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Purification and Characterization of Superoxide Dismutase Isolated From Sewage Isolated *E. coli*

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Abstract

Superoxide dismutase (SOD), which plays a very important role in protecting organisms from oxygen toxicity, has therapeutic importance. It was purified from sewage isolated *E. coli* and characterized. Eukaryotic cells also produce SOD but culturing and maintenance of eukaryotic cells for production of SOD is costly as well as difficult. Using prokaryotic cells i.e. bacteria, production cost can be reduced. A rich bacterial source was identified. Bacterial membrane was ruptured in the presence of lysozyme and glass bead. Following ammonium sulphate precipitation, SOD-containing solution was applied to DEAE-cellulose and then Sephadex G- 75 gel columns. SOD was purified 63.91-fold with a specific activity of 3835U/ mg. The molecular weight was estimated to be 35.713 kDa by SDS-PAGE gel. Maximum SOD activity was observed between pH 7.0 to 7.5 at temperature range 37–50°C. This enzyme has fair thermal stability. The enzyme was found to be stable in presence of 1% salt only. The activity found to be gradually reduced approximately 50% at higher concentrations. It was totally inactivated above 9% salt concentration.

Keywords: SOD, purification, characterization, sewage isolate *E. coli*

Introduction

The superoxide radical is an intermediate reduction product of oxygen produced in a variety of biological reactions. The superoxide radical ($O_2 \cdot^-$) and other reactive derivatives in cells have received recent attention as agents of oxygen toxicity. Most organisms produce defense systems, such as metallo-enzymes, to protect themselves from reactive oxygen species. Metallo-enzymes that catalyze the dismutation of superoxide free radicals ($O_2 \cdot^-$) to hydrogen peroxide (H_2O_2) and oxygen (O_2), are known as superoxide dismutases (SODs). SODs play an important role to protect the 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 (·OH), formation of which, via the Fenton reaction, is favored by excess superoxide [1-5]. SODs are divided into three classes on the basis of their active site metals: copper and zinc (Cu/Zn-SOD), manganese (Mn-SOD)/ iron (Fe-SOD) and in some bacteria one more variety is found named as Ni-SOD [6]. CuZn-SOD was found widely in the cytoplasm and certainly in the mitochondrial intermembrane space of the eukaryotic cells and chloroplasts of plants. It is also reported in bacteria like *S. aureus*, *Pseudomonas spp*., *E. coli* etc [7-10] Mn-SOD are located in prokaryotes and in the mitochondria of eukaryotes [11], while Fe-SOD has been found in bacteria, blue-green algae and protozoa [12,13]. Recent reports also indicated that the enzyme was present in higher land plants [2].

Materials and Methods

Materials

Pure /reference SOD (Sigma) Coomassie brilliant blue G, gel filtration and electrophoresis molecular weight marker kits, Sephadex G-75 was purchased from Sigma and DEAE-cellulose from Hi-media. Pure /reference SOD isolated from *E. coli* was purchased from sigma. All other chemical reagents used were of analytical grade. Optical measurements were achieved with a spectrophotometer (UV-1800, Shimadzu, Japan) at 560 nm and 650 nm. Electrophoresis was performed with Edvotek M-36 during experimental studies.

Isolation and purification of superoxide dismutase

Scientist showed the presence of SOD in *S. aureus* [8] and *P. aeruginosa* [9]. It was decided to choose these bacteria for production of SOD. Total 64 samples were used for SOD testing. Urine [14] and pus [15], suspected for *S. aureus* were collected from various pathological laboratories and hospitals (CPR Hospital, Computek laboratory, Micropath lab, Shivtej laboratory etc. in Kolhapur and confirmed by morphological and biochemical testing) 10 non pathological samples were also used . Total 26 samples *Pseudomonas spp* were tested. Urine [12] and pus [6], suspected for *Pseudomonas spp* were collected from various pathological laboratories and hospitals (further confirmed by morphological and biochemical testing). 8 non pathological spp were also used. Among these 8 samples of *S. aureus* and 12 samples of *P. aeruginosa* showed presence of SOD. Sewage samples were also used for analysis. From 6 different sites around Kolhapur, sewage samples were collected. A loopful of each sample was streaked on SRBM (Sulphate reducing bacterial medium) as well as on Nutrient agar using four quadrant method. SRBM incubated anaerobically to obtain sulphur reducing bacteria, while nutrient agar incubated aerobically for 24 hr at 37°C to get aerobic flora. 12 samples of sewage isolate were selected for SOD assay.

Assay for estimation of SOD activity [16]

The method is based on comparison of yellow colored nitroblue tetrazolium to blue colored farmazone by O_2. In this method O_2 is

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generated by addition of riboflavin. Also the reaction is initiated by addition of riboflavin. Illumination of solutions containing riboflavin, methionine, and NBT resulted in a linear accumulation of the blue formazan, whether oxygen was present or not. In the absence of oxygen, superoxide dismutase was without influence whereas, under aerobic conditions, superoxide dismutase inhibited the reduction of NBT. Thus, illumination of reaction mixtures which contained 0.4 ml riboflavin (60 µM), 1.2 ml methionine (130 mM), 0.6 ml NBT (750 µM), and 3.4 ml phosphate buffer (0.2 M pH7.8), 0.3 ml EDTA (10 mM) and 0.1 ml suspension of respective bacteria or enzyme solution caused an increase in absorbance at 560 nm. Control tube was wrapped with black cloth. The assay tubes were exposed to UV light (30 cm length, 2.5 cm diameter) for 30 min. The non-irradiated mixture was served as blank. The cell suspensions were prepared from all test samples having same absorption value at 620 nm. Assay run in triplicate and average of three was considered for results.

Purification of SOD

After SOD assay, it was found that sewage isolates produces more SOD than non sewage isolates as per Table 1. Among them highest producer RJ-2-1 was selected for further work i. e. production and partial purification of SOD. This bacterium was showing characteristics like *E. coli* 4157 confirmed by biochemical tests and 16s r-RNA analysis. Selected bacterium was cultured on Nutrient agar plates and suspended in 0.1 M phosphate buffer (pH 7.5) by scrapping them with the help of sterile lancet. Cells were collected by centrifugation (10000 g, 10 min), then washed twice with 0.1 M phosphate buffer (pH 7.5). The suspension was ground in mortar with glass beads and vortexed for 5 min. in presence of lysozyme. The disrupted product was centrifuged (10000 g, 10 min) and supernatant was re-centrifuged (25000 g, 30 min). Then supernatant was 30% saturated with solid ammonium sulfate and resulting precipitate was discarded. More ammonium sulfate was added to the supernatant to reach 80% saturation. The precipitate was dissolved in 0.1 M phosphate buffer (pH 7.5) and dialyzed against the same buffer overnight. Then content of dialysis bags was centrifuged (25000 g, 20 min). Supernatant containing SOD activity was applied to a DEAE-cellulose column (40×1.6 cm), which was equilibrated with 0.05 M phosphate buffer (pH 7.4) at 400ºC. Elution of the enzyme was achieved by establishing a linear gradient with a 0.06-0.140 M phosphate buffer (pH 7.4) at a flow rate of 0.5 ml/min. Fractions containing SOD activity were loaded onto a Sephadex G-75 (90×1.6 cm) column equilibrated with 0.05 M phosphate buffer (pH 7.4) and eluted with the same buffer at a flow rate of 0.2 ml/min.

	O.D. at 550 nm					
Sr. No	***S. aureus***		***P. aeruginosa***		**Sewage isolate**	
1	Comp-U-15	0.22	SANJ-U-1	0.87	RJ-1-1	0.15
2	Comp-P-4	0.33	SANJ-U-2	0.82	RJ-1-2	0.13
3	Comp-P-15	0.30	MPL-U-6	0.67	RJ-2-1	0.03
4	MPL-U-3	0.19	MPL-U-8	0.48	RJ-2-2	0.05
5	CPR-U-9	0.29	MPL-U-9	0.62	RK-1-2	0.17
6	NP-3	0.42	MPL-U-11	0.48	RK-2-1	0.25
7	NP-7	0.58	MPL-U-13	0.54	RK-2-2	0.16
8	NP-8	0.41	COM-P-1	0.42	SRW-1-2	0.21
9	--	--	COM-U-1	0.72	SRW-1-3	0.07
10	--	--	CPR-U-1	0.66	SRW-2-1	0.12
11	--	--	NP-4	0.49	BP-1-2	0.14
12	--	--	NP-6	0.57	BP-1-4	0.18

Abbreviations used - CPR Hospital –CPR; Sanjivani Hospital – SANJ; Saraswati Hospital – SAR; Computek laboratory- COMP; Micropath laboratory – MPL; Shivtej laboratory – SVT; Rajendra nagar – RJ; Ratnappa Kumbhar nagar – RK; Sarnaubatwadi – SRW; Bapat camp – BP; Urine sample – U; Pus sample- P; Non pathological sample – NP

Table 1: SOD activity/ml of bacterial culture.

Molecular weight determination

The apparent molecular weight of the purified SOD was determined by SDS-PAGE. The molecular weight of purified SOD was estimated using the calibration curve. SDS-polyacrylamide gel electrophoresis was performed according to the Westermeier method using a horizontal slab gel apparatus. The following proteins were used as SDS-PAGE electrophoresis molecular weight determination.

Standards: BSA (66 kDa), Trypsin (MW- 23.3 KD) Pure /reference SOD (MW- 35.713 KD) and isolated SOD were dissolved in 0.0625 M Tris HCl buffer (pH 6.8) containing 5% β-mercaptoethanol and denatured by 4 min incubation in boiling water.

The 40 µl marker proteins were loaded in first, third and fourth well and the sample was loaded in the second well. Electrophoresis was performed according to discontinuous method with 12.50% separating and 2.50% stacking polyacrylamide gels at room temperature at 30 mA for 4 h. Gel was stained with Coomassie brilliant blue R-250 dye reagent overnight.

Determination of type of SOD

Type of SOD was confirmed by performing inhibition assay. In this experiment standard assay was run in presence of specific SOD inhibitors. Blue color of farmazone is formed after activity of $O_2^{\cdot-}$, SOD is dismutating $O_2^{\cdot-}$ so in presence of SOD blue color is not formed but if SOD is inhibited by specific inhibitor blue color i.e. farmazone is not formed

Protein determination

During the purification steps, protein levels were determined spectrophotometrically according to the Folin Lowry method, using bovine serum albumin as the standard. Protein amounts in column fractions were observed via absorbance variations at 650 nm.

The effect of pH on SOD activity

The pH activity profile of SOD was studied at standard assay conditions by using 0.05 M phosphate buffer having pH values between 6.0 and 8.0 and incubated for 2 h at 250ºC. After incubation, the activity of enzyme preparations was measured under standard assay conditions. pH stability being expressed as mean enzyme activity.

Estimation of temperature stability

The optimum temperature for enzyme activity was measured by incubating the enzyme at different temperatures. Enzyme was incubated for 2 h in 0.05 M phosphate buffer (pH 7.4) at temperature values between 15 and 700ºC. After incubation, the activity of enzyme preparations was measured under standard assay conditions. Temperature stability was expressed as mean enzyme activity.

Estimation of NaCl conc. on SOD activity

Reaction mixture was incubated in presence of 2 ml of respective salt concentration. After incubation, the activity of enzyme preparations was measured under standard assay conditions.

Storage stability

The purified SOD enzyme (70 µg/ml) was stored in 0.0 M phosphate buffer (pH 7.4) at 40ºC. No loss of activity was observed over 3 months.

Results

SOD purification

The isolation of SOD from bacterial culture was performed by the disintegration of the membrane by mechanical, enzyme solubilization and freeze-thaw methods. 5 min grinding was applied with mortal and pastel. The disrupted product was centrifuged (10000 g, 10 min). The supernatant was re-centrifuged (25000 g, 30 min) and the activity of SOD in supernatant was measured 60 U/mg. while the specific activity of SOD with cell suspension was found to be 69.61 U/mg. As SOD is membrane bound enzyme, activity may be decreased after the disintegration of the cell membrane.

The first purification step for obtaining SOD from the crude extract was achieved by fractional precipitation of proteins by using ammonium sulphate (30-80%). The best precipitation yield was obtained by adding four volumes of ethanol per volume of sample in the bottom precipitate phase. The precipitate having the highest SOD activity was resuspended in 0.05 M potassium phosphate buffer, pH 7.4. The samples were stirred for 15 min and the insoluble material removed by centrifugation at 4,000 g for 15 min. Activity of SOD at this stage was found 580 U/mg.

Using DEAE cellulose column the unbound proteins were eluted by equilibration buffer. The bound proteins having SOD, were eluted via a linear gradient method by using 0.06-0.140 M phosphate buffers at pH 7.4 having specific activity 1976 U/mg.

Fractions having SOD were applied to a Sephadex G-75 gel chromatography column for further purification. SOD activity was eluted between fractions 9–13. A 63.91-fold purification of the enzyme with a 3835 U/mg specific activity was achieved. The results of the purification procedure are summarized in Table 2.

SDS PAGE analysis

The apparent molecular weight of enzyme was 35.71 kDa by SDS PAGE gel electrophoresis (Figure 1)

Type of SOD

In inhibition assay blue color was formed in presence of H_2O_2. H_2O_2 is Cu/Zn- SOD inhibitor. It indicates that type of isolated purified SOD was Cu/Zn- SOD.

The effect of pH, temperature, salt concentration on SOD activity

The effect of pH on enzyme stability was determined under standard assay conditions after 2 h pre-incubation using 0.05 M phosphate buffer, (pH 6.0-8.0) at 250°C (Table 3). The purified SOD was found to be stable at pH 7.5 under the conditions studied (Figure 2). Activity loss was more than 40% at pH 6 and 8, while 11% and 26% at pH 7 and 6.5 respectively. The thermal stability of the SOD was also investigated between 15-700°C for 2 h incubation period in 0.05 M phosphate buffer of pH 7.4 (Table 4). This SOD showed rather fair thermal stability. Activity loss was only 8% at 500°C but 80% at 700°C after 2 h incubation (Figure 3). The enzyme was found to be stable in presence of 1% salt only (Table 5). The activity found to be gradually reduced approximately 50% at higher concentrations (Figure 4). The purified SOD enzyme (70 µg/ml) was stored in 0.05 M phosphate buffer (pH 7.4) at 40°C. No loss of activity was observed over 3 months.

Purification steps	Total proteins mg/ml	Total activity U	Specific activity (U/mg)	Fold purification	% yield
Crude extract	6024.28	361456	60	1	100
Precipitation	620.42	359843	580	9.66	99.55
DEAE-cellulose chromatography	160.14	316436	1976	32.99	87.54
Sephadex G-75 chromatography	70.86	271748	3835	63.91	75.18

Table 2: Purification steps of SOD from sewage isolate bacteria.

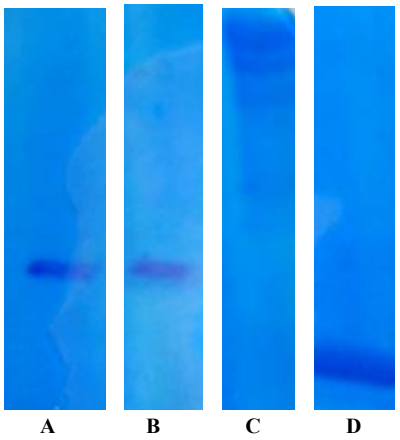

Figure 1: SDS PAGE Analysis. **A:** Pure SOD (MW- 35.713 KD). **B:** Isolated SOD (MW- 35.713 KD). **C:** Trypsin (MW- 23.3 KD). **D:** BSA (MW- 66 KD).

pH	Mean enzyme activity (U/ml)
6	6.738 ± 0.371915
6.5	8.530 ± 0.36680
7	10.30 ± 0.529739
7.5	11.499 ± 0.463334
8	6.634 ± 0.357867

Table 3: The effect of pH on SOD activity.

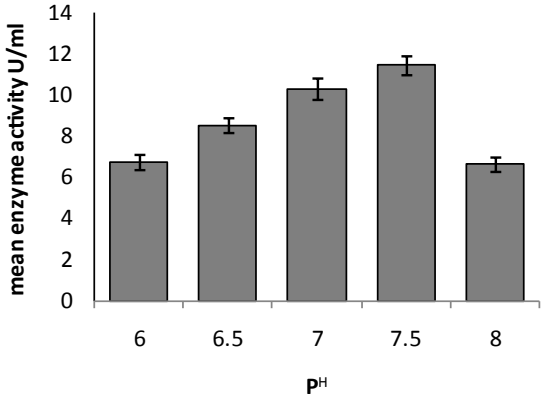

Figure 2: The effect of pH on SOD activity.

Discussion

Sewage isolated bacterium showed maximum amount of SOD in suspension form. Among them *E. coli* was higher producer. SOD purified from sewage isolated *E. coli* has optimum conditions at temperature 370°C and pH 7.5 same as standard/reference SOD

Temperature ºC	Mean enzyme activity (U/ml)
15	5.789 ± 0.492239
25	7.56 ± 0.62038
37	12.76 ± 0.579137
50	11.08 ± 0.522814
70	2.522 ± 0.534557

Table 4: The effect of temperature on SOD activity.

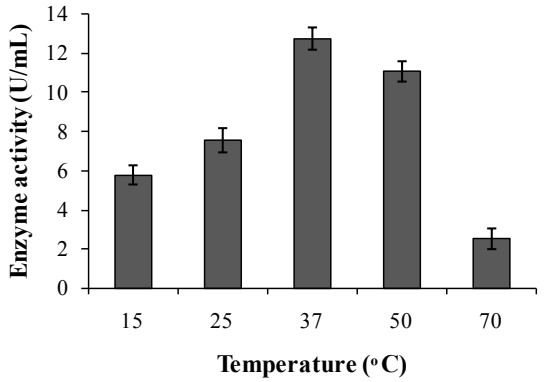

Figure 3: The effect of Temperature on SOD activity.

Salt concentration (%)	Mean enzyme activity (U/ml)
1	5.48 ± 0.22
3	2.25 ± 0.34
5	1.08 ± 0.28
7	0.39 ± 0.18
9	0

Table 5: The effect of salt concentration on SOD activity.

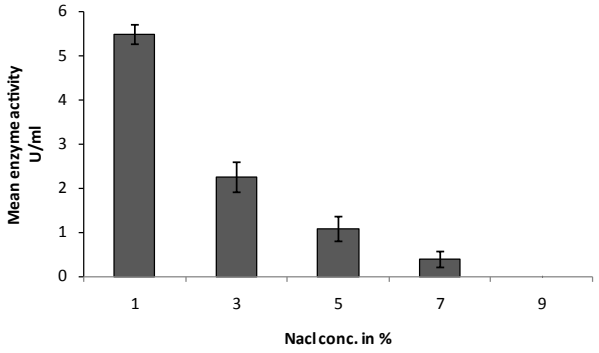

Figure 4: The effect of NaCl concentration (%) on SOD activity.

(Sigma). But specific activity found to be different, isolated enzyme has 3835U/mg specific activity at 63.91-fold purification while standard/reference SOD has 4285 U/mg specific activity. Our enzyme has 28% more specific activity than standard/reference SOD. In *E. coli* 474B5, 17 U/mg of SOD is reported [17] and in *E. coli* LE392, 12 U/mg of SOD [18] is reported in suspension form while our bacterium was showing presence of 69.61 U/mg of SOD in suspension form. Molecular wt. of Mn SOD from *Escherichia coli B* was reported 39000 kD [19].While Mn SOD isolated from K12 strain have molecular wt 24 kD and pH 6.88 [20]. SOD isolated from Aspergillus species showed (3,294-3,531 U/mg) activity [21]. Superoxide dismutase purified from the photoautotrophic cyanobacterium *Gloeocapsa spp*. has a molar mass of 38 kDa, as estimated by gel filtration [6]. SOD separated from *Thermothrix spp* isolated from thermal spa water in Serbia, showed high superoxide dismutase activity. The enzyme molecular weight determined by gel chromatography is 37 kD. While the optimum pH for enzyme activity was in the range of 8 to 10. The optimum temperature for SOD activity was 600ºC [22]. SOD purified and partially characterized from chicken erythrocytes. The apparent molecular weight of the chicken erythrocyte SOD was found to be 30.6 kDa by gel filtration on a Sephadex G-75 column and 15 kDa by SDS slab gel electrophoresis. Maximum activity was observed between pH 7.0 to 9.0 at 250ºC [23]. SOD of Neurosporo crassa [24], and yeast [14,25] had a molecular weight of about 32 kDa. Generally, Cu- Zn SODs are stable at neutral pH [11,14] The pH dependence of the Fe-SOD stability of *Aerobacter aerogenesis*, remained comparatively stable at alkaline pH 7.0-11.0, but was rapidly inactivated below pH 7.0 [26]. The activity of Cu-Zn SODs is dependent on ionic strength and alkaline pH in a way that typically reflects the functional role of charged amino acid residues, in particular lysine [20,27]. At neutral pH, however, the radical exists mainly as $O_2^{\cdot -}$, and the dismutation reaction is relatively slow due to electrostatic repulsion of negatively charged radicals [28]. In similar conditions, the thermal, pH and storage stability of our SOD is better than that of Cu-Zn SOD purified from the venom of the H. fulvipes scorpion [29], *Aerobacter aerogenesis* [26] and cabbage [30].

Acknowledgments

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Conflict of Interest Disclosure

There are no any potential sources of conflict of interest.

References

1. Lumsden J, Cammack R, Hall DO (1976) Purification and physicochemical properties of superoxide dismutase from two photosynthetic microorganisms. Biochim Biophys Acta 438: 380–392.
2. Giannopolitis CN, Ries SK (1977) Superoxide dismutases: I. Occurrence in higher plants. Plant Physiol. 59: 309–314.
3. Fridovich IA (1978) The biology of oxygen radicals. Science 201: 875-880.
4. Kanematsu S, Asada K (1979) Ferric and manganic superoxide dismutases in *Euglena gracilis*. Arch Biochem Biophys 195: 535–545.
5. Baum JA, Chandlee JM, Scandalions JG (1983) Purification and Partial Characterization of a Genetically-Defined Superoxide Dismutase (SOD-1) Associated with Maize Chloroplasts. Plant Physiology 73: 31-35.
6. Hammouda O (1999) Purification and identification of the type of superoxide dismutase from *Goeocapsa sp*. Folia Microbiologica 44: 32-36.
7. Carlioz A, Touati D (1986) Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life? EMBO Journal 5: 623-630.
8. Clements MO, Watson SP, Foster SJ (1999) Characterization of the Major Superoxide Dismutase of Staphylococcus aureus and Its Role in Starvation Survival, Stress Resistance, and Pathogenicity. J. Bacteriology 181: 3898-3903.
9. Britigan BE, Miller R A, Hassett DJ, Pfaller MA, McCormick M L, Rasmussen GT (2001) Antioxidant enzyme expression in clinical isolates of *Pseudomonas aeruginosa*: identification of an atypical form of manganese superoxide dismutase. Infection and Immunology 69: 7396–7401.
10. Kluytmans J, Van Belkum A, Verbrugh H (1997) *Staphylococcus aureus*: epidemiology and underlying mechanisms, and associated risks". Clinical Microbiology 10: 505–520.
11. Fridovich I (1974) Superoxide dismutases. Adv. Enzymol 41: 35-97.
12. McCord JM, Fridovich I (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem 244: 6049–6055.

13. Gregory EM, Fridovich I (1973) Oxygen toxicity and the superoxide dismutase. J Bacteriol. 114: 1193–1197.

14. Beauchamp CO, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem 44: 276-287.

15. Jackson C, Dench J, Moore AL, Halliwell B, Foyer CH, Hall DO (1978) Subcellular localisation and identification of superoxide dismutase in the leaves of higher plants. Eur J Biochem. 91: 339–344.

16. Scott MD, Meshnickq SR, Eaton JW (1987) Superoxide Dismutase-rich Bacteria paradoxical increase in oxidant toxicity. J Biol Chem 262: 3640-3645.

17. Keele BB, Jr, McCord JM, Fridovich I (1970) Superoxide dismutase from Escherichia coli B. A new manganese-containing enzyme. J. Biol. Chem 245: 6176–6181.

18. www.uniprot.org/uniprot/P00448

19. Henry LE, Cammack R, Schwitzguebel JP, Palmer JM, Hall DO (1980) Intracellular localization, isolation and characterization of two distinct varieties of superoxide dismutase from Neurospora crassa. Biochem J. 187: 321–328.

20. Svetlana E, Ljubinkagligi, Eljkaradulovi, Ratko MJ (2004) Purification and partial characterization of superoxide dismutase from the thermophilic bacteria *Thermothrix sp*. J.Serb.Chem.Soc 69: 9–16

21. Aydemir T, Tarhan T L (2001) Purification and Partial Characterisation of Superoxide Dismutase from Chicken Erythrocytes Turk J Chem 25: 451 – 459.

22. Rigo A,Viglino P, Radilio G, Tomat R (1975) FEBS Lett 50: 86-88.

23. Goscin SA, Fridovich I (1972) The purification and properties of superoxide dismutase from *Saccharomyces cerevisiae*. Biochim Biophys Acta. 289: 276–283.

24. Weser U, Fretzdorff A, Prinz R (1972) Superoxide dismutase in baker's yeast. FEBS Lett 27: 267-269

25. Kim SW, Lee SW, Lee TH (1991) Purification and characterization of superoxide dismutase from *Aerobacter aerogenes*. Agric. Biol. Chem 55: 101-108.

26. Salin ML, Bridges SM (1981) Absence of the iron-containing superoxide dismutase in mitochondria from mustard (*Brassica campestris*). Biochem J 195: 229-33.

27. Emanuele Argese, Adelio Rigo, Paolo Viglino, Emilio Orsega, Franco Marmocchi, et al. (1984) A study of the pH dependence of the activity of porcine Cu, Zn superoxide dismutase. Biochimica et Biophysica Acta 787: 205-207.

28. Ramanaiah M, Venkaiah B (1992) Characterization of superoxide dismutase from south Indian scorpion venom. Biochem Int 26: 113-123.

29. Walker JL, McLellan KM, Robinson DS (1991) Isolation and purification of superoxide dismutase from Brussels sprouts. Food Chemistry 41: 1-10.

30. Asada K, Urano M, Takahashi M (1973) Subcellular Location of Superoxide Dismutase in Spinach Leaves and Preparation and Properties of Crystalline Spinach Superoxide Dismutase. Eur J Biochem 36: 257-266.