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IDENTIFICATION AND PURIFICATION OF FE CONTAINING SUPEROXIDE DISMUTASE FROM LEISHMANIA DONOVANI

Bikramjit Raychaudhury*[1], R. Jyoti[1], Kakuli Chakraborty[1], Anindita Chakraborty[2], Moushree Palroy[1] and Rajen Haldar[3]

[1]Department of Physiology, Ananda Chandra College, Jalpaiguri 735101.
[2]Department of Physiology, ABN Seal College, Coochbehar 736101.
[3]Department of Physiology, University Colleges of Science and Technology, University of Calcutta, Kolkata, India.

***Corresponding Author: Bikramjit Raychaudhury**

Department of Physiology, Ananda Chandra College, Jalpaiguri 735101.

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ABSTRACT

Leishmaniasis is a parasitic disease which infects as many as 400,000 people per year. Because the infective agent- a protozoan-inhabits phagolysosomes in host macrophages, the parasites are partially protected from chemotherapeutic agents. Thus, treated patients often relapse or experience toxic reactions to the drugs. In order to develop new leishmanicidal agents, studies have been conducted to understand the interactions of the parasite with the macrophage. Ordinarily, microorganisms which are taken up by macrophages are destroyed by oxygen-dependent and oxygen-independent antimicrobial systems. The oxygen-dependent antimicrobial activity of macrophages is dependent on the generation of superoxide (O_2^-) by the one-electron reduction of molecular oxygen. This O_2^- can then undergo a series of reactions to produce hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$), and perhaps, singlet oxygen ($^1O_2$), which may be responsible for destroying the ingested microorganism. Some microorganisms, such as Toxoplasma gondii, block the generation of superoxide by macrophages in order to survive phagocytosis. It is found that this was not the case for Leishmania. The uptake of Leishmania tropica promastigotes by mouse peritoneal macrophages was accompanied by the generation of high levels of superoxide and other activated forms of oxygen. The possibility that parasites might survive phagocytosis by efficient enzymatic decomposition of H_2O_2 and O_2^- has also been investigated. The parasites were found to lack or contain low levels of enzymes capable of decomposing H_2O_2 (glutathione peroxidase and catalase) but to contain relatively high levels of superoxide dismutase, which degrades O_2^-. Evidence was obtained that the leishmanial superoxide dismutase was substantially different from the superoxide dismutase found in its host and therefore made a potential target for the design of parasite-specific enzyme inhibitors. The leishmanial superoxide dismutase activity was found to be insensitive to inhibition by cyanide, but sensitive to inhibition by azide and peroxide, properties which suggest an Fe containing superoxide dismutase. In contrast, the mammalian host has only Cu/Zn-containing and Mn-containing superoxide dismutases. In order to further characterize the leishmanial superoxide dismutase, we have isolated superoxide dismutase from *Leishmania donovani*.

KEYWORDS: Leishmania donovani, Superoxide dismutase, Antioxidant.

INTRODUCTION

The superoxide radical is an intermediate reduction product of oxygen produced by a variety of biological reactions. The superoxide radical (O_2) and other reactive derivatives have received recent attention as agents of oxygen toxicity in cells. Most organisms, therefore, have defense systems, such as metallo-enzymes, to protect themselves from toxic oxygen species. Metallo-enzymes that catalyze the disproportionation of superoxide free radicals (O_2) to hydrogen peroxide (H_2O_2) and oxygen (O_2), are known as superoxide dismutases (SODs). SODs play an important role in the protection of cells from the oxidative damage of superoxide radicals. Cell damage may also be due to the superoxide itself or, indirectly, even more reactive oxygen species, such as hydroxyl radicals ($^{\cdot}OH$), formation of which, via the Fenton reaction, is favoured by excess superoxide[1] SODs have subunit molecular weights ranging from 16 kDa to 26 kDa and are divided into three classes on the basis of their active site metals: copper and zinc (Cu/Zn-SOD), manganese (Mn-SOD), and iron (Fe-SOD). Cu/Zn-SOD is found widely in the cytoplasm and certainly in the mitochondrial inter-membrane space of the eukaryotic cells and chloroplasts of plants. Mn-SOD is located in prokaryotes and in the mitochondria of eukaryotes[2] while Fe-SOD has been found in bacteria, blue-green algae and protozoa[3] Recent reports also indicated that the enzyme was present in higher land plants[4] In addition, Ni-SOD has been isolated from some microorganisms.[5]

NADPH oxidase, also referred to as phox, is expressed in professional phagocytes such as neutrophils and macrophages. It catalyzes the production of large amounts of $O_2^{\cdot-}$ (~10 mM inside the phagosome) upon activation. The neutrophil is particularly efficient and produces levels 2-3 times higher than those of macrophages. The important role of phox to the immune defense in exemplified by the fact that a defective enzyme results in chronic granulomatous disease (CGD)**[6].** People with CGD have a genetic deficiency of phox components and suffers from recurrent infections. There are also other cellular sources of O_2, including xanthine oxidoreductase and homologues of the gp91phox subunit of phox. In addition, the mitochondria and certain metabolic pathways generate O_2.

Superoxide dismutases (SODs) represent a family of metalloenzymes, found in organisms ranging from bacteria to humans.[7] SODs exist in several different isoforms that differ in their structure and prosthetic ion/ions but all isoforms catalyze the dismutation of O_2^- to hydrogen peroxide (H_2O_2) and oxygen (O_2). Iron-SOD (Fe-SOD) and Manganese-SOD (Mn-SOD) are located in the cytoplasm of prokaryotes whereas Cu/Zn-SOD is located in the periplasm of gram-negative bacteria, anchored to the outer membrane, or secreted[8] Thus, Cu/Zn-SOD in bacteria has the potential to protect against reactive oxygen species (ROS) generated from external sources. Accordingly, it has also been shown to be a virulence mechanism of many bacteria. The amino acid sequences of Cu/Zn-SOD from different bacterial species show extensive variation[9] and is predicted to affect the architecture of the active site channel and subunit assembly and, hence, enzyme activity. Thus, the SOD activity may vary substantially between bacterial species.

MATERIALS AND METHODS

Reagents

Brain heart infusion broth was obtained from Acumedia Manufactures, Baltimore, Maryland, USA. Bactoagar was procured from DIFCO laboratories, Detroit, Michigan, USA. The culture media Medium 199, fetal bovine serum, penicillin-streptomycine, HEPES buffer and L-glutamine were purchased from GIBCO Laboratories, Grand Island, New York, USA. Alkaline phosphatase conjugated anti-rabbit and mice IgG (whole molecule) were from Sigma Immunochemicals. TRITC and FITC coupled anti rabbit and mice IgG were obtained from Bangalore Genei, India. Other reagents were of highest purity available from Sigma Chemical Company, St. Louis, Missouri, USA.

DEAE-52 was purchased from Whatman, and Sephadex G-75 from Amersham Pharmacia Biotech Corporation (Sweden). Molecular weight markers for gel electrophoresis were obtained Amersham Pharmacia Biotech Corporation (Sweden). The other chemicals were analytical reagents.

Parasite

Leishmania donovani strain MHOM/IN/AG/83 was obtained from kala-azar patient[10] and maintained by intracardial passage in every 8 weeks in Syrian golden hamster. Promastigotes were obtained by transforming amastigotes isolated from infected spleen[11] and maintained in medium -199 supplemented with 10% fetal calf serum *in vitro*. The strain was also maintained at 22° C in modified Rays medium.[12]

Purification of Fe-SOD from *Leishmania donovani*

Preparation of cell lysate

A total of 25 gm Leishmania cells were taken at this stage for purification of Fe-SOD. Cells were dissolved in x 2 volume ice-cold buffer A containing 50 mM potassium phosphate, 10mM TES, 1mM EDTA, 0.5 mM phenylmethylsulfonyl (in 0.1% ethanol) fluoride and 0.25 mM leupeptin at pH 7.6 and were sonicated by giving 5-6 strokes of 20 sec in an MSE sonicator at 4°C on a setting of 6 and centrifuged at 100,000g for 60 min in a Sorvall centrifuge. The supernatant cell free extract obtained was then used in subsequent steps of purification. .

$(NH4)_2SO_4$ precipitation

The cell-free extract was treated with $(NH4)_2SO_4$ in three steps. First, solid $(NH4)_2SO_4$ was added to the extract to 30% saturation at 4ºC, then the mixture was stirred for 15 min at 4ºC, and left at 4ºC for 60 min. The precipitate was removed by centrifugation at 9,000 rpm for 10 minutes. In the second step, the supernatant was treated with solid $(NH4)_2SO_4$ to 60% saturation at 4ºC, stirred for 15 min at 4ºC, and left at 4ºC for 60 min. The precipitate was removed by centrifugation at 9,000 rpm for 10 minutes. In the third step, the supernatant was treated with solid $(NH4)_2SO_4$ to 90% saturation at 4ºC, stirred for 15 min at 4ºC, and left at 4ºC for 60 min. The precipitate with SOD activity was centrifuged at 9000 rpm for 10 min (Hettich, Universal 30RF centrifuge) and then dissolved in a minimal volume of 20 mM potassium phosphate (pH 7.6 buffer B) and dialyzed overnight at 4ºC against the same buffer. The SOD solution was concentrated to 0.5 ml by a Amicon PM – 30 (30,000-molecular weight cut off).

Ion-exchange Chromatography

The concentrated enzyme solution was loaded on to a DEAE-52 column (2.6 cm x 60 cm) and equilibrated with buffer B for 5 h at 0.25 ml / min. The column was eluted with a 0–500 mM NaCl linear gradient and fractions of 3 ml were collected. The SOD active fractions were pooled, concentrated by Amicon PM–30 (30,000-molecular weight cut off) at 4ºC to 10 ml and dialyzed against 10 mM phosphate buffer (buffer C, pH 7.6) for 24 h (4ºC).

Gel Chromatography

The concentrated enzyme solution was applied on to a Sephadex G-75 column (2.6 cm x 100 cm), equilibrated with PBS at pH 7.2. The column was eluted with the

same buffer at a flow rate of 0.25 ml/min. The fraction with SOD activity was eluted with 55 ml PBS and dialyzed against 0.01M phosphate buffer pH 7.2 over night and concentrated with Amicon PM–30 (30,000-molecular weight cut off) and stored at -20°C until used.

SOD activity assay

Superoxide dismutase activity was assayed after each purification step by measuring the inhibition of pyrogallol autoxidation rate.[13] The assay mixture contained 0.2mM pyrogallol in air equilibrated 50mM tris–cacodylic acid buffer, pH 8.2, and 1mM diethylene triamine penta acetic acid. The rate of autoxidation was obtained by monitoring the increase in absorbance at 420 nm in a Hitachi spectrophotometer, No U2000. SOD has the ability to inhibit the autoxidation and the extent of inhibition is taken as the measure of enzymic activity.

Molecular Weight Determination

The determination of the molecular weight by gel filtration was carried out on Sephadex G-200 column equilibrated with 0.05 M phosphate buffer pH 7.2, and calibrated with the following molecular weight standards: immunoglobulin G (160 000 D), human serum albumin (67 000 D), ß-lactoglobulin (35 000 D), cytochrome c (12 400 D), vitamin B12 (1355 D), cytidine (246 D).

Protein Estimation

Proteins were determined by the method of Bradford,[14] using bovine serum albumin as the standard.

Activity staining for SOD

Purified SOD was separated on a 10% non-denaturing polyacrylamide gel[15] for activity staining[16] Solution A was prepared by dissolving 10 mg nitroblue tetrazolium and 4 mg riboflavin in 10 ml glass distilled water. Solution B contained 600 µl TEMED in 10 ml glass distilled water. At the end of electrophoresis, gels were first incubated in Solution A for 20 min and then in Solution B for another 20 min to finally illuminate till the appearance of white bands against a blue background was observed.

This assay depends on the ability of SOD to inhibit the reduction of nitroblue tetrazolium to blue coloured formazan by O_2^- generated by reoxidation of photochemically reduced riboflavin. Thus regions containing active SOD appear colourless against a blue background. The band intensity is roughly proportional to the amount of SOD protein.[16]

SDS-Polyacrylamide gel electrophoresis

SDS-PAGE of different fractions was done as described by Laemmli.[15]

RESULTS AND DISCUSSION

Purification of SOD

After each purification step, the protein content and the enzyme activity were determined. According to our results, ammonium sulphate precipitation to 30% and 60% saturation eliminated 50% of the blast protein, without loss in SOD activity and the third precipitation step at 90% ammonium sulfate saturation resulted in a SOD preparation of higher specific activity.

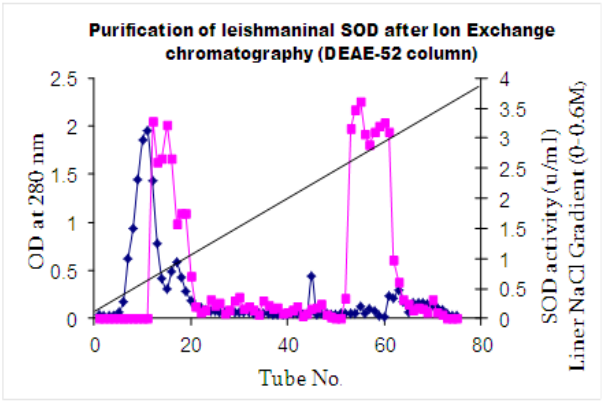

Fig. 1: Ion-exchange chromatography on a DEAE-52 column; protein concentration (-▲-), superoxide dismutase activity (–●–), concentration of NaCl (—).

After that, ion-exchange chromatography on DEAE-52 column with a concentration gradient of NaCl (0–0.6 M) was performed which resulted in elimination of 90% of the blast protein with a loss of SOD activity of about 57 %. Results of this chromatographic procedure are shown in Fig. 1.

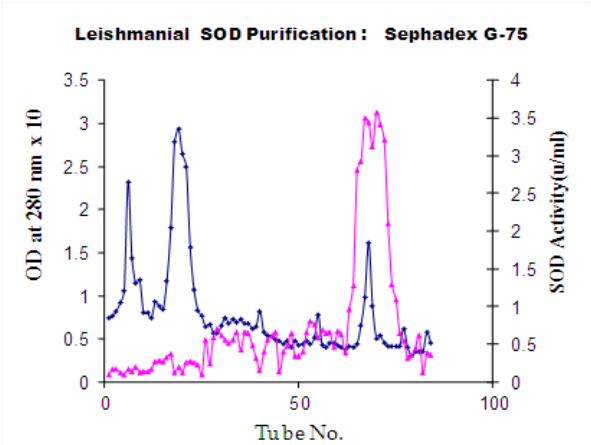

Fig. 2: Gel chromatography on a Sephadex G-75 column; protein concentration (-▲-), superoxide dismutase activity (–●–).

In Ion-exchange chromatography, the 28-44 eluted fraction detected at 280 nm presented an SOD activity and corresponds therefore to the SOD fractions. [Column Volume: 88 ml, Sample charged: 2 ml, Protein content of the sample: 0.47mg/ml, Active Fraction: Tube No. 65 to 72, Flow Rate: 1ml/min, Vol. of each tube: 2 ml].

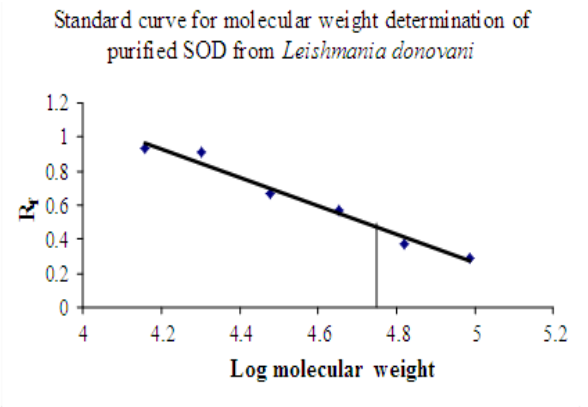

Fig 3: Plot for determination of molecular weight of leishmanial SOD.

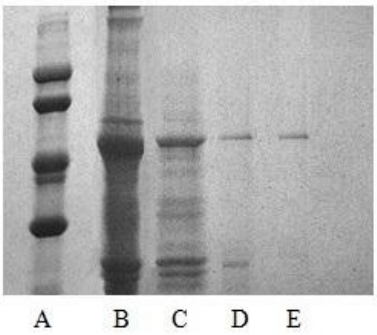

Figure 4: *SDS PAGE : A–Protein markers, B–Leishmania cell-free extract, C–Pellet after ammonium sulfate precipitation at 90%saturation, D–Fractions with SOD activity after ion-exchange chromatography, E-Fractions with SOD activity after gel chromatography*

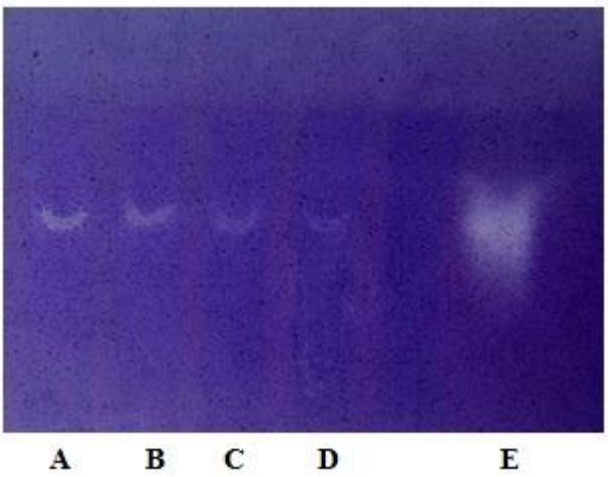

Fig 5: Activity staining of leishmanial SOD after separating by native PAGE in a 10% gel. 60 micrograms of protein was loaded in each lane. A-Leishmania cell-free extract, B-Pellet after ammonium sulfate precipitation at 90%saturation, C-Fractions with SOD activity after ion-exchange chromatography, D-Fractions with SOD activity after gel chromatography, E-Pure Fe SOD.

The SOD was then eluted from a Sephadex G-75 column. Figure 2 presents the results of the gel chromatography on Sephadex G-75 column. The pooled fraction had a volume of 20 ml and was concentrated to a 1.5 ml volume. The SOD fraction showed only one band of SDS-PAGE after gel chromatography. It can be concluded that the SOD was purified to homogeneity after three-step ammonium sulphate precipitation, ion exchange chromatography and gel chromatography. The final sample was applied on SDS-PAGE to determine its molecular weight (Fig 3). The molecular weight of SOD by SDS-PAGE was 37200 dalton. Figure 4 shows the electrophoretic pattern of SOD obtained during different steps of SOD purification. The total purification achieved by the procedure was 105 fold over the first soluble extract, with a yield of 15%. The specific activity of the purified enzyme was 9191 U/mg. Figure 5 shows the activity staining of leishmanial SOD after separating by native PAGE in a 10% gel. Activity staining of native polyacrylamide gels clearly showed the SOD activity obtained during different steps of SOD purification. SOD catalyzes dismutation of toxic superoxide radicals.[17] It is one of the key enzymes of oxygen defence system is known to be an essential factor in mediating normal cellular functions.[18] As a result, the enzyme has been found to be targeted for the treatment of several diseases.[19-21] SOD also plays a vital role during host–parasite interaction.

Its activity is elevated when Leishmania parasite infects host cells.[22] In a recent report, SOD has been demonstrated to be a key enzyme to play a vital role in the survival of intracellular parasites.[23] Importance of this enzyme in the host–parasite interaction was established by generating SOD-deficient *Leishmania donovani*. An earlier report suggested that the enzyme that was present in leishmanial glycosomes isolated by the classical sucrose gradient technique was of the Cu/Zn type.[16] In this present work, SOD was purified from *Leishmania donovani* promastigotes. The total purification achieved by the procedure was 105 fold over the first soluble extract, with a yield of 15%. The specific activity of the purified enzyme was 9191 U/mg. The molecular weight was estimated as 37200 dalton. SOD activity was observed in crude extracts of *Leishmania donovani* cells grown anaerobically. This finding indicates that these enzymes are being constitutively expressed during the growth of this organism in the absence of oxygen. Results elucidated the important roles of Fe-SOD in the cellular stress responses and anti-oxidative processes of the parasite *Leishmania donovani*. Future studies will be necessary to investigate functions of more antioxidant enzymes to gain a better understanding of the antioxidant mechanism in the Leishmania species under various stresses.

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