Partial purification of superoxide dismutase and peroxidase from ber (*Zizyphus mauritiana* Lamk.) fruit using anion exchange chromatography

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ABSTRACT

Partial purification of the two enzymes *i.e.* superoxide dismutase (SOD) and peroxidase (POX) from ber pulp has been obtained by passing the ammonium sulphate fraction through a diethyl amino ethyl-cellulose (DEAE-Cellulose) column. The fractions showing SOD and POX activity were pooled separately and passed through a Sephadex G100 column for further purification. SOD was purified 12.2 fold with 12.6% yield while POX was purified 15.6 fold with 19.3% yield. Approximate molecular mass for SOD and POX, as judged by gel filtration method was 35.6 and 81.5 kDa, respectively. [**Physiol. Mol. Biol. Plants** 2008; 14(3) : 167-172] E-mail : sunilsaini2007@rediffmail.com

Key words : Ber fruit, superoxide dismutase, peroxidase, DEAE-cellulose chromatography, fold purification, yield, *Zizyphus mauritiana* Lamk.

Plants face a common problem of oxidative stress experienced by all aerobic organisms. On the one hand, ground state of molecular oxygen is essential to many indispensable metabolic processes of all aerobic life forms. On the other hand, necessary presence of oxygen leads to multifarious risks of oxidative damage due to the formation of reactive oxygen species (ROS) such as $\bullet\text{O}_2^-$, H_2O_2 , $\bullet\text{OH}$ and $^1\text{O}_2$, which are produced as byproduct of normal cell metabolism (Foyer, 1997; Allen, 1995). In addition, ROS production has also been found to be stimulated by various abiotic (Foyer *et al.*, 1997) and biotic factors (Low and Merida, 1996). Accumulation of ROS is linked to various cellular injuries including membrane damage, lipid peroxidation, DNA mutation, protein denaturation and enzyme inactivation (Regoli and Winston, 1999). However, plants possess an array of defense mechanisms against oxidative stress damage. These include naturally occurring small antioxidant

molecules like α -tocopherol, β -carotene, uric acid, ascorbic acid and glutathione and antioxidative enzymes such as superoxide dismutase, catalase, peroxidase, glutathione reductase etc. (Foyer *et al.*, 1994).

The antioxidative enzymes, SOD ($\bullet\text{O}_2^- : \bullet\text{O}_2^-$ oxidoreductase EC 1.15.1.1) and peroxidase (H_2O_2 oxidoreductase EC 1.11.1.7) are of paramount physiological importance as the former catalyses the dismutation of superoxide radical to molecular oxygen and H_2O_2 while the latter converts H_2O_2 to water and harmless molecular oxygen (McCord and Fridovich, 1969). Both enzymes run in a cyclic manner to counter oxidative stress.

Ber (*Zizyphus mauritiana* Lamk.) is one of the most ancient fruits of India and is of world wide economic importance. It is an ideal crop for growing in the arid and semi-arid zones like Punjab, Haryana, Rajasthan, Western U.P. and Delhi. (Naik and Rokhode, 1997; Aulakh, 1998). It is considered to be a poor man's apple as it prevents chronic diseases such as diabetes and inhibits oxidation of human low density lipoproteins (Piga *et al.*, 2003). Though ber tree is hardy and native

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to hot and arid climate, fruit is highly perishable and undergoes cell wall degradation and fruit softening during ripening and storage (Kohar, 2003). In our laboratory, investigations have been carried out on these aspects in tomato (Mondal *et al.*, 2003, 2004). In continuation of these studies, attempts were made to purify SOD from ber fruits. While passing the ammonium sulfate fraction through DEAE-cellulose column, it was found that SOD and POX could be separated in two different fractions, which were further purified by molecular sieving. We report here the results obtained during purification of SOD and POX from ber fruit variety Umran (a late maturing variety).

MATERIALS AND METHODS

Plant material and chemicals

Ber fruits of Umran variety were harvested at mature green stage from ten-year old trees grown at Horticulture farm, CCSHAU, Hisar and stored at -70°C until used. All chemicals used during present investigations were of analytical grade and obtained from Sigma Chemical Company, St. Louis, M.D., USA (DEAE- Cellulose, Sephadex G100); Sisco Research Laboratories Pvt. Ltd., Bombay (Riboflavin, L-methionine, nitroblue tetrazolium, guaiacol, EDTA, PVP); E. Merck (β -mercaptoethanol) and Himedia Laboratories Ltd., Bombay (Tris-HCl, CaCl_2).

Enzyme extraction

Preliminary experiments were conducted to optimize the extraction conditions with respect to pH, molarity and type of buffer, concentration of stabilizing agent(s) and the other constituents of the extraction medium. Finally, one gram of fresh tissue (fleshy part) was homogenized with 3.0 ml of cold 0.1M Tris- HCl buffer (pH 7.5) containing 1mM EDTA, 3% polyvinyl pyrrolidone and 1mM CaCl_2 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 10,000xg for 20 min in a refrigerated centrifuge (Hitachi, Japan) at 4°C. The supernatant so obtained was referred to as crude enzyme preparation.

Assay of SOD activity

Superoxide dismutase was assayed by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) adopting the method of Beauchamp and Fridovich (1971). The reaction mixture (3.0 ml) contained 50 mM Tris-HCl (pH 7.8), 14 mM L-methionine, 60 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 40 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 560 nm. Percent inhibition was calculated by the following formula of Asada *et al.* (1974) :

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

Where,

V = Rate of assay reaction in absence of SOD.

v = Rate of assay reaction in presence of SOD.

One enzyme unit was defined as the amount of enzyme, which could cause 50 percent inhibition of the photochemical reduction of NBT (McCord and Fridovich, 1969). However, for the purpose of kinetic and regulatory properties, the enzyme activity was expressed in terms of units ml^{-1} and calculated by the commonly used formula of Giannopolitis and Ries (1977):

$$\text{SOD units ml}^{-1} = \frac{V - V_0}{V_0} \times \text{Dilution factor}$$

Assay of POX activity

Peroxidase was assayed by determining the rate of guaiacol oxidation in the presence of H_2O_2 at 470 nm (Rao *et al.*, 1996). Reaction mixture (3.0 ml) contained 2.15 ml of 0.1 M Tris HCl buffer (pH 7.0), 0.6 ml of 1% guaiacol, 0.1 ml of enzyme extract and 0.15 ml of 100 mM H_2O_2 . H_2O_2 was added in the end, mixed and absorbance was recorded at 470 nm at 15s interval up to 3 min. The linear portion of O.D. change was taken to calculate enzyme units using a molar extinction coefficient of 26.6 $\text{mM}^{-1} \text{cm}^{-1}$ for guaiacol oxidation. One unit of peroxidase activity was defined as nmol of guaiacol oxidized $\text{min}^{-1} \text{ml}^{-1}$.

Purification of SOD and POX

Unless stated otherwise, all steps of enzyme purification were carried out at 0-4°C. Each experiment was repeated at least twice and the estimations further made in duplicate. Solid ammonium sulfate was added to crude extract to 0-35% saturation and left for 5h. After 5h, the

solution was centrifuged and the precipitate discarded, as it had negligible activities of SOD and POX and the resulting supernatant was brought to 85% $(\text{NH}_4)_2\text{SO}_4$ saturation. The precipitate was collected by centrifugation (10,000 $\times$ g, 20 min), dissolved in 0.05M Tris HCl buffer (pH 7.5) and dialyzed for 24 h against the same buffer with repeated changes of buffer. The dialyzed protein was concentrated by osmosis against solid sucrose and loaded onto DEAE- cellulose column (3 $\times$ 42cm) pre-equilibrated with 0.05 M Tris HCl buffer (pH 7.5). The enzyme was eluted first by the above buffer and then by a linear gradient of 0.0 to 0.4 M KCl with a flow rate of 32 ml h⁻¹. Fractions of 3.0 ml each were collected and analyzed for protein at 280 nm and for activity of SOD and POX. The active fractions giving clear cut activities for SOD and POX were pooled separately and again concentrated against sucrose. SOD active concentrated fraction was applied to a Sephadex G100 column pre-equilibrated with 0.05 M Tris HCl (pH 7.5) for 10 h. The enzyme was eluted with same buffer at a flow rate of 18 ml h⁻¹. The active fractions of 3.0 ml each were pooled and concentrated using sucrose and used as purified SOD. The column was washed with same buffer for 24 h for reuse. The POX fraction eluted from DEAE-column and concentrated against sucrose was loaded onto the same Sephadex G100 column. The enzyme was eluted at a flow rate of 18 ml h⁻¹ and the active fractions of 3.0 ml each were pooled, concentrated and used as purified POX. The concentration of protein at every step was determined by the method of Lowry *et al.* (1951), using bovine serum albumin (BSA) as a

standard. Molecular weight of the two partially purified enzymes was determined by gel filtration on a Sephadex G-100 column. The column was calibrated for molecular weight with standard molecular weight markers; cytochrome-C (12.4 kDa), β -lactoglobulin (18.0 kDa), carbonic anhydrase (29.0 kDa), pepsin (34.7 kDa), ovalbumin (45.0 kDa), albumin bovine (66.0 kDa) and alcohol dehydrogenase (150.0 kDa). The molecular weights were determined using a plot of v_e/v_o vs. log molecular weights of standard proteins, according to the method of Whitaker (1963). A common factor $y = -0.008x + 2.511$ was computed statistically and used to know the log M_r of purified proteins (Fig. 5).

RESULTS

The crude extract prepared by homogenizing 100 g of ber fruit pulp in 500 ml Tris-HCl buffer (0.1M, pH 7.5) containing 1 mM CaCl_2 gave substantial amount of SOD and POX activity. These were sequentially purified by ammonium sulfate fractionation (35-85% saturation), DEAE-cellulose column chromatography and then through gel filtration in cold chamber (LKB, USA). The results of the enzyme purification are given in Table 1. The initial specific activity in crude extract was 6.9 and 51.7 for SOD and POX, respectively. Elution profile of SOD, POX and protein on DEAE-cellulose column chromatography is shown in Fig. 1 and 2. Protein was distributed randomly throughout all the fractions, whereas SOD and POX activities were present in fraction

Table 1. Summary of purification of superoxide dismutase and peroxidase from ber (*Zizyphus mauritiana* Lamk.) fruit

Purification step	Enzyme	Total Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Fold purification	Yield
Crude extract	SOD	510.0	234.0	1607.0	6.9	1.0	100.0
	POX	–	–	12087.0	51.7	1.0	100.0
35-85% Ammonium Sulfate saturation	SOD	42.0	48.0	400.0	8.3	1.2	24.9
	POX	–	–	7373.5	153.6	3.0	61.0
DEAE-fraction	SOD	20.0	7.7	238.0	30.9	4.5	14.8
	POX	54.0	6.1	2703.3	443.8	8.6	22.4
Sephadex G100 fraction	SOD	55.0	2.4	202.0	84.2	12.2	12.6
	POX	32.5	2.9	2334.0	804.9	15.6	19.3

Fig. 1. Elution profile of DEAE-cellulose column along with SOD activity.

number 17-23 and 111-130, respectively. Fractions with SOD and POX activities were pooled separately and then passed individually through Sephadex G100 column (1.5x58 cm). SOD fraction eluted at 120 ml volume from the Sephadex G100 column (Fig. 3). DEAE eluted POX fraction was also eluted through Sephadex G100 column and it eluted at 75 ml (Fig. 4).

The sequential purification procedures and the results for SOD and POX are given in Table 1. The SOD was purified about 12.2 fold with 12.6% recovery while POX was purified about 15.6 fold with 19.3% yield. The specific activities of purified SOD and POX were 842 and 2334 units mg^{-1} protein, respectively. Molecular weight as determined by gel filtration was found to be 35.6 kDa for SOD and 81.5 kDa for POX (Fig. 5).

DISCUSSION

Preliminary experiments were conducted to standardize the conditions in such a way that both enzyme activities could be recovered in the same fraction. Incorporation of CaCl_2 in extraction buffer was found to be an essential requirement in order to have both activities in good

Fig. 2. Elution profile of DEAE-cellulose column along with POX activity.

Fig. 3. Elution profile of Sephadex G100 column along with SOD activity.

Fig. 4. Elution profile of Sephadex G100 column along with POX activity.

amount in the same extraction. Kumar *et al.* (2004) reported that Ca^{2+} ions have no effect on SOD activity while Agostini *et al.* (1997) observed that CaCl_2 enhances activity of peroxidases. Both the enzymes could be precipitated in 35-85% $(\text{NH}_4)_2\text{SO}_4$ saturation. These results are in agreement with those of Shannon *et al.* (1966) and Kumar *et al.* (2004), who have reported SOD and POX to be present in 35-75% ammonium sulfate fraction.

Fig. 5. Standard curve of standard proteins as eluted through Sephadex G100 column.

Elution profile of the two enzymes on DEAE-cellulose column revealed that SOD did not bind with DEAE-cellulose, as it was eluted in fractions 17–23. Misra and Fridovich (1972) purified SOD to 60.2 fold with 14% recovery by ethanol and acetone precipitation. Several workers have reported the purification of SOD from different plant species using techniques of ammonium sulphate fractionation, anion exchange chromatography and gel filtration. Sevilla *et al.* (1980) purified SOD from *Pisum sativum* with 2.3% yield and Duke and Salin (1985) from *Ginkgo biloba* with 8% yield. Four isoenzymes of SOD have been observed in groundnut (*Arachis hypogea*) seedlings which were purified with 20.12, 5.06, 6.8 and 5.3% recovery, respectively (Sulochana and Venkaiah, 1990). Kumar *et al.* (2004) purified CuZn SOD from tomato (*Lycopersicon esculentum* Mill.) to 22 fold with 44% yield. Several workers have reported the molecular weight of SOD between 31–39 kDa (Misra and Fridovich, 1972; Beauchamp and Fridovich, 1973; Lumsden and Hall, 1974; Rabinowitch and Sklan, 1981 and Shivaprakasam *et al.*, 2004). Federico *et al.* (1985) isolated and purified SOD from *Lens esculenta* cotyledons. Gel filtration gave a molecular weight of 33 kDa. Kumar *et al.* (2004) predicted molecular weight of 36.3 kDa for SOD in tomato. These reports are in fair agreement with our results.

Peroxidase eluted late (fraction 111–130) through DEAE column. With a large number of isozyme species and substrates, peroxidases have been difficult to purify (Jen *et al.*, 1980). Several workers have purified peroxidases from different sources yielding enzyme with a range of molecular weights. Deits *et al.* (1984) purified an ovoperoxidase from the egg of sea urchin, *Strongylocentrotus purpuratus*, using ammonium sulphate precipitation, DEAE-Sephacel chromatography in the absence of divalent cations and finally by CM-Sephadex chromatography to 1.46 fold with 52% recovery. The molecular weight was 70 kDa as judged by SDS-PAGE. Urek and Pazarliolu (2004) purified a Mn peroxidase with 23.08% recovery with a fold purification of 5.8. Gel filtration indicated a molecular weight of 45 kDa. Mdulili (2005) partially purified a polyphenol oxidase (PPO) and peroxidase from Marula fruit by a combination of temperature induced phase separation in Triton X-114, DEAE-cellulose and Sephadex G100 gel chromatographies. PPO activity was purified 58 fold with 75% recovery while the fold purification for POX was 19 with 25% recovery. Both shared the same molecular weight 71 of kDa. From the results presented here, it could be suggested that though fold purification and protein yield are not very high yet it can pave a way to

have multiple purifications from a single column by judicious adjustments of standard conditions required by different enzymes.

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REFERENCES

- Agostini, E., deForchetti, S.M. and Tigier, H.A. (1997). Production of peroxidases by hairy roots of *Brassica napus*. *Plant Cell, Tissue Organ Culture*. 47: 177–182.
- Allen, R.D. (1995). Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiol.* 107: 1049–1054.
- Asada, K., Takahashi, M. and Nagate, M. (1974). Assay and inhibitors of spinach superoxide dismutase. *Agril. Biol. Chem.* 38: 471–473.
- Aulakh, P.S. (1998). Performance of ber cultivars under the rainfed conditions in the Shivalik foothills of Punjab. *Haryana J. Hort. Sci.* 27(4): 230–231.
- Beauchamp, C.O. and Fridovich, I. (1971). Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* 44: 276–287.
- Beauchamp, C.O. and Fridovich, I. (1973). Isozymes of superoxide dismutase from wheat germ. *Biochimica Et Biophysica Acta* 317: 50–64.
- Deits, T., Farrance, M., Kay, E.S., Medill, L., Turner, E.E., Weidman, P.J. and Shapiro, B.M. (1984). Purification and properties of ovoperoxidase, the enzyme responsible for hardening the fertilization membrane of the sea urchin egg. *J. Biol. Chem.* 259: 13525–13533.
- Duke, M.V. and Salin, M.L. (1985). Purification and characterization of an iron-containing superoxide dismutase from a eukaryote, *Ginkgo biloba*. *Arch. Biochem. Biophys.* 243: 305–314.
- Federico, R., Medda, R. and Floris, G. (1985). Superoxide dismutase from *Lens esculenta*: Purification and properties. *Plant Physiol.* 78: 357–358.
- Foyer, C.H. (1997). Oxygen metabolism and electron transport in photosynthesis. In: *Scandalios J. (ed) Molecular biology of free radical scavenging systems*. Cold Spring Harbor Laboratory Press, New York, Pp. 587–621.
- Foyer, C.H., Descourvieres, P. and Kunert, K.J. (1994). Protection against oxygen radicals: an important defense mechanism studied in transgenic plants. *Plant Cell Environ.* 17: 507–523.
- Foyer, C.H., Lopez, D.H., Dat, J.F. and Scott, I.M. (1997). Hydrogen peroxide and glutathione-associated mechanisms of acclamatory stress tolerance and signaling. *Physiol. Plant.* 100: 241–254.
- Giannopolitis, C.N. and Ries, S.K. (1977). Superoxide dismutase. I. Occurrence in higher plants. *Plant Physiol.* 59: 309–314.

- Jen, J.J., Seo, A. and Flurkey, W.H. (1980). Tomato peroxidase: Purification via hydrophobic chromatography. *J. Food Sci.* 45(1): 60-63.
- Kohar, S. (2003). Biochemical studies on fruit ripening in ber. *M.Sc Thesis submitted to CCS Haryana Agricultural University, Hisar.*
- Kumar, S., Dhillon, S., Singh, D. and Singh, R. (2004). Partial purification and characterization of superoxide dismutase (SOD) from tomato fruit. *J. Food Sci. Nutr.* 9: 283-288.
- Low, P.S. and Merida, J.R. (1996). The oxidative burst in plant defense: function and signal transduction. *Physiol. Plant.* 96: 533-542.
- Lowry, D.H., Rosenbrough, N.J., Farr, A.L. and Randall, J.L. (1951). Protein measurement with Folin-phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Lumsden, J. and Hall, D.O. (1974). Soluble and membrane bound superoxide dismutase in a blue-green alga (*Spirulina*) and spinach. *Biochem. Biophys. Res. Comm.* 58: 35-41.
- McCord, J.M. and Fridovich, I. (1969). Superoxide dismutase: An enzymic function for erythrocyte (Hemocytin). *J. Biol. Chem.* 244: 6049-6055.
- Mdluli, K.M. (2005). Partial purification and characterization of polyphenol oxidase and peroxidase from marula fruit (*Sclerocarya birrea* subsp. Caffra). *Food Chem.* 92 (2): 311-323.
- Misra, H.P. and Fridovich, I. (1972). The purification and properties of superoxide dismutase from *Neurospora crassa*. *J. Biol. Chem.* 247: 3410-3414.
- Mondal, K., Sharma, N.S., Malhotra, S.P., Dhawan, K. and Singh, R. (2003). Oxidative stress and antioxidant systems in tomato fruits during storage. *J. Food Biochem.* 27: 515-527.
- Mondal, K., Sharma, N.S., Malhotra, S.P., Dhawan, K. and Singh, R. (2004). Antioxidant system in ripening tomato fruits. *Biol. Plant.* 48: 49-53.
- Naik, K.R. and Rokhade, A.K. (1997). Storage studies in ber cultivars. *Karnataka J. Agric. Sci.* 10 (2): 383-386.
- Piga, A., Caro, A.D. and Corda, G. (2003). From plums to prunes: Influence of drying parameters on polyphenols and antioxidant activity. *J. Agric. Food Chem.* 51: 3675-3681.
- Rabinowitch, H.D. and Sklan, D. (1981). Superoxide dismutase activity in ripening cucumber and pepper fruit. *Physiol. Plant.* 52: 380-384.
- Rao, M.V., Paliyath, G. and Ormrod, D.P. (1996). Ultraviolet-B- and ozone induced biochemical changes in antioxidant enzymes of *Arabidopsis thaliana*. *Plant Physiol.* 110: 125-136.
- Regoli, F. and Winston, G.W. (1999). Quantification of total oxidant scavenging capacity of antioxidants for peroxynitrite, peroxy radicals and hydroxyl radicals. *Toxicol. Appl. Pharmacol.* 156: 96-105.
- Sevilla, F., Gorge, J.L., Gomez, M. and Rio, L.A.D. (1980). Manganese superoxide dismutase from a higher plant: Purification of a new Mn-containing enzyme. *Planta* 150: 153-157.
- Shannon, L.M., Kay, E. and Lew, J.Y. (1966). Peroxidase isozymes from horseradish roots: Isolation and physical properties. *J. Biol. Chem.* 241(9): 2166-2172.
- Sivaprakasam, G., Singh, D., Dhillon, S., Malhotra, S.P., Ahlawat, T.R. and Singh, R. (2004). Purification and characterization of superoxide dismutase from guava (*Psidium guajava* L.). *Physiol. Mol. Biol. Plants* 10: 59-64.
- Sulochana, K.N. and Venkaiah, B. (1990). Purification of isoenzymes of superoxide dismutase from groundnut (*Arachis hypogea*) seedlings. *Biochem. Int.* 22: 133-140.
- Urek, R.O. and Pazarliolu, N.K. (2004). Purification and partial characterization of manganese peroxidase from immobilized *Phenerochaete chrysosporium*. *Process Biochem.* 39 (12): 2061-2068.
- Whitaker, J.R. (1963). Determination of molecular weights of proteins by gel filtration on Sephadex. *Anal. Chem.* 35: 1950-1953.