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Partial Purification and Characterization of Superoxide Dismutase from Tomato (*Lycopersicon esculentum*) Fruit

Sunil Kumar, Santosh Dhillon[1†], Dharam Singh and Randhir Singh

Department of Biochemistry, College of Basic Sciences and Humanities,
[1]Department of Biotechnology and Molecular Biology, College of Basic Sciences and Humanities CCS Haryana Agricultural University, Hisar-125004, India

Abstract

Superoxide dismutase (SOD) from tomato (*Lycopersicon esculentum* Mill.) fruit was purified by ammonium sulphate precipitation, Sephadex G-100 and DEAE-cellulose column chromatographies. A 22 fold purification and an overall yield of 44% were achieved. The purified enzyme was a homodimer with Mr 37.1 kDa and subunit Mr 18.2 kDa as judged by SDS-PAGE. SOD showed K_m values of 25×10^{-6} M and 1.7×10^{-6} M for nitroblue tetrazolium (NBT) and riboflavin as substrates, respectively. The enzyme was thermostable upto 50°C and exhibited pH optima of 7.8. The effect of metal ions and some other compounds on enzyme activity was studied. Co^{2+} and Mg^{2+} were found to enhance relative enzyme activities by 27% and 73%, respectively, while Mn^{2+} inhibited the SOD activity by 64%. However, Ca^{2+} and Cu^{2+} had no effect on enzyme activity. Other compounds like H_2O_2 and NaN_3 inhibited enzymatic activities by 60% and 32%, respectively, while sodium dodecyl sulphate (SDS), chloroform plus ethanol and β-mercaptoethanol had no effect on the activity of SOD.

Key words: inhibitors, nitroblue tetrazolium, purification, superoxide dismutase, thermostability, tomato fruit

INTRODUCTION

In the plant system, reactive oxygen species (ROS) such as superoxide radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\cdot OH$) are generally formed due to leakage of electrons into molecular oxygen from the electron transport activities of chloroplasts, mitochondria and plasma membranes (1,2). In addition, ROS production has been found to be stimulated by various environmental stresses (3,4) as well as by some biotic factors such as invasion of various pathogens (5). The ROS are highly toxic to cells as they cause lipid peroxidation of biomembranes, mutations, protein denaturation and enzyme inactivation (6). However, antioxidants such as ascorbate and glutathione and antioxidative enzymes namely superoxide dismutase, catalase, ascorbate peroxidase etc. are present in plants to scavenge ROS and give protection against oxidative stress (7).

Of these antioxidative enzymes, SOD ($\cdot O_2^-:\cdot O_2^-$ oxidoreductase EC 1.15.1.1) is of immense physiological importance as it catalyses the dismutation of superoxide radical to molecular oxygen and H_2O_2, which is the first toxic product generated during oxidative stress (8) and should be decomposed to prevent generation of other harmful ROS.

SOD has been purified and characterized from a number of organisms such as plants (9-11), animals (12) and microorganisms (13-15). It is a metalloenzyme and, based on the metal ion present as the prosthetic group, three different forms have been characterized: CuZn-SOD, Mn-SOD and Fe-SOD. CuZn-SOD is sensitive to cyanide and H_2O_2 but not to chloroform plus ethanol. Mn-SOD is denatured by chloroform plus ethanol, whereas Fe-SOD is insensitive to chloroform plus ethanol as well as cyanide but inactivated by H_2O_2 (16,17).

Tomato is a major fruit crop of world wide economic importance. In tomato, high light intensity is known to cause extensive damage to fruits by ROS, called 'sunscald'. This stress is known to be lessened by SOD (18, 19). In the present study, we describe the partial purification of SOD from tomato fruit and its characterization for kinetic and regulatory properties.

MATERIALS AND METHODS

Plant material and chemicals

Tomato (*Lycopersicon esculentum* Mill.) fruits harvested at yellowish orange stage were purchased from a local market and stored at -70°C until used. All chemicals used during present investigation were of analytical

[†]Corresponding author. E-mail: santosh_dhillon@hau.ernet.in
Phone: +91-1662-232386, Fax: +91-1662-284334

grade and obtained from Sigma Chemical Company, St. Louis, MD., USA (DEAE-cellulose, Sephadex G-100, PVP); SISCO Research Laboratories Pvt. Ltd., Bombay (Riboflavin, L-methionine, Nitroblue tetrazolium, EDTA); E. Merck (β-mercaptoethanol) and HiMedia Laboratories Ltd., Bombay (Tris-HCl, mono and dibasic phosphates).

Enzyme extraction

One gram of fresh tissue (tomato pericarp) was homogenized with 5 mL of cold 0.1 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 2% polyvinyl pyrollidone and 10 mM β-mercaptoethanol in a pre-chilled pestle and mortar using acid washed sand as an abrasive. The homogenate was filtered through four layers of cheesecloth and the filtrate centrifuged at 10,000 × g for 20 min in a refrigerated centrifuge at 4°C. The supernatant obtained was used as crude enzyme solution.

Assay for SOD activity

The enzyme activity was estimated by its ability to inhibit the photochemical reduction of nitroblue tetrazolium using the method of Beauchamp and Fridovich (20). The reaction mixture (3.0 mL) contained 50 mM Tris-HCl (pH 7.8), 14 mM L-methionine, 120 μM NBT, 3 μM riboflavin, 0.1 mM EDTA and 0.1 mL of enzyme extract. Riboflavin was added at the end. The tubes were properly shaken and placed 30 cm below light source consisting of three 20 W-fluorescent lamps (Phillips, India). The reaction was started by switching on the light and terminated after 45 min of incubation by switching off the light. After terminating the reaction, the tubes were covered with black cloth to protect them from light. A non-irradiated reaction mixture that did not develop colour served as the control. The reaction mixture developed maximum colour without enzyme extract and its absorbance decreased with the addition of enzyme. The absorbance was recorded at A_{560}. Per cent inhibition was calculated by the following formula of Asada et al. (21):

$$\text{Percent inhibition} = \frac{V - v}{V} \times 100$$

V = Absorbance at A_{560nm} in absence of SOD.
v = Absorbance at A_{560nm} in presence of SOD.

For the purpose of kinetic and regulatory studies, the enzyme activity was expressed according to the method given by Giannopolitis and Ries (22).

$$\text{SOD units mL}^{-1} = \frac{V - v}{v} \times \text{Dilution factor}$$

Purification of SOD

All steps of purification were carried out at 4°C. Solid ammonium sulphate was added to crude extract to 0~35% saturation and left for 5 hr. The supernatant was collected by centrifugation and brought to 75% saturation in the same manner. The precipitate was collected by centrifugation, dissolved in 0.1 M phosphate buffer (pH 7.5) and dialyzed for 24 hr against the same buffer with repeated changes of buffer. The dialyzed enzyme was applied to a Sephadex G-100 column (3 × 60 cm) pre-equilibrated with 0.1 M phosphate buffer (pH 7.5) for 14 hr. The active fractions were eluted with the same buffer, pooled and concentrated using sucrose. The enzyme solution was then applied to DEAE-cellulose column (3 × 34 cm) pre-equilibrated with 0.1 M phosphate buffer (pH 7.5). After the column was washed with the above buffer, a linear gradient of 0.0 to 0.4 M KCl was applied. The active fractions were pooled, concentrated and used as purified enzyme. The concentration of protein was determined by the method of Lowry et al. (23), using bovine serum albumin (BSA) as standard.

Electrophoresis

Native polyacrylamide gel electrophoresis (PAGE) was carried out using a 10% gel according to the method of Davis (24) while for SDS-PAGE method of Laemmili (25) was followed. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 and then destained in the same solution without the dye. For molecular weight determination, a calibration curve was prepared using standard marker proteins viz. alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), pepsin (34.7 kDa), β-lactoglobulin (18 kDa), lysozyme (14.3 kDa) and cytochrome C (12.4 kDa).

RESULTS AND DISCUSSION

Purification of SOD

Crude enzyme preparation from tomato fruit was sequentially purified by ammonium sulphate fractionation and two successive chromatographies using Sephadex G-100 and DEAE-cellulose. Elution profiles of the last two steps using chromatography are shown in Figs. 1 and 2, respectively. On the elution profile of DEAE-cellulose, the enzyme peak based on the activity coincided with a protein peak. Purity of each eluate during purification process was examined by electrophoresis on 10% native PAGE. The active fraction from DEAE-cellulose ion-exchange chromatography showed a single major (along with some minor) protein band on native page. The purification procedures and the results are summarized in Table 1. The SOD was concentrated about 22 fold with 44% recovery yielding a partially

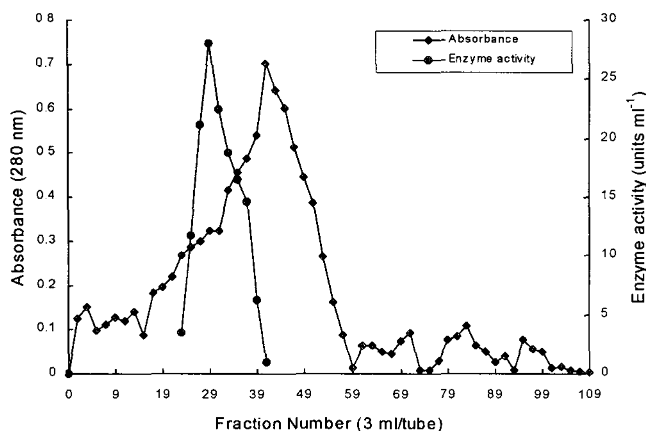

Fig. 1. Elution profile of superoxide dismutase on Sephadex G-100.

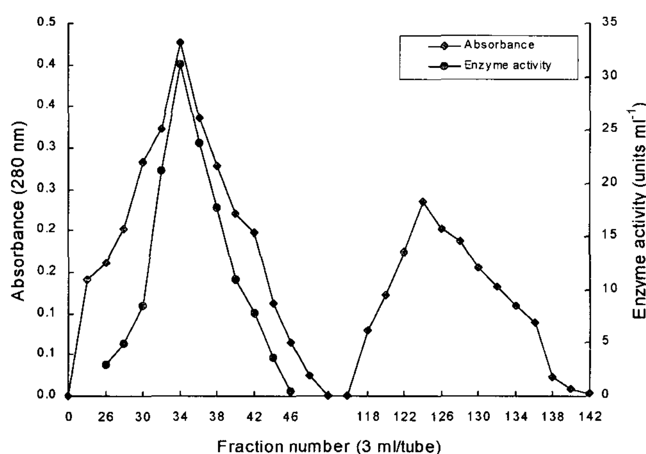

Fig. 2. Elution profile of superoxide dismutase on DEAE-cellulose.

purified SOD enzyme with a specific activity of about 122.74 units mg^{-1} protein.

Molecular weight of SOD

The partially purified enzyme preparation showed a single major band of 37.1 kDa and 18.2 kDa on native and SDS-PAGE, respectively, suggesting that the enzyme was a homodimer of two identical subunits (Figs. 3 and 4). Several workers have reported the purification of SOD from different plant species. Sevilla et al. (26) purified SOD from *Pisum sativum* with 2.3% yield and specific activity of 2632 units mg^{-1} protein. SOD from Brussells sprouts (*Brassica oleracea* L. var. bullata) has been purified to 36.9 fold with 24% yield and the specific activity of 4801 units mg^{-1} protein by Walker et al. (27). Kwiatowski and Kaniuga (28) purified cytosolic and chloroplastic isozymes of CuZn-SOD from tomato leaves whose relative molecular masses were about 33 and 30 kDa, respectively. Federico et al. (29) purified and characterized SOD from *Lens esculenta* cotyledons and shoots having molecular weight of 32 kDa each along with subunit molecular weight of 15.9 kDa. Clarkson et al. (30) purified SOD from soybeans having molecular weight of 36 kDa, a homodimer with two subunits of 18 kDa each. Bueno and Rio (31) found that glyoxysomal CuZn-SOD from watermelon cotyledons had molecular weight of 33 kDa and was composed of 2 equal subunits of 16.5 kDa.

Effect of substrate concentration

Different concentrations of NBT ranging from 5 to 70 μM were included in the assay mixture to study their effect on enzyme activity. Maximum activity was observed at 60 μM NBT. From a double reciprocal plot

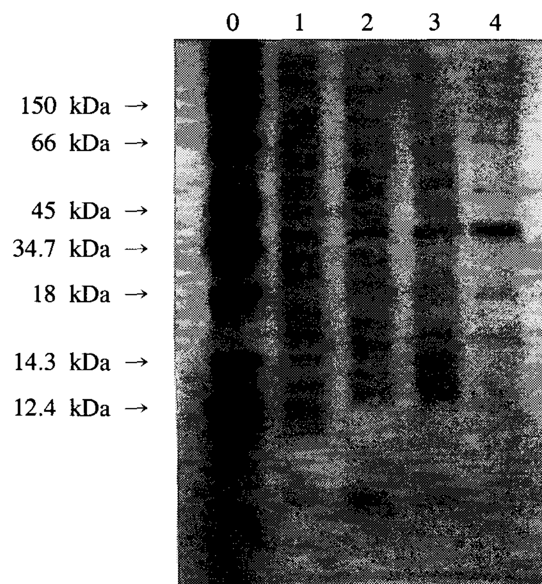

Fig. 3. Native-PAGE pattern of purified preparation of superoxide dismutase from tomato fruit.
Lane 0: Standard markers.
Lane 1: Crude extract.
Lane 2: (35~75%) $(NH_4)_2SO_4$ fraction.
Lane 3: Sephadex G-100 column fraction.
Lane 4: Purified enzyme.

Table 1. Summary of purification of superoxide dismutase from tomato fruit

Purification step	Total protein (mg)	Total activity (units)	Sp. activity (units mg^{-1} protein)	Yield (%)	Purification (fold)
Crude extract	416.0	2348.0	5.6	100.0	1.00
$(NH_4)_2SO_4$ fraction (35~75%)	66.3	1569.4	23.7	66.9	4.20
Sephadex G-100	27.3	1309.0	48.0	55.8	8.50
DEAE-cellulose	8.4	1032.2	122.7	44.0	21.76

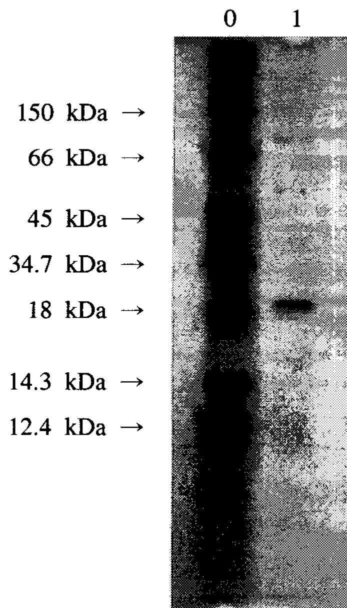

Fig. 4. SDS-PAGE pattern of purified preparation of superoxide dismutase from tomato fruit.
Lane 0: Standard markers.
Lane 1: Purified enzyme.

(Fig. 5), the K_m of enzyme for NBT was found to be 25×10^{-6} M. The enzyme activity was also determined in the presence of different concentrations of riboflavin (0.5 to 4.0 µM). Maximum SOD activity was observed at 3.0 µM riboflavin and K_m for riboflavin observed to be 1.7×10^{-6} M as shown in Fig. 6. Pruthi (11) reported higher K_m values of 310×10^{-6} M and 41×10^{-6} M for its substrates NBT and riboflavin, respectively for SOD purified from *Brassica juncea* (cv. Varuna). Misra and Fridovich (32) reported that SOD from *E. coli* had K_m value of 6.3×10^{-6} M for riboflavin.

Effect of varying enzyme concentration

Enzyme concentration was varied between 25 to 300

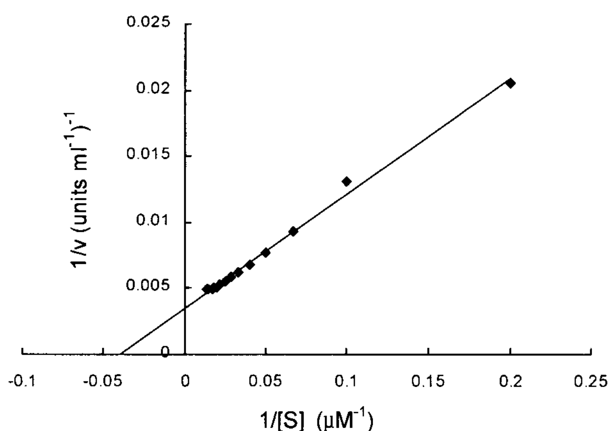

Fig. 5. Lineweaver-Burk plot showing K_m value for SOD a function of NBT concentration.

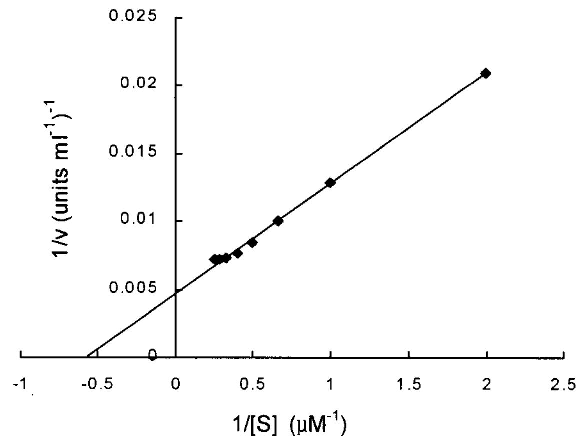

Fig. 6. Lineweaver-Burk plot showing K_m value for SOD as function of riboflavin concentration.

µL. Percent inhibition of NBT photoreduction increased almost linearly with increasing enzyme concentration upto 68%. A similar effect of enzyme concentrations has earlier been reported by Gianopolitis and Ries (22) in maize where they observed linearity between enzyme concentrations versus percent inhibition upto 70%.

pH and thermostability

The enzyme activity was measured over a range of pH 5.7 to 9.0. As shown in Fig. 7, the maximum activity was observed at pH 7.8. However, the enzyme was active over a wide range. Fridovich (33) reported that eukaryotic CuZn-SOD was unaffected by pH in the range of 5.5 to 10.0. Ravindranath and Fridovich (34) isolated and characterized Mn-SOD from yeast with pH optima of 7.8 and the activity decreased as the pH was raised above 7.8. Thermal stability was checked over a range of 20 to 90°C. The enzyme was stable up to 50°C and thereafter enzyme activity decreased sharply (Fig. 8). At 70°C, only 25% of the maximum activity retained and at 90°C, no enzyme activity was observed. Superoxide dismutase

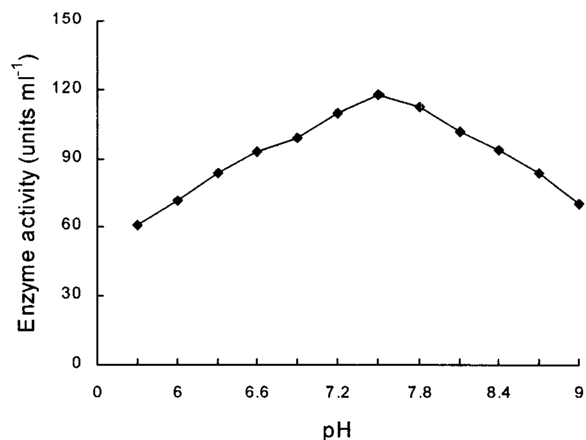

Fig. 7. Effect of pH on activity of purified superoxide dismutase.

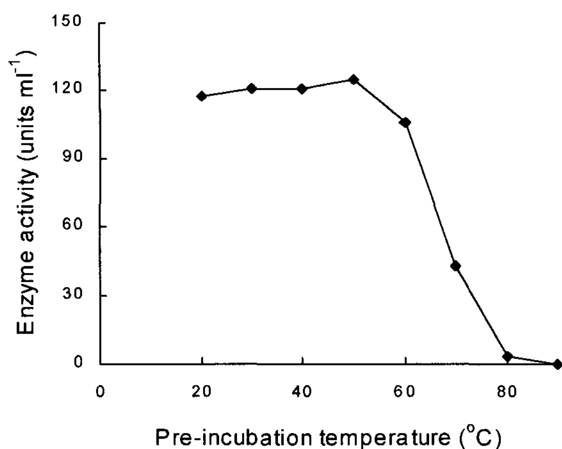

Fig. 8. Thermostability of purified superoxide dismutase.

from rice was stable at 50°C for 1 hr; at 60°C, half of the activity was lost after 1 hr, and at 70°C, initially enzyme inactivation was 20~30% but remaining activity decreased very slowly (13). Kanematsu and Asada (35) reported inactivation of CuZn-SOD-I and II after heating above 70°C.

Effect of metal ions, inhibitors, activators and stabilizers

Effect of various metal ions, inhibitors, activators and stabilizers on the enzyme activity was investigated (Table 2). The enzyme was pre-incubated with various reagents at concentrations given in Table 2 for 45 min during enzyme assay. Enzyme was activated by Co^{2+} and Mg^{2+} and inhibited strongly by Mn^{2+} while Ca^{2+} and Cu^{2+} had no effect. The reason for this unexpected behaviour of divalent cations is uncertain. Similarly, β-mercaptoethanol, SDS and chloroform + ethanol had no effect on enzyme activity. On the other hand, sodium azide (NaN_3) and H_2O_2 were found to be the potent inhibitors of enzyme activity. Since SOD in the present study was a homodimer of 18.2 kDa that was inhibited by H_2O_2, but not by chloroform + ethanol, it was concluded that the partially purified enzyme is of the cupero-zinc nature. Baum and Scandalios (36) reported increase in relative activity of maize CuZn SOD in presence of 1 mM Mg^{2+}. Bakardjieva et al. (37) reported that at 20 and 50°C, *in vitro* added Ca^{2+} increased SOD activity in four plant species (*Taxus baccata, Pinus sylvestris, Medicago rigidula* and *Zea mays*) to different levels. Fridovich (33) reported that Cu^{2+} is tightly bound and cannot be replaced by the same or any other metal ion, suggesting no effect on relative enzyme activity by exogenous Cu^{2+} ions. Trant et al. (38) studied the effect of 5 mM NaN_3 on enzyme activity of two isozymes (designated SOD-2 and SOD-3) of Fe-SOD purified from *Crithidia fasciculata* and observed inhibition of both isoforms upto 33% of their original activity, which is similar to our observations in CuZn-SOD from tomato.

Table 2. Effect of metal ions, inhibitors, activators and stabilizers on activity of superoxide dismutase purified from tomato fruit

Reagents	Concentration	Relative activity (%)
Control	-	100
Co^{2+}	5 mM	127
Mn^{2+}	5 mM	36
Mg^{2+}	5 mM	173
Ca^{2+}	25 mM	100
Cu^{2+}	5 mM	100
H_2O_2	5 mM	40
β-ME	10 mM	100
SDS	1%	100
NaN_3	5 mM	68
$CHCl_3 + C_2H_5OH$	1:1	97

REFERENCES

1. Foyer CH. 1997. Oxygen metabolism and electron transport in photosynthesis. In *Molecular biology of free radical scavenging systems*. Scandalios J, ed. Cold Spring Harbor Laboratory Press, New York. p 587-621.
2. Allen RD. 1995. Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol* 107: 1049-1054.
3. Foyer CH, Lopez DH, Dat JF, Scott IM. 1997. Hydrogen peroxide and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. *Physiol Plant* 100: 241-254.
4. Boo YC, Jung J. 1999. Water deficit-induced oxidative stress and antioxidant defenses in rice plants. *J Plant Physiol* 155: 255-261.
5. Low PS, Merida JR. 1996. The oxidative burst in plant defense: function and signal transduction. *Physiol Plant* 96: 533-542.
6. Regoli F, Winston GW. 1999. Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxyl radicals and hydroxyl radicals. *Toxicol Appl Pharmacol* 156: 96-105.
7. Foyer CH, Descourvieres P, Kunert KJ. 1994. Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. *Plant Cell Environ* 17: 507-523.
8. McCord JM, Fridovich I. 1969. Superoxide dismutase: An enzymic function for erythrocuprein (Hemocuprein). *J Biol Chem* 244: 6049-6055.
9. Ishikawa Y. 1995. Purification and immunological characterization of superoxide dismutase of onion maggot, *Delia antiqua. Arch Insect Biochem Physiol* 28: 387-396.
10. Zaki SN. 1999. Purification and characterization of the cytosolic CuZn-superoxide dismutase (CuZn-SOD) from rocket (*Eruca sativa*) leaves. *J Medical Res Inst* 20: 149-158.
11. Pruthi V. 2002. *Alternaria brassicae* induced alterations in enzymatic activities and polypeptide patterns in *Brassica juncea. PhD Thesis.* CCS Haryana Agricultural University, Hisar, India.
12. Weisiger RA, Fridovich I. 1973. Superoxide dismutase: organelle specificity. *J Biol Chem* 248: 3582-3592.
13. Kanematsu S, Asada K. 1978. Superoxide dismutase from an anaerobic photosynthetic bacterium, *Chromatium vin-*

osum. Arch Biochem Biophys 185: 473-482.
14. Pavlina DA, Maria A, Ljubka G, Stanka S, Wolfgang V. 1999. A novel Cu, Zn-superoxide dismutase from the fungal strain *Humicola lutea* 110: Isolation and physicochemical characterization. *Spectrochimica Acta Part-A Molecular and Biomolecular Spectroscopy* 55: 2249-2260.
15. Barkely KB, Gregory EM. 1990. Tetrameric manganese superoxide dismutases from anaerobic *Actinomyces. Arch Biochem Biophys* 280: 192-200.
16. Bray RC, Cockle SA, Feilden EM, Roberts PB, Rotilio G, Calabrese L. 1974. Reduction and inactivation of superoxide dismutase by hydrogen peroxide. *Biochem J* 139: 43-48.
17. Fridovich I. 1978. The biology of oxygen radicals. *Science* 201: 875-879.
18. Rabinowitch HD, Sklan D. 1980. Superoxide dismutase: A possible protective agent against sunscald in tomatoes (*Lycopersicon esculentum* Mill.) *Planta* 148: 162-167.
19. Rabinowitch HD, Sklan D, Budowski P. 1982. Photoxidative damage in ripening tomato fruit: Protective role of superoxide dismutase. *Physiol Plant* 54: 369-374.
20. Beauchamp CO, Fridovich, I. 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44: 276-287.
21. Asada K, Takahashi M, Nagate M. 1974. Assay and inhibitors of spinach superoxide dismutase. *Agril Biol Chem* 38: 471-473.
22. Giannopolitis CN, Ries SK. 1977. Superoxide dismutases. I. Occurrence in higher plants. *Plant Physiol* 59: 309-314.
23. Lowry DH, Rosenbrough NJ, Farr AL, Randall JL. 1951. Protein measurement with Folin-phenol reagent. *J Biol Chem* 193: 265-275.
24. Davis BJ. 1964. Disc electrophoresis II. Method and application to human serum proteins. *Annu NY Acad Sci* 121: 404-427.
25. Laemmili UK. 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T_4. *Nature* 277: 680-685.
26. Sevilla F, Gorge JL, Gomez M, Rio LAD. 1980. Manganese superoxide dismutase from a higher plant: Purification of a new Mn-containing enzyme. *Planta* 150: 153-157.
27. Walker JL, McLellan KM, Robinson DS. 1991. Isolation and purification of superoxide dismutase purified from Brussels sprouts (*Brassica oleracea* L. var. bullata subvar. gemmifera). *Food Chem* 41: 1-9.
28. Kwiatowski J, Kaniuga Z. 1986. Isolation and characterization of cytosolic and chloroplastic isoenzymes of CuZn-superoxide dismutase from tomato leaves and their relationships to other CuZn-superoxide dismutases. *Biochim Biophys Acta* 874: 99-115.
29. Federico R, Medda R, Floris G. 1985. Superoxide dismutase from *Lens esculenta*: Purification and properties. *Plant Physiol* 78: 357-358.
30. Clarkson SP, Large PJ, Bamforth CW. 1989. Purification of a cyanide-sensitive superoxide dismutase from soybeans: a food-compatible enzyme preparation. *J Sci Food Agric* 48: 87-97.
31. Bueno P, Rio LAD. 1992. Purification and properties of glyoxysomal cuprozinc superoxide dismutase from watermelon cotyledons (*Citrullus vulgaris* Schrad). *Plant Physiol* 98: 331-336.
32. Misra HP, Fridovich I. 1977. Superoxide dismutases: A photochemical augmentation assay. *Arch Biochem Biophys* 181: 308-312.
33. Fridovich I. 1975. Superoxide dismutases. *Ann Rev Biochem* 44: 147-159.
34. Ravindranath SD, Fridovich I. 1975. Isolation and characterization of manganese containing superoxide dismutase from yeast. *J Biol Chem* 250: 6107-6112.
35. Kanematsu S, Asada K. 1989. CuZn-superoxide dismutases from the fern *Equisetum arvense* and the green alga *Spirogyra* sp.: occurrence of chloroplast and cytosol types of enzyme. *Plant Cell Physiol* 30: 717-727.
36. Baum JA, Scandalios JG. 1981. Isolation and characterization of the cytosolic and mitochondrial superoxide dismutases of maize. *Arch Biochem Biophys* 206: 249-264.
37. Bakardjieva NT, Christov KN, Christova NV. 2000. Effect of calcium and zinc on the activity and thermostability of superoxide dismutase. *Biol Plant* 43: 73-78.
38. Trant NL, Meshnick SR, Kitchener K, Eaton JW, Cerami A. 1983. Iron containing superoxide dismutase from *Crithidia fasciculata*: Purification, characterization and similarity to leishmanial and trypanosomal enzymes. *J Biol Chem* 258: 125-130.

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