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Partial purification and characterization of ascorbate peroxidase from ripening ber (*Ziziphus mauritiana* L) fruits

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Ascorbate peroxidase (EC 1.11.1.11; APX) was purified from ripe ber (*Ziziphus mauritiana* L.) fruits var. Illaichi using conventional techniques of ammonium sulphate fractionation, gel filtration through Sephadex G-100 and ion-exchange chromatography on DEAE-cellulose. The enzyme was purified about 47.4 fold with 34.6% recovery. The molecular weight as determined by gel filtration was found to be 58.08 kDa. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) yielded a single major protein band with molecular weight of 29.79 kDa indicating that the enzyme was a homodimer. Native PAGE revealed a single prominent band suggesting that enzyme was purified to near homogeneity. The optimum pH for APX was found to be 7.8. It exhibited the Michaelis-Menten kinetics with *Km* values for ascorbate and H_2O_2 of 1.82 and 2.85 mM, respectively. Mn^{2+}, NO_3^-, SO_4^{2-} and Co^{2+} were found to be potent inhibitors of APX while K^+, Na^+, Ca^{2+} and Cl^- stimulated the enzyme activity. Diethylpyrocarbonate (DEPC), dithiothreitol (DTT), $NaBH_4$ and mercaptoethanol inhibited the enzyme activity while iodoacetate and 5, 5'-dithiobis-2-nitrobenzene (DTNB) had no inhibitory effect. Based on the inhibition studies, histidine and tryptophan have been suggested to be present at the active site.

Key words. Fruit ripening, *Ziziphus mauritiana,* ascorbate peroxidase, purification.

INTRODUCTION

Fruit ripening is a genetically programmed, highly co-ordinated physiological process of organ transformation from unripe to ripe stage. It is affected inevitably by oxidative stress created by over accumulation of reactive

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oxygen species (ROS) such as superoxide radical, hydrogen peroxide, and lipid peroxides produced during the process. Like other aerobic organisms, fruits also possess ROS-scavenging systems consisting of multiple defense enzymes like superoxide dismutase (SOD), peroxidase, catalase and ascorbate peroxidase (APX: EC 1.11.1.11) (Yadav et al., 2014). Ascorbate peroxidase has been found in higher plants, algae (Shigeoka et al., 1980), and some cyanobacteria (Tel-Or et al., 1986), but not in animals. APX protects the cell constituents from damage caused by hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH) that is produced from hydrogen peroxide and superoxide. It is a hydrogen peroxide-scavenging enzyme with high specificity for ascorbate as reductant. The enzyme catalyzes the reaction:

$$2\ \text{Ascorbate} + H_2O_2 \longrightarrow 2\ \text{monodehydroascorbate (MDHA)} + H_2O$$

APX has been purified from roots of Japanese radish (Ohya et al., 1997); spinach chloroplasts (Nakano and Asada, 1987), rice seedlings (Sharma and Dubey, 2004), root nodules (Dalton et al., 1987), *Pallavicinia lyelli* (Rajan and Murugan, 2010) and plastids of tobacco (Madhusudhan et al., 2003), and its characteristics have been widely investigated. Increased activity of APX in response to environmental stresses such as salinity, chilling, drought, heat, etc. has been reported in different plant species which suggests its possible role in protecting cells from oxidative damages under stress conditions (Davis and Swanson, 2001). However, information in fruits is very scanty and only bell pepper (Schantz et al., 1991) and strawberry fruits (In-Jung and Won-Il Chung, 1998) have been investigated for the expression of APX gene during fruit ripening.

We have been working with the oxidative stress and antioxidative system during ripening and post-ripening of various fruits for the last ten years (Mondal et al., 2004, 2006, 2008, 2009; Kumar and Malhotra, 2008; Kumar et al., 2011a, 2011b; Yadav et al., 2012). Ber is a tropical fruit tree species belonging to the family *Rhamnaceae*. It is very popular among consumers due to its high nutritive value and comparatively lower market price. Superoxide dismutase and peroxidase have been purified and characterized in our laboratory from guava (Sivaprakasam et al., 2004) and ber (Kumar and Malhotra, 2008) fruits. To continue work in the same direction, it has become imperative for us to purify and characterize APX from ber fruit. We report here the extraction, partial purification and characterization of APX from ber fruits.

MATERIALS AND METHODS

Fruit samples

Ber fruits (varieties Umran and Illaichi) were harvested at different stages of maturity viz. immature green (IG), mature green (MG), colour turning (CT), ripe (R) and over-ripe (OR) from the orchards of CCS Haryana Agricultural University, Hisar, India for the purpose of studying the APX profile since Illaichi fruits harvested at ripe stage were used for the purpose of enzyme purification.

Chemicals

All chemicals and biochemicals used during the present course of investigations were of analytical grade and high purity. They were obtained from Sigma Chemical Company (St. Louis, USA), E. Merck (Bombay, India), Himedia Laboratories Limited (Bombay, India) and Sisco Research Laboratories Pvt. Ltd. (Bombay, India).

Enzyme assay

The enzyme was assayed by the method of Nakano and Asada (Nakano and Asada, 1981). The reaction mixture (3.0 ml) contained 95 mM potassium phosphate buffer (pH=7.0), 0.5 mM L-ascorbate, and 0.5 mM H_2O_2. The reaction was initiated by the addition of 50 µL of enzyme extract. The decrease in absorbance at 290 nm which corresponded to the oxidation of ascorbic acid was recorded spectrophotometrically for 2 min against reagent blank. The enzyme activity was calculated using the molar extinction coefficient of 2.8 $mM^{-1}\ cm^{-1}$ for ascorbate. One enzyme unit was expressed as amount of enzyme required to oxidise one nmol of ascorbate per min.

Enzyme purification

Unless stated otherwise, all steps of enzyme purification were carried out at 0-4°C.

Preparation of crude extract

Preliminary experiments were conducted to optimize the extraction conditions with respect to pH, molarity and type of buffer, concentration of stabilizing agent(s) and other constituents of extraction medium to achieve maximum extraction of the enzyme from ber (*Ziziphus mauritiana* L.) fruits. Two hundred grams of ripe fruits was ground with 500 ml of potassium phosphate extraction buffer (pH=7.5, 0.1 M) containing 3% PVP, 1 mM EDTA and 1 mM $CaCl_2$ in a pre-chilled pestle and mortar using acid wash quartz sand as abrasive. The homogenate was filtered through four layers of cheese cloth and the filtrate centrifuged at 15,000 × *g* for 20 min in a refrigerated centrifuge at 4°C. The supernatant was carefully decanted and used as crude enzyme preparation.

$(NH_4)_2SO_4$ fractionation

To the crude extract (515 ml), solid ammonium sulphate (0 to 35 % saturation) was added with constant stirring and left for 5 h. The solution was centrifuged at 10,000 × *g* for 20 min and the precipitate discarded, as it had negligible activity of APX. The resulting supernatant was brought to 65% $(NH_4)_2SO_4$ saturation and left for 5 h. The precipitate was collected by centrifugation (10,000 × *g*, 20 min), dissolved in 95 mM potassium phosphate buffer (pH=7.0), and dialyzed against the same buffer (diluted 10 times) for 24 h with repeated changes of the buffer. The dialyzed $(NH_4)_2SO_4$ fraction was concentrated against solid sucrose and used for gel filtration chromatography.

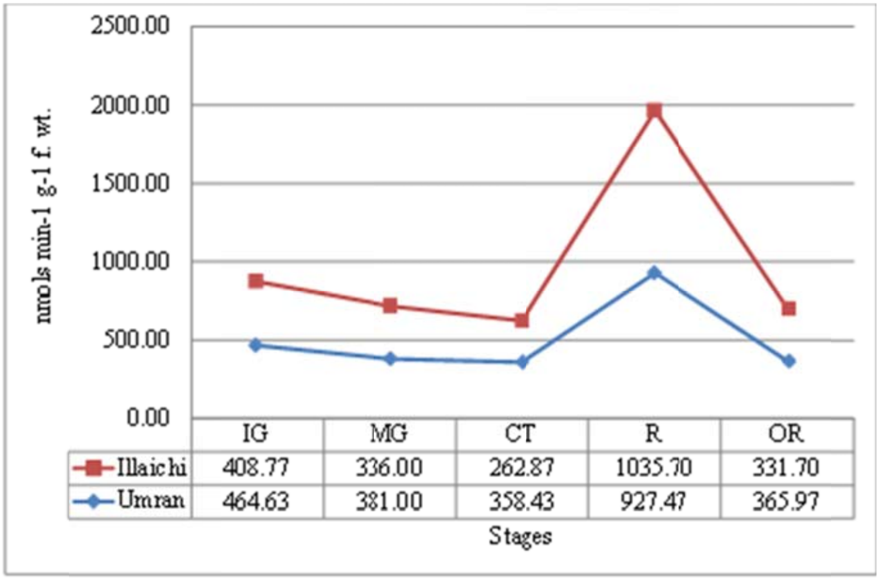

Figure 1. APX activity during ripening of ber fruits. IG, Immature green; MG, mature green; CT, color turning; R,Ripe; OR, Over ripe.

Gel filtration

The concentrated enzyme was loaded onto a Sephadex G-100 column (dimensions 100 × 1.5 cm) pre-equilibrated with 95 mM potassium phosphate buffer (pH 7.0). After complete sedimentation of the gel, the effective length of the column was 65 cm. The void volume was calculated by passing blue dextran (2 mg/ml) through the column. The column was eluted with 95 mM potassium phosphate buffer (pH=7.0) at a flow rate of 15 ml/h. The fractions of 3.0 ml each were collected and analyzed for protein (A_{280}) and APX activity. The active fractions were pooled and concentrated against solid sucrose and subjected to ion exchange chromatography.

Ion exchange chromatography

The concentrated fraction obtained after gel filtration was loaded over the top of the DEAE cellulose column (60 × 3 cm) previously equilibrated with 95 mM potassium phosphate buffer (pH=7.0) and eluted first with 95 mM potassium phosphate buffer (pH=7.0) and then by a linear gradient of 0.0 to 0.4 M KCl in the same buffer with a flow rate of 28 ml/h. The fractions of 3.0 ml each were collected and analyzed for protein (A_{280}) and APX activity. The active fractions were pooled and concentrated against solid sucrose stored at 4°C and used to study enzyme characteristics.

Protein estimation

Protein in crude extract and subsequent enzyme preparations at various stages of purification was quantitatively estimated by the method of Lowry et al. (1951).

Determination of purity and molecular weight

Purity of APX was checked by native-PAGE (10 %) using anionic system of Davis (1964). The molecular mass of the purified enzyme was estimated by gel filtration through a column of Sephadex G-100 calibrated with standard molecular weight markers viz., cytochrome-C (12.4 kDa), carbonic anhydrase (29.0 kDa), bovine serum albumin (66.0 kDa), alcohol dehdyrogenase (150.0 kDa) and ß-amylase (200.0 kDa). Subunit composition of the enzyme was determined by performing denaturating SDS-PAGE following the method of Laemmli (1970).

RESULTS AND DISCUSSION

Purification of ascorbate peroxidase

Figure 1 shows the profile of APX activity during ripening of ber fruit cv. Illaichi and Umran. The activity increased drastically up to ripe stage and then decreased at OR stage in both the varieties. Illaichi had significantly higher activity than Umran at all the stages of fruit ripening. Hence, the ber fruits of cv. Illaichi harvested at ripe stage were used for the purification purpose. The elution profile of sephadex G-100 (Figure 2) revealed that the enzyme was eluted as single peak with 20 fold purification and 47.2% recovery. The concentrated enzyme was then loaded onto a column of DEAE-cellulose, previously equilibrated with 95 mM potassium phosphate buffer (pH=7.0) and eluted with 0 to 0.4 M linear gradient of KCl in potassium phosphate buffer applied after 144 ml of eluent. Fractions of 3 ml each were collected and tested for protein and enzyme activity. The enzyme got eluted as a single peak between fractions 86 to 101 (Figure 3). The final enzyme preparation exhibited 47.4 fold purification with specific activity of 97619.4 units/mg protein and with 34.6% recovery (Table 1). Native PAGE

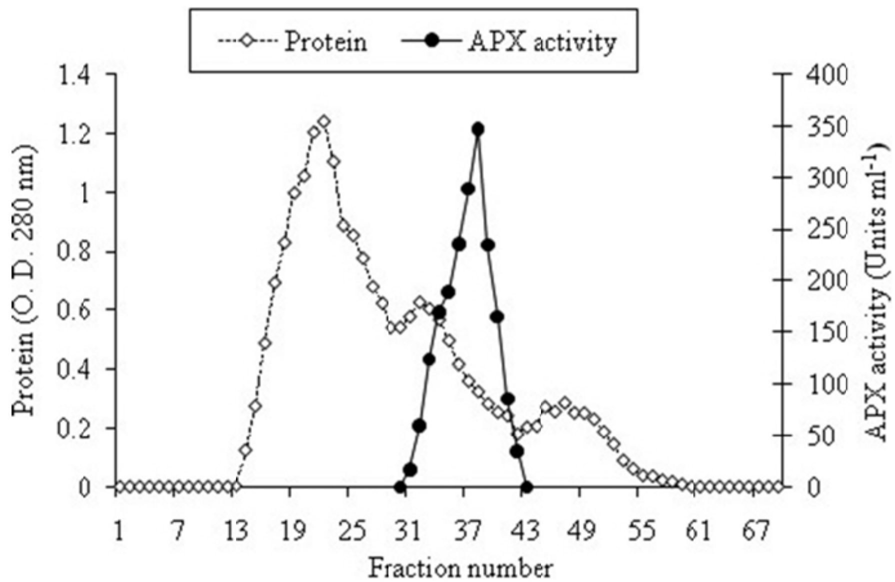

Figure 2. Elution profile of ascorbate peroxidase on sephadex G-100 column.

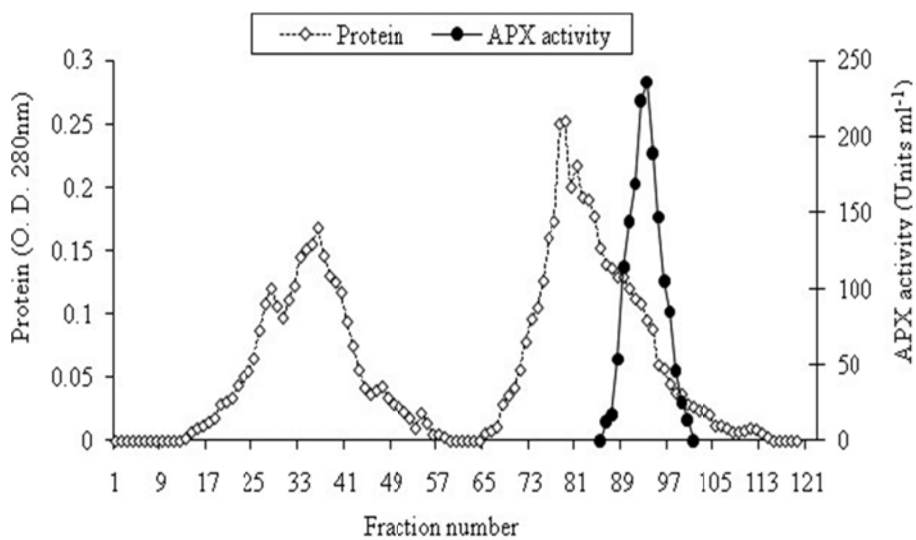

Figure 3. Elution profile of ascorbate peroxidase on DEAE column.

(Plate 1) revealed that number of bands decreased with every purification step and the final enzyme preparation gave one major band suggesting that the enzyme was purified to near homogeneity. Since this is the first report of purification of ber APX, the results could not be compared with the literature values.

Characterization of APX

The molecular weight of APX as determined by gel filtration through Sephadex G-100 column was 58.08 kDa (Figure 5 A). SDS-PAGE yielded a single major protein band (Figure 4 B), with molecular weight of 29.79 kDa (Figure 5 B), suggesting that the enzyme from ber fruit is composed of two identical subunits i.e. the enzyme is homodimer. These results are in accordance with those of Mittler and Zilinskas (1991) who purified APX from 14-day old pea (*Pisum sativum* L.) shoots and found that the enzyme was a homodimer with molecular weight of 57.5 kDa and it was composed of two identical subunits with molecular weight of 29.50 kDa each. Lu et al. (2005) also predicted molecular weight 56 kDa for APX from rice

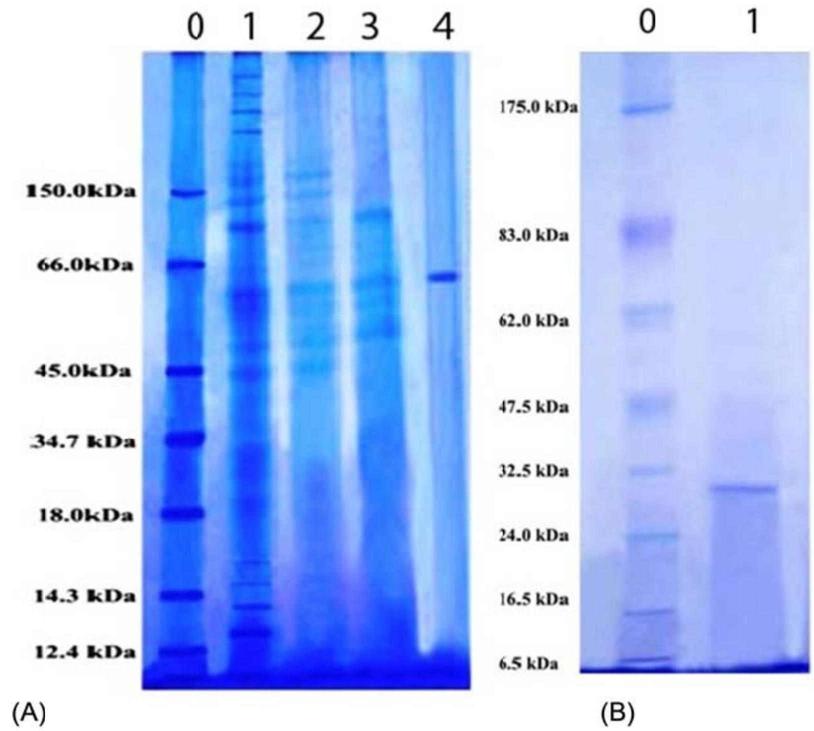

Figure 4. (A) Native-PAGE pattern of purified APX from ber (*Ziziphus mauritiana* L.) fruit. Lane 0, standard markers; Lane 1, crude extract; Lane 2, 35-65% $(NH_4)_2 SO_4$ fraction; Lane 3, sephadex G-100 fraction; Lane 4, purified enzyme. **(B)** SDS-PAGE pattern of purified APX from ber (*Ziziphus mauritiana* L.) fruit. Lane 0, standard markers; Lane 1, purified enzyme.

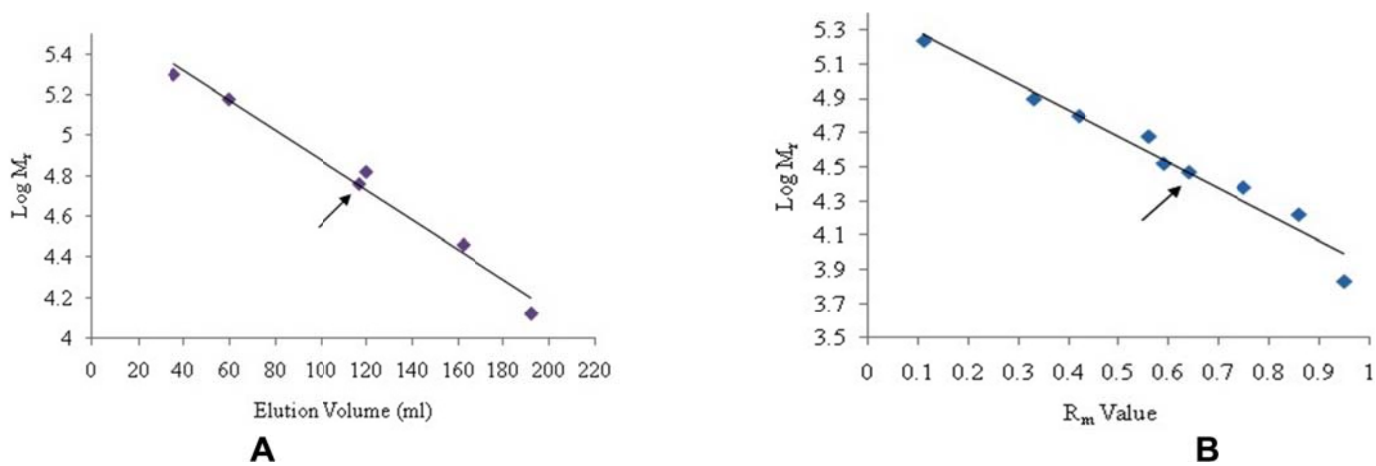

Figure 5. Determination of molecular weight of APX by (A) gel filtration (Markers: 12.4, 29, 66, 150 and 200 kDa). **(B)** SDS-PAGE (markers: as listed in figure1 B).

(*Oryza sativa* L.). Dalton et al. (1987) however, found that APX purified from the root nodules was a heme protein with molecular weight of 30.0 kDa as determined by SDS-PAGE.

The enzyme activity as determined over a range of pH 5.0 to 9.0 (Figure 6) increased from 78.3 units/ml at pH=5.0 to 406.6 units/ml at pH=7.8. Thereafter, it decreased sharply, thus, clearly indicating, 7.8 to be the

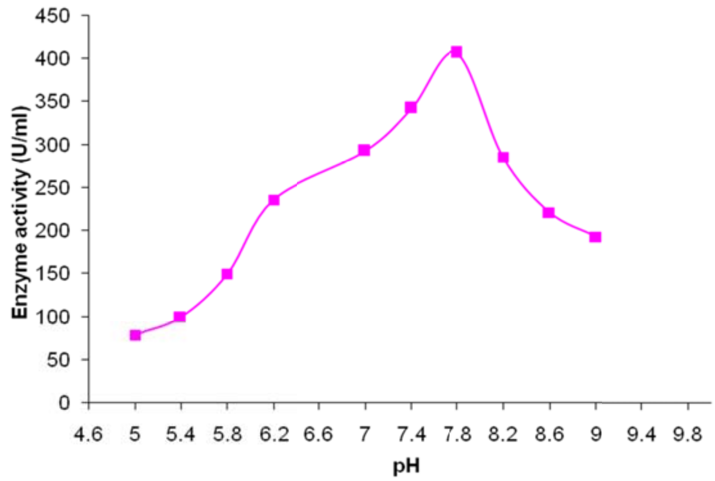

Figure 6. Effect of pH on purified APX activity.

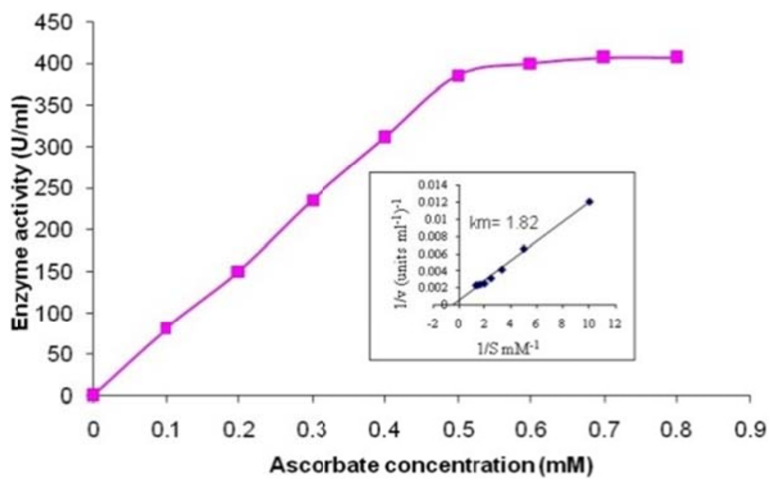

Figure 7. Effect of ascorbate concentration on activity of purified APX.

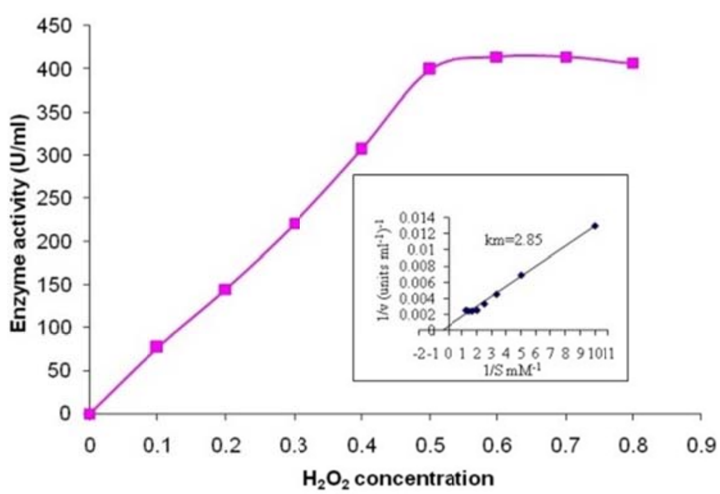

Figure 8. Effect of H_2O_2 concentration on activity of purified APX.

Table 1. Summary of purification of ascorbate peroxidase from ber (*Ziziphus mauritiana* Lamk.) fruits.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (Units) ×10^3	Specific activity (Units mg^{-1} protein)	Fold purification	Yield (%)
Crude extract	515	288	592.2	2055.5	_	100
35-65% $(NH_4)_2SO_4$ fractionation	30	61	310.4	5081.9	2.47	52.3
Sephadex G-100	35	6.8	280.1	41176.4	20.0	47.2
DEAE - Cellulose	46	2.1	205.9	97619.4	47.4	34.6

Table 2. Effect of various metal ions on the activity of purified APX.

Ion (1 mM)	Enzyme activity (% of control)
Control	100
K^+	113.5
Na^+	118.2
Mn^{2+}	64.8
Ca^{2+}	114.5
NO_3^-	83.4
Cl^-	109.6
SO_4^{2-}	76.1
Co^{2+}	90.7

optimum pH (potassium phosphate buffer). Similar pH optima have been reported for cytosolic APX from pea (*Pisum sativum* L.) shoots (1991) and from Japanese radish (Ohya et al., 1997). However, APX from rice showed broad pH optima between pH=6.0 to pH=7.0 (Lu et al., 2005).

Since the enzyme uses two substrates *viz*. ascorbate and H_2O_2, *Km* was determined for both the substrates Figure 7 and 8. The plot of enzyme velocity *vs*. substrate concentration showed a typical hyperbolic curve for both the substrates, thus, clearly indicating that the enzyme followed Michaelis-Menten Kinetics. From double reciprocal plots, *Km* of enzyme for acorbate and H_2O_2 was found to be 2.5 and 2 mM respectively. Lu et al. (2005) reported similar Michaelis-Menten Kinetics for APX from rice (*Oryza sativa* L.) with Km values of 4 mM and 0.3 mM for ascorbate and H_2O_2, respectively. From the Lineweaver-Burk plots of the purified APX from Japanese radish root, the apparent Km values for ascorbate and H_2O_2 were determined to be 770 and 130 μM, respectively (Ohya et al., 1997). However, Mittler and Zilinskas (1991) reported that Lineweaver-Burk plots of APX were not linear, thus indicating that the reaction does not follow Michaelis-Menten kinetics. Plots of ascorbate concentration versus velocity consisted of a sigmoidal saturation curve indicating a co-operative binding of ascorbate to APX from pea (*P. sativum* L.) shoots. However, plots of H_2O_2 concentration versus velocity were hyperbolic.

The effect of various metal ions on APX activity was studied by incubating the enzyme with various cations and anions at a concentration of 1 mM each at pH=7.5 (Table 2). Mn^{2+}, NO_3^-, SO_4^{2-} and Co^{2+} were found to be inhibitory as they inhibited the enzyme activity by 35.2, 16.6, 23.9 and 9.3%, respectively whereas K^+, Na^+, Ca^{2+} and Cl^- stimulated the enzyme activity and the respective stimulation was 13.5, 18.2, 14.5 and 9.6%. Dalton et al. (1987) found that APX purified from the root nodules was inhibited by KCN, NaN_3, CO and C_2H_2 were potent inhibitors of enzyme.

Activity of purified APX was observed in the presence of different concentrations of various inhibitors of specific groups with a view to identify the amino acid present at the active site (Markovic and Jornvall, 1986). Data presented in Table 3 shows that DEPC, DTT, $NaBH_4$ and mercaptoethanol inhibited the purified enzyme activity. However, the reaction rates with iodoacetate and DTNB were approximately equal to that of control. From the data of inhibition studies, it could be suggested that reducing agents such as mercaptoethanol and DTT could inhibit APX activity via hydrolysis of the disulphide bridges in the structure. DEPC inhibited the enzyme activity, suggesting that a functional histidyl residue may be essential for the catalytic activity of the enzyme and

Table 3. Effect of various inhibitors on the activity of purified APX (Units ml^{-1}).

Inhibitor	1mM	2mM	5mM
Mercaptoetanol	97.7	0	0
DTT	122.8	33.3	0
Iodoacetate	392.2	394.4	387.6
DEPC	0	0	0
N-Bromosuccinamide	320.4	272.9	210.3
DTNB	367.6	332.1	326.8
$NaBH_4$	0	0	0
Control	399.6		

that this residue is most likely to be present at or near the active site. Iodoacetate and DTNB, however, had no effect on enzyme activity thus ruling out the possibility of SH group involvement in the activation. N-bromo-succinamide slightly inhibited the enzyme activity at higher concentrations (2, 5mM). It suggests that, tryp-tophan residues are likely to be involved in the active site of the enzyme. Ohya et al. (1997) also reported that purified ascorbate peroxidase from roots of Japanese radish was not inhibited by the DTNB (0.1 mM) while DTT inhibited (50%) the enzyme activity. Similar results have been reported by Leonardis et al. (1995) who observed that iodoacetate had no effect on activity of purified ascorbate peroxidase from potato tuber mitochondria.

Conclusions

The aim of this study was to purify and characterize APX from ripening ber fruit. The enzyme was purified about 47.4 fold with 34.6% recovery. Our results show that the enzyme is a homodimer and 7.8 is the optimum pH. It is tentatively proposed that enzyme has histidine and tryptophan at the active site. Such studies will be invaluable in elucidating the function and role of APX in fruit ripening process.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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