported to increase gradually during the life cycle of *S. mansoni,* reaching maximum in adults (Hong *et al* 1992). However, microfilaria of two other filarial parasites *D. immitis* and

Table 4. IgG and IgM levels to SOD in human filariasis sera.

Group	IgM		IgG	
	OD 492	Positive (%)	OD 492	Positive (%)
Endemic normals (EN)	0·06 ± 0·01	20·0	0·10 ± 0·06	33·0
Asymptomatic microfilaraemic carriers (AS)	0·10 ± 0·04	60·0	0·20 ± 0·12	76·6
Chronic filarial patients (CP)	0·16 ± 0·06	100·0	0·26 ± 0·14	100
Non-endemic normals	0·02 ± 0·01	0	0·04 ± 0·02	0

n = 30 in each group were tested in ELISA at a fixed dilution (1 : 200). The threshold for positivity (> mean A492 + 3 SD) for IgM and IgG was 0· 08 and 0 ·10 respectly.

O. cervicelis are reported to have considerable SOD levels (Callahan *et al* 1988) It is possible that these disparate results reflect differences in the microfilarial stages of parasites. The distribution pattern of SOD activity in different fractions of adult parasite is different. An increase of SOD activity in detergent extractable fraction containing surface components of parasites over the aqueous soluble fraction indicates its role in parasitic defence.

Treatment with organic solvents inactivates Mn-SOD leaving Cu-Zn SOD activity unaffected (Paoletti and Mocali 1990). 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.

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* L_3 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 (Chandler 1935). Antibodies capable of inhibiting enzyme (proteolytic, lipolytic) activity were detected in bovine lungworm *Dictyocalus viviparus* infection (Britton *et al* 1992) and in *Ascaris suum* infection (Knox and Kennedy 1988). However it should be mentioned that numerous instances are also known where antibodies to parasite enzymes do not neutralize the activity (Kumar 1993).

Antibody positivity to SOD enriched fraction are 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 (Koraput) of Orissa suggests that the antibody response that is reported here is specific for filarial infection. Many parasitie enzymes have been implicated as immunodiagnostic antigens (Kennedy 1991). 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. In 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 enzymic activity. The most significant finding is the inhibition of SOD enzyme by human sera from filariasis patients. As the anti-oxidant enzymes are crucial for parasite survival, immune inhibition of SOD activity would have deleterious effect for the parasite in hosts. Superoxide dismutase of the parasite could be a target for future vaccinaiion studies in filariasis.

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