Turk J Chem
24 (2000) , 109 – 116.

Some Properties of Cu, Zn-Superoxide Dismutase from Sheep Erythrocyte

Leman TARHAN
Department of Chemistry, Faculty of Arts and Science,
Dokuz Eylül University 35150 Buca, Izmir-TURKEY
M. Nalan TÜZMEN
Department of Chemistry, Faculty of Arts and Science,
Süleyman Demirel University Isparta-TURKEY

Received 15.05.1998

Superoxide dismutase (SOD) was isolated from sheep erythrocyte. SOD activity was measured under the optimized assay conditions by observing the variations of autoxidation rate of 6-hydroxydopamine (6-OHDA). The enzyme was characterized as containing copper and zinc and was insensitive to chloroform-ethanol mixture but inhibited by cyanide and hydrogen peroxide.

The activity variations and stability properties of sheep erythrocyte Cu, Zn-SOD were investigated under the optimized activity assay conditions by observing inhibition change at the autoxidation rate of 6-OHDA. The optimum pH and temperature of sheep erythrocyte Cu, Zn-SOD were found to be 9.4 and 30°C respectively. The enzyme showed high pH- and thermal-stability properties around neutral pH and up to 37°C after 2.5 h incubation. Variations in the inhibition percentage of autoxidation were investigated in 0.2-0.9 mM range of 6-OHDA. The same procedures were repeated by adding catalase also.
Key Words: Superoxide radical, Cu, Zn-superoxide dismutase, 6-hydroxydopamine, dismutation

Introduction

Superoxide dismutases (SODs) are oxidoreductase enzymes (EC 1.15.1.1) catalzing the disproportionation of highly reactive superoxide anion radicals ($O_2^{\cdot-}$) in living cells to molecular oxygen and hydrogen peroxide[1−3]. The $O_2^{\cdot-}$ is an intermediate reduction product of oxygen produced by a variety of biological reactions. The major process for production of reactive oxygen species under physiological conditions include the following: electron transport chain of mitochondria, lipid peroxidation, radiation, metabolism of quinones by redox-recycling and reactions of enzymes xanthine oxidase, amino oxidase, cytochrome P-450 and prostaglandine synthase[4−6]. Superoxide radicals have been shown to cause damage to nearby cells by peroxidation of membranes, proteins and DNA and also by inhibition of some critical enzyme activities. Thus, it is very important to have SOD activity which can remove the toxic radicals.

Based on their metal content and on sequence similarities the superoxide dismutases are divided into three groups: the Cu, Zn-SOD found in the cytosol of eukaryotes, in chloroplasts and also in some bacteria,

the Mn-SOD occurring in prokaryotes and in the mitochondrial matrix and Fe-SOD found in prokaryotes and some plant families[7−10].

Quite a few methods such as pyrogallol, cytochrome c, 6-hydroxydopamine and nitro blue tetrazolium regarding the determination of the activity of superoxide dismutases were developed[11−15]. However, while studying the determination of kinetic parameters of the enzymes, some difficulties were encountered[16]. In this study the determination of some kinetic parameters of superoxide dismutase from sheep erythrocyte was accomplished by exploiting the autoxidation of 6-hydroxydopamine to form $O_2^{\cdot-}$ and its conversion into quinone derivatives through semiquinone radicals under modified and optimized assay conditions.

Experimental

Chemicals: 6-Hydroxydopamine (6-OHDA; 2, 4, 5 trihydroxyphenylethylamine), catalase (CAT; E. C.1.11.1.6 from *Aspergillus niger*), H_2O_2, pyrogallol, chloroform, ethanol, ethylenediamine tetraacetic acid (EDTA) were supplied from Sigma, St. Louis USA. All chemicals used were analytical grade.

SOD Activity Assay

The activity of SOD was measured by a modification of the method of Heikkila and Cabbat. The method is based on the mesurement of the inhibitory effect of SOD on the spontaneous autoxidation of 6-hydroxydopamine (6-OHDA)[17].

Stock solution of 6-OHDA was prepared daily in 1 mM KC1, at pH 2.0. The soluble oxygen was removed from the stock solution by passing pure N_2 through pyrogallate solution. During the experiments stock solution was reserved in N_2 media at +4°C. Autoxidation rate of 6-OHDA (0,4mM) in 0.1 M phosphate buffer pH 7.4 which was saturated by air O_2 (8.2 mg/l) at 20°C was determined by observing absorbance changes depending on time at 490nm and 20°C. 1 IU of superoxide dismutase activity is the amount of superoxide dismutase required for 50 % inhibition of the initial rate of 6-hydroxydopamine autoxidation.

Catalase Activity Assay

The activity was calculated by measuring the period of the time in seconds necessary for a decrease in optical density of a 10.5 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0 from 0.450 to 0.400 at 240 nm and 25°C [18]. 1 IU is the enzyme activity which decomposes 1 μmol H_2O_2 per minute at 25°C under strictly defined conditions, especially with regard to the H_2O_2 concentration.

Preparation of the Crude Enzyme Extract from Sheep erythrocytes

The blood samples were supplied from Tansaş-İzmir. 25 ml of venous sheep blood (9.26 Red Blood Cells $\times 10^{12/L}$) was collected in a tube containing 2.5 m1 of EDTA (185 mM) and then centrifuged at 2000 rpm for 10 e min. In equal volume of 0.9 % NaCl (w/v) was added to the rest after removing the plasma phase at 4°C. The mixture was turned upside down and then centrifuged at 2000 rpm for 5 min. This procedure was repeated three times. Stock hemolysate was obtained by fivefold dilution of packed erythrocytes with bidistilled water and kept at 4°C. Volumetric activity of SOD and CAT in sheep erythrocytes were determined to be 145 IU/ml min and 1200 IU/ml min respectively. Hemoglobin was precipitated from hemolysate by

addition of an equal volume of chloroform/ethanol (1/5, v/v) pre- cooled in the deep-freeze. No activity variations were observed after removing the hemoglobin.

Results and Discussion

Superoxide dismutase from sheep erythrocyte was not susceptible to denaturation by chloroform-ethanol. Hemoglobin was removed from hemolysate with full activity yield. According to the reports, Cu, Zn-SOD and Fe-SOD are resistant to organic solvent[19].

Complete loss of SOD activity of hemoglobin free hemolysate was obtained by adding 3 mM KCN to the assay medium. Cu,Zn-SOD can be distinguished from the Mn-SOD and Fe-SOD by the inhibition of only Cu,Zn-SOD in the presence of mM levels of cyanide[20,21]. It can be said that sheep erythrocyte SOD is a Cu,Zn metallo-enzyme.

Effect of H_2O_2 Concentration on the Cu,Zn-SOD Activity

The effect of various H_2O_2 concentrations on the residual activity of sheep erythrocyte Cu,Zn-SOD was investigated in 0.5-2.0 mM concentration range under standard activity assay conditions. Two mM azide was added into the reaction mixture in order to inhibit the catalase activity (Figure 1).

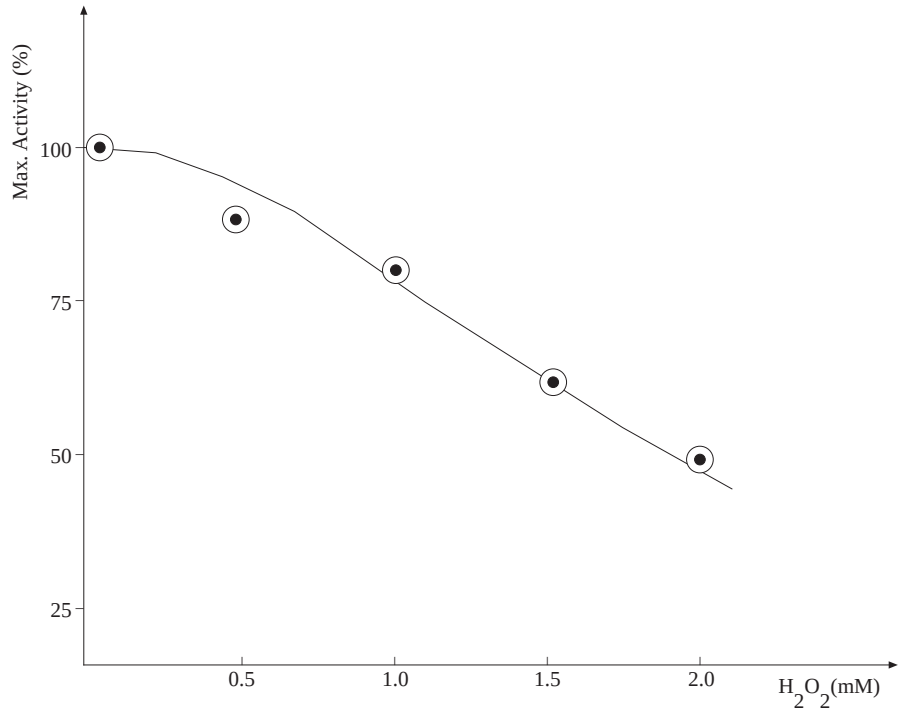

Figure 1. The inhibition effect of H_2O_2 on sheep erythrocyte Cu,Zn-SOD in 0.1 M phosphate buffer pH 7.4 and at 20°C.

Increasing inhibition of Cu,Zn-SOD activity was observed by increasing H_2O_2 concentration. Activity decrease against H_2O_2 concentration was almost linear within the concentration range studied. The inactivation of the Cu,Zn-SOD has been attributed to the reduction of the enzyme-bound Cu^{+2} to Cu^{+1} by H_2O_2, followed by a Fenton's type reaction of the Cu^{+1} with additional H_2O_2 to form Cu^{+2}-OH. The modification

of tryptophan, histidine and cystein residues has also been reported previously for the possible explanation of the inactivation of Cu,Zn-SOD and Fe-SOD by H_2O_2 treatment[22−25].

pH and Temperature Activity Profiles of Cu,Zn-SOD Activity

pH-activity profile of sheep erythrocyte Cu,Zn-SOD was investigated in the range of 7.0 to 10.0 at 20°C. At pH values 7.0-8.0, 0.1 M phosphate buffer and at pH values 8.0-10.0, 0.1 M borate buffer were used. The pH activity profile of enzyme was estimated at the varying concentration of 6-OHDA that provided the same initial absorbance value at 90 sec.

Optimum pH of sheep erythrocyte Cu,Zn-SOD was found to be 9.4, similar to bovine erythrocyte Cu,Zn-SOD (Figure 2)[26]. This value has been reported as 10.2 for Ascaris suum Cu,Zn-SOD[27].

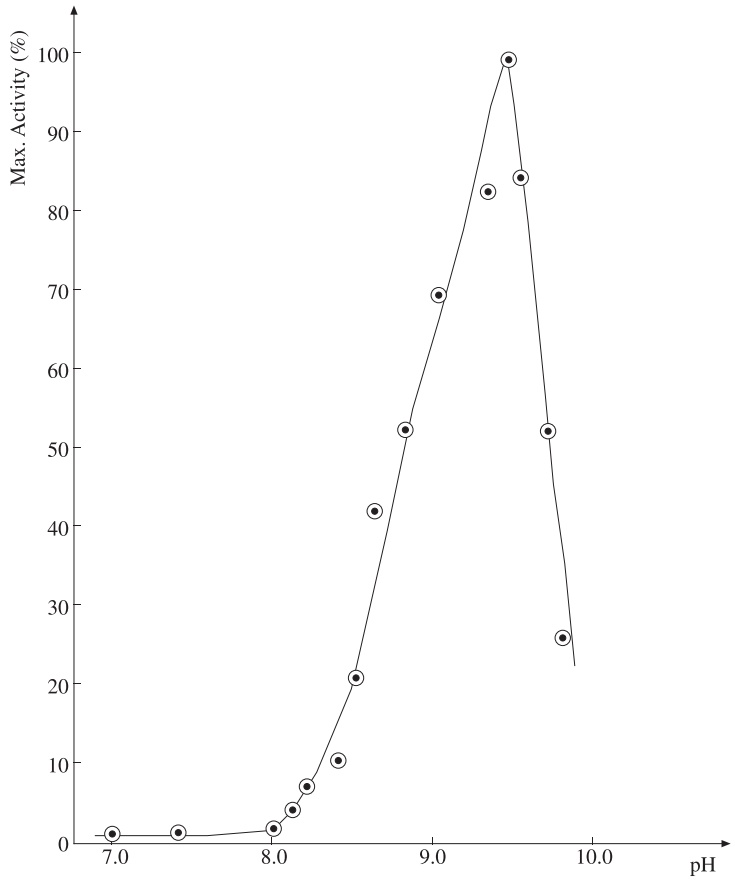

Figure 2. The pH-activity profile for sheep erythrocyte Cu,Zn-SOD at $[0_2] = 8.2$ mg/L and 20°C.

As can be seen from Figure 2, the catalytic activity of sheep erythrocyte Cu,Zn-SOD was very low and pH independent in the pH range 7.0 to 8.0. At pH values higher than 9.4 a sharp decrease was observed in the rate of dismutation reaction with O_2^-. The possible reason of this decrease may be the loss of positive charges on the surface and in the active center of SOD when pKa values of amino acids such as lysine and arginine are considered[27,28]. The existence of the positively charged resudies on the surface, in combination with the electrostatic repulsive effect of the negatively charged residues, serves the activity to guide the anionic substrate O_2^- radicals to the active site of the enzyme.

Temperature-activity profile of enzyme was investigated in the range of 20 to 40°C in 0.1 M phosphate buffer at pH 7.4 and constant O_2 concentration (8.2 mg/l). The solubility of oxygen at different temperatures was controlled by O_2-meter by passing pure O_2 or N_2 through the buffer solution before adding 6-OHDA.

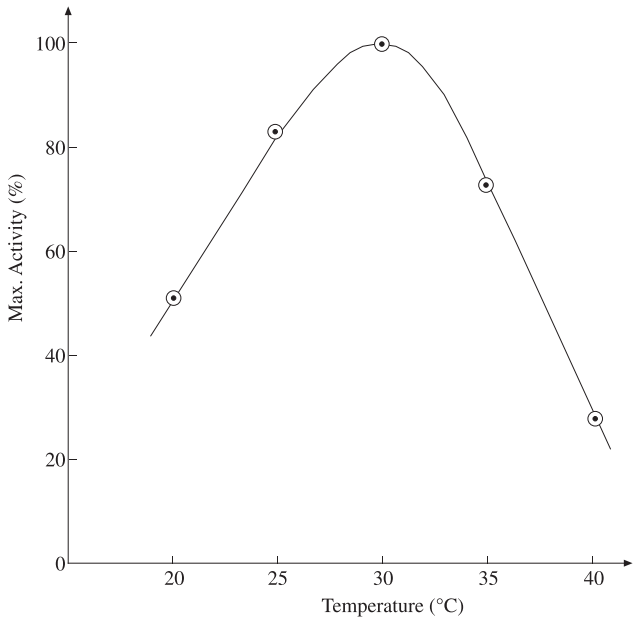

Figure 3. Temperature activity profile for sheep erythrocyte in 0.1 M phosphate buffer pH 7.4 and at $[O_2] = 8.2$ mg/l.

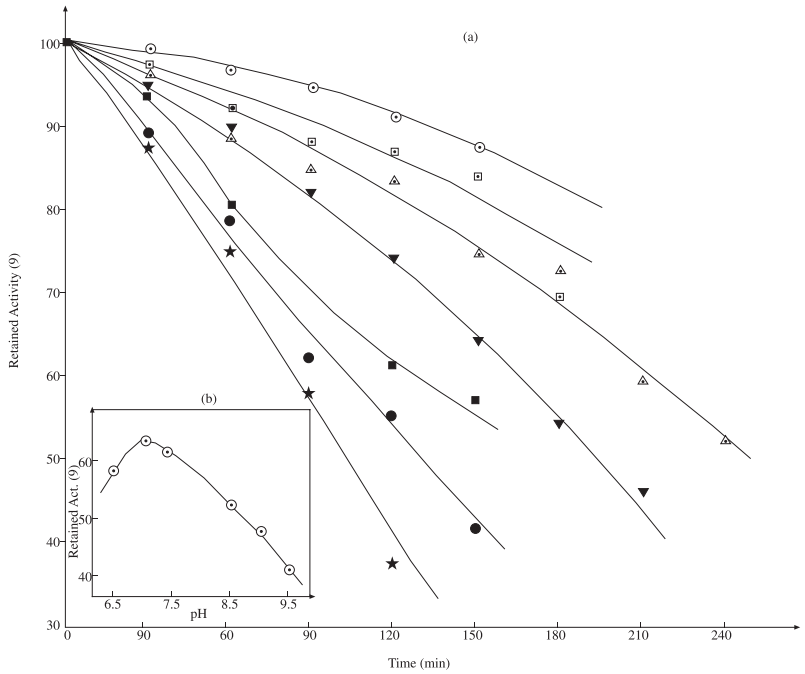

Figure 4. Time dependent pH-stability variations of sheep erythrocyte Cu,ZnSOD incubated at 30°C: ◬pH 6.5, ⊙ pH 7.0, ⊡ pH 7.4, ▼ pH 8.5, ■ pH 9.0, • pH 9.5 ∗ pH 10.0 (a) pH-dependent stability incubated at 30°C for 2.5 h (b).

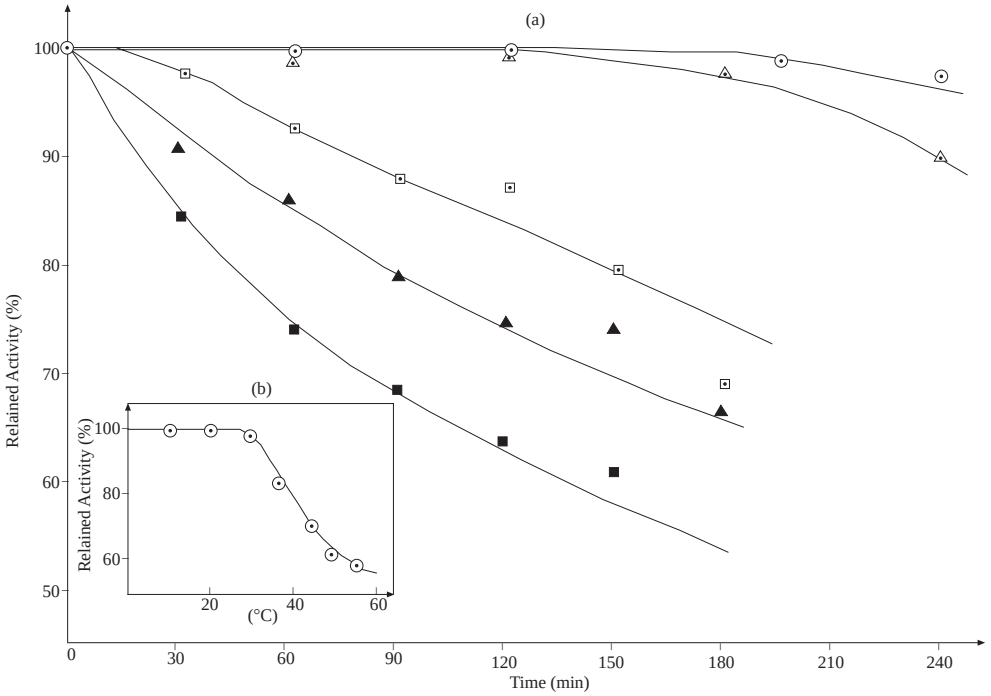

Figure 5. Time dependent thermal-stability variations of sheep erythrocyte Cu,ZnSOD incubated in 0.1 M phosphate buffer pH 7.4: ⊙ 20°C, △30°C, ⊡ 37°C, ▼ 45°C, ■ 55°C (a) and temperature-dependent stability incubated in 0.1 M phosphate buffer pH 7.4 for 2.5 h (b) ⊡.

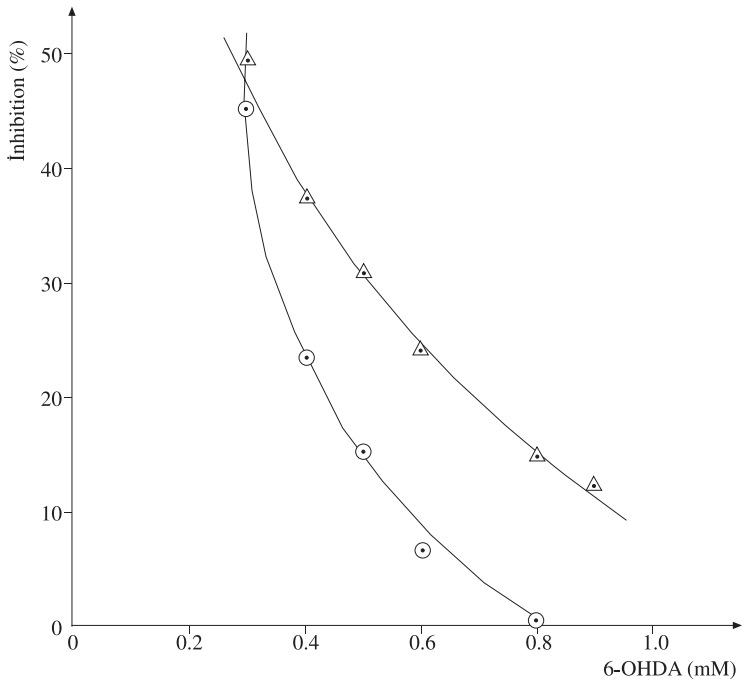

Figure 6. Concentration dependent inhibition variations of 6-OHDA autoxidation: ⊙ sheep erythrocyte Cu,Zn-SOD and △Cu,Zn-SOD+CAT

The Effect of Cu,Zn-SOD Inhibition on the 6-OHDA Autoxidation

The inhibitory effect of Cu,Zn-SOD on the autoxidation of the different concentrations of 6-OHDA in the range of 0.2-0.9 mM were investigated at the constant concentration of crude enzyme extract (95 μl) in standard assay medium. The inhibition measurements were repeated by adding 10 μl catalase from *Aspergillus niger* (2500 IU/ml) to each reaction medium (Figure 6).

As can be seen from Figure 6, the decreasing percent of inhibition caused by Cu,Zn-SOD was observed at the increasing concentration of 6-OHDA. Addition of catalase to the reaction medium increased the inhibitory effect of Cu,Zn-SOD in the autoxidation of 6-OHDA.

The increase of the inhibition percentage was the result of the reduction of Fe^{+3}-catalase to Fe^{+2}-catalse by reacting with $O_2^{\cdot-}$ and the transformation into the inactive compound-III (Figure 6). The experiments performed by increasing SOD concentration suggest that the efficiency of this conversion, of which the mechanism is explained by Fenton reactions, decreased due to the competition with catalase [30−31].

Acknowledgement

We are grateful to Ass. Arzu Bozkaya and Raziye Öztürk for skilled technical assistance and to Dokuz Eylül university (Grant No: 0922.96.01.23) for financial support.

References

1. D. E. Edmondson, D. R. Groeseneken, R. A. Lontie, R. E. Lynch, F. Müller and G. Tollin, **"Radicals in Biochemistry"** 1st ed. Springer Verlag, (1983).
2. J. Fehér, G. Csomós, A. Vereckei, **"Free Radical Reactions in Medicine"** 1st ed Springer Verlag, 1987.
3. L. Packer, **"Oxygen Radicals in Biological Systems",** in **Methods in Enzymology**, Vol.233, Part C, eds. J. N. Abelson, M. I. Simon, Academic Press, (1994).
4. H. Sies, **"Oxidative Stress"**, 1st ed. Academic Press, London, (1985).
5. H. Sies, **Am. J. Med., 91:** 3C-31S (1991).
6. A. Blast, R. M. M. Guido, M. M. Haenen, J. A., Cees and J. A. Doelman, **Am. J. Med. 91:** 3C-2S (1991).
7. H. M. Steinman, in W. Oberley (Ed.), **"Superoxide Dismutases: Protein Chemistry and Structure Function Relationships"**, in **Superoxide Dismutase,** Vol. 1, pp. 11-69, CRC Press, Boca Raton, FL., (1982).
8. J. K. Donnelly, K. M. McLellan, J. L. Walker and D. S. Robinson, **Food Chem. 33** 243-270 (1989).
9. Y. M. M. Bettaiello, J. Cocoreca-Crespo, P. L. Ho, A. Carvalho and I. Raw, **Biochem. and Mol. Biol. Int., 30**, 1, 45-51 (1993).
10. Z. Hong, D. J. Kosman, A. Thakur, D. Rekosh and P. T. LoVerde, **Inf. Imm. 60, 9,**3641-3655 (1992).
11. T. Bilinski, J. Litwinska, M. Blaszezynski et. al. **Biochim. Biophys. Acta 1001** 102 (1989).
12. P. Caliceti, O. Schiavon, A. Mocali, and F. M. Veronese, **Farmaco, 44** 711 (1989).
13. I. Fridovich, **Arch. Biochem. and Biophys., 247** 1 (1986).

14. R. Heikkila and G. Cohen, **Sci., 172** 1257 (1971).
15. D. Mascone, **Anal. Chim. Acta, 211** 195 (1988).
16. M. D. Holdom, R. J. Hay and A. J. Hamilton, **Infect. Immun., 64,** 3326-3334 (1996).
17. H. E. Aebi, **"Catalase"** in **Methods of Enzymatic Analysis,** 3rd ed., Vol.3, ed. H? U. Bergmeyer, pp 273-286, V. C. H. (1987).
18. J. V. Bannister and W. H. Bannister, **"Isolation and characterization of Superoxide Dismutase"** in **Methods of Enzymology** Vol. 105, 88-93 (1984).
19. S. Kanematsu and K. asada, **Arch. Biochem. Biophys., 1856** 473-482 (1978).
20. R. A. Weisinger and I. Fridovich, **J. Biol. Chem., 248,**3582-92 (1973).
21. F. Yamakura, D. Ohmori and K. Suzuki, in **Superoxide Dismutase and Superoxide Dismutase in Chemistry, Biology and Medicine** G. Rotilio (Ed.), pp. 121-214, Elsevier Sci. Pub. B. V., 1986.
22. D. M., Blech, Jr. C. L. Borders, **Arch. Biochem. Biophys., 224,**579-586 (1983).
23. F. Yamakura, **Biochem. Biophys. Res. Commun. 112,**635-641 (1984).
24. E. K., Hodgson, I. Fridovich, **Biochemistr y 24,** 5294-5298 (1975).
25. N. Tüzmen and L. Tarhan, **Chim. Acta Turc.,** in press (1998).
26. Sanchez-Moreno, M. Garcia-Ruiz, A. Sancez-Naves and M. Monteoliva, **Comp. Biochem. Physiol. 92B,** 4, 737-740 (1989).
27. A. Cudd and I. Fridovich, **J. Biol. Chem. 257,** 11443-11452 (1982).
28. J. L. Walker, K. M. McLellan and D. S. Robinson, **Food Chem. 23,**245-256 (1987).
29. N. Shimuzu, K. Kobayashi and K. Hayashi, **J. Biol. Chem., 259,** 7, 4414-(1984).
30. Y. Kono and I. Fridovich, **J. Biol. Chem., 257,** 10, 5751-(1982).