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Research Article

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Partial Purification and Characterization of Peroxidase from Seedlings of Tomato

Ami Lokhandwala and Madhumati Bora*

Natubhai V Patel College of Pure and Applied Sciences, Vallabh Vidyanagar – 388 120, Gujarat, India
E-mail address: drmadhumatibora@gmail.com

ABSTRACT

Germination an important mechanism in seed physiology begins by imbibition followed by rapid increase in oxygen uptake and oxidative phosphorylation processes for which high energy cost is a prerequisite. Mobilization of food storage along with oxidative phosphorylation generates reactive oxygen species (ROS). Enzymes responsible for ROS scavenging are consequently of particular importance for the completion of seed germination process. Peroxidases (E.C.1.11.1.7) have been reported to have various physiological roles such as oxidation of wide range of biomolecules by accumulation of active forms of oxygen. In present study, Peroxidase was partially purified 1.42 fold from Lycopersicon esculentum Mill seedlings with 1.3% yield by 70% ammonium sulphate precipitation and dialysis. The substrate specificity was checked with Pyrogallol (1, 2, 3-trihydroxybenzene), o-dianisidine (4-(4-amino-3-methoxyphenyl)-2 methoxyaniline) and TMB (3, 3', 5, 5'-Tetramethylbenzidine) substrates. Km and Vmax values with all the three substrates were calculated from Lineweaver- Burk graphs. Among the substrates tested, highest specificity constant and rate of reaction was obtained by oxidation of o-dianisidine which is 181800 µM and 90.9µmoles/min/ml respectively. Optimum pH, optimum temperature, optimum ionic strength, pH stability, temperature stability conditions determined for o-dianisidine/H_2O_2 substrate pattern were found to be 6.0, 50° C, 0.1, 9.0 and 25° C to 50° C respectively.

Keywords- *Peroxidase, Lycopersicon esculentum Mill, enzyme partial purification, enzyme characterization*

Abbreviations *– Peroxidase/s (POD/s), TMB (3, 3', 5, 5'-Tetramethylbenzidine)*

INTRODUCTION

Seed germination and seedling development are two vital stages in crop establishment[1]. Various processes like membrane reorganization and metabolic reactivation that occur at this stage can have profound influences on seed germination[2]. PODs (E.C.1.11.1.7), enzymes categorized under oxidoreductases are haem proteins and contain iron (III) protoporphyrin IX (ferriprotoporpyrin IX) as the prosthetic group. Oxidation of a variety of organic and inorganic compounds and reduction of peroxides, such as hydrogen peroxide is catalyzed by PODs[3]. Antioxidative POD enzyme system catalyzes peroxidative damage of cell. PODs play significant physiological roles in plants, animals and microorganisms[3-5]. Upon infection or wound in plants, POD participates in the formation of phenolic polymers such as lignins, suberins etc. [6-7] and also in formation of lignins in the secondary cell walls during normal growth[8].

Applications of POD have been recommended in the medicinal, chemical and food industries[9]. POD is also widely used for clinical diagnosis and microanalytical immunoassays because of its high sensitivity. High thermo stability and involvement in the oxidation of many organic compounds make POD to establish comfortably in many plant based foods[10]. Duarte-Vazquez[11] reported its profitable use in ELISA.

Some of the novel applications of PODs include synthesis of various aromatic compounds and removal of peroxide from food stuff and industrial wastes[12]. POD is also implicated in fruit ripening and enzymatic

browning, either or together with polyphenol oxidase activity. For a more effectual management of these adverse reactions specifically in heat processed food where residual POD is a regular observation, a well defined understanding of POD is desirable[7, 13]. The major source of commercially available POD is roots of horseradish but it is also present in varied number of plant species. So, availability of PODs with different specificity would promote the development of new analytical methods and potential industrial processes[7].

Tomato is the world's largest vegetable crop because of its wide spread production and as protective food because of its special nutritive value. Tomato is one of the most important vegetable crops cultivated for its fleshy fruits. Earlier, POD have been partially purified and characterized from roots of tomato[14], ripe and unripe fruits of tomato[15], skin of maturing tomato fruit[16]. A single POD in tomato fruit extracts also exhibiting some IAA oxidase activity[17, 18] has been implicated in the production of ethylene[19-20]. Plant resistance mechanisms are frequently associated with up-regulation or down-regulation of oxidative defense enzymes such as superoxide dismutase, catalase, and POD[21-29].

Many reports have suggested that host-pathogen interaction results in increased POD activity followed by non-specific induction of plant resistance[30].

The objective of present study was partial purification and characterization of POD from 2- week- old seedlings of *Lycopersicon esculentum* Mill and to check its substrate specificity.

MATERIALS AND METHODS

Plant material and chemicals

Seeds of *Lycopersicon esculentum* Mill were procured from Seedco Company, Jalna, India and were stored at 4° C until used. All the chemicals used were of analytical grade obtained from HiMedia Laboratories, Mumbai, India.

Seed germination

Tomato seeds were grown in autoclaved sand containing 0.5x MS salts[31]. Sowing of seeds was done in medium sized plastic cup at 0.5 inch distance in autoclaved sand. These cups were incubated in dark until they start germinating. Seeds were allowed to grow for 2 weeks in natural conditions. Two week- old-seedlings were harvested for further experiments.

Preparation of homogenate

The crude extract for POD was prepared using 0.2gm/ml of plantlet[32]. For this, 2- week-old 20 g Lycopersicon *esculentum* Mill seedlings were homogenized in 100 ml ice-cold 0.1 M sodium phosphate buffer (pH 6.0) in a chilled pestle and mortar using sand as an abrasive. The homogenate was strained through two folds of muslin cloth and centrifuged at 16,000 x g for 20 min at 4^{o} C in Remi made C-24 BL cooling centrifuge. Supernatant thus obtained was used as enzyme source.

Ammonium sulphate fractionation and dialysis

Crude homogenate was subjected to 0-80% (w/v) saturation with ammonium sulphate at cold conditions. The saturated solution was left overnight at 4° C and the precipitated protein was sedimented by centrifuging at 7000 x g for 10 min at 4° C. The pellet was dissolved in 2 ml of phosphate buffer (pH 6.0, 0.1 M). The concentrated sample with maximum specific activity was dialyzed for 8 h against phosphate buffer (pH 6.0, 0.1 M) for further use[3].

Protein determination

Total protein was estimated quantitatively by absorbance measurements at 550 nm following Lowry's method[33] with Bovine serum albumin as standard[34-35].

POD assay

The POD activity in *Lycopersicon esculentum* Mill seedlings was measured using o-dianisidine as substrate[32]. The homogenate was prepared as above mentioned method. The assay mixture contained 1ml of 0.01 M o-dianisidine solution, 200 µl of enzyme extract, 0.5 ml of 0.02 M Hydrogen Peroxide and 1 ml of Potassium Phosphate buffer (pH 6.0, 0.1 M). The amount of oxidized o-dianisidine released was monitored spectrophotometrically every 30 seconds for 3 min at 430 nm. Optical absorption was recorded

on UV-VIS 1800 spectrophotometer (Shimadzu). One unit of enzyme activity is defined as µmoles of enzyme used per µl of substrate per min.

Native PAGE electrophoresis

Native Polyacrylamide Gel Electrophoresis (PAGE) was performed according to modified Laemmli's procedure[37] under native conditions. The experiment was conducted using 5% stacking gel, 10% separating gel and Tris/Glycine (25mM, pH 8.3) as electrode buffer. Samples were prepared by mixing 20 ul of homogenate prepared by above mentioned method and 20 µl of loading dye(3.5 ml D/W, 1.25 ml 0.5 M Tris HCl, 2.5 ml Glycerol, 2 ml 10% SDS and 0.2 ml 0.1% BPB). 10 ul of these samples were loaded. Electrophoresis (Bio-Rad made Mini-PROTEAN Tetra cell) was performed at 100 V until the dye migrated at the distance of 1 cm from bottom. After electrophoretic run, gel was stained separately with all the three substrates: o-dianisidine, pyrogallol and TMB. For o-dianisidine the gel was incubated in 0.1 M Acetate buffer (pH 4.6) containing 10% o-dianisidine for 30 min and then transferred to 0.1 M Acetate buffer (pH 4.6) containing 0.03% hydrogen peroxide until brownish red coloured bands appear[36] (modified method). For TMB, gel was incubated in 3 mM TMB, 75 mM sodium acetate and 30% methanol (pH 5.2) for 45 min and then H_2O_2 to final concentration of 30 mM was added and incubated till blue bands appear[38]. For pyrogallol, gel was incubated in 50 mM sodium phosphate buffer (pH 7.0) for 30 min and then H_2O_2 to final concentration of 4 mM and pyragallol to final concentration of 20 mM was added in the same buffer and incubated till bands appeared[39].

Molecular weight determination

Molecular weight of POD isoenzymes was determined by graphical method [40]. The standard proteins used for Native PAGE were Ovalbumin (43 KDa), Trypsin Soyabean Inhibitor (20.1 KDa) and Lactoglobulin (18.4 KDa). The protein bands were stained with Coomassie Brilliant Blue R-250. The results obtained were co-related to the Molecular weight determined by AlphaEaseFC software.

Kinetic studies

Optimum pH and pH stability profile

The optimum pH value for partially purified POD activity was estimated by assaying enzyme activity at different pH levels. The test was carried out in the presence of buffers with different pH such as 0.2 M Glycine-HCl buffer (pH 2.8 and 3.6), 0.1 M Sodium Phosphate buffer (pH 5.8 to 8.0) and 0.2 M Glycine-NaOH buffer (pH 9 and 10) separately in an assay mixture. The pH stability for POD was assayed for pH ranging from 2.8 to 10 for 8 days. For this 0.5 ml of enzyme extract and 0.5 ml of respective buffer was incubated. Residual enzyme activity was measured by above mentioned method at every 24 hrs [41] (modified method).

Optimum temperature and temperature stability profile

The enzyme activity for partially purified POD was measured at different temperatures in the range of 10° C to 80° C. In order to determine thermal stability, 500 µl of partially purified POD was assayed at fixed time intervals up to 3 hours for above mentioned temperatures. Residual activity was assayed by above mentioned method and compared with unheated enzyme[42].

Effect of ionic strength

The effect of ionic strength was assayed using sodium phosphate buffer (pH 6.0) of different molarities (0.05M to 3 M) [41].

Substrate specificity

Under optimal conditions, the efficiency of catalytic oxidation of o-dianisidine[32], pyrogallol[42] and TMB[16] by hydrogen peroxide in presence of POD was evaluated. Km and Vmax values were calculated for each substrates using Lineweaver-Burk transformation of the Michaelis-Menten equation. Specificity constant (Vmax/Km) for each substrate was also calculated.

All the experiments were performed in triplicates.

RESULTS

Ammonium sulphate fractionation and dialysis

The fraction containing 70% ammonium sulphate showed maximum specific activity of 1381.3 µmoles /min/ml. This primary purification step resulted in 1.06 fold purification. The specific activity increased to 1970.0µmoles/min/ml after 8 hours of dialysis with1.42 fold purification **(Table 1).**

Table 1 Level of partial purification of *Lycopersicon esculentum* Mill POD obtained after the application of different purification steps.

Purification steps	Total enzyme volume (ml)	Enzyme activity (µmoles/min)	Total Enzyme activity µmoles/min/ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (µmoles/min/ml/mg of protein)	Yield (%)	Purification fold
Crude Homogenate	100	859.225	859225	0.622	62.2	1381.3	100	1
Ammonium sulphate precipitation and Dialysis	6	1895.7	11374.2	0.962	5.772	1970	1.3	1.42

Native PAGE electrophoresis and Molecular weight determination

Equal number of bands appeared for o-dianisidine and TMB, while no bands appeared with pyrogallol. So, partial purification was carried out using o-dianisidine as substrate as it was more specific than TMB. Partially purified POD showed 3 bands in comparison to 6 bands from crude sample **(Figure 1).** The electrophoretic pattern of Native standard markers is shown in Lane 1, 2 and 3 of **Figure 1.**Graph of R_f calculated from gel and Log MW of standard proteins was plotted. The molecular weight of all the three isoenzymes were calculated using the formula obtained from the graph:

R_f= -0.8448 x Log $_{MW}$ + 1.880 R^2 = 0.9614

Molecular weights obtained by graphical method were 25 KDa; 44 KDa and 49 KDa, while using the same gel pattern calculated from AlphaeaseFC software **(Figure 2)** also were 24.56 KDa, 42.98 KDa and 49.70 KDa. Molecular weights of all the three POD isoenzymes obtained from the above method and from the AlphaeaseFC software support with each other **(Table 2).**

Figure: 1. Native PAGE of Crude POD and Partially Purified POD from *Lycopersicon esculentum* Mill. Lane 1- Lactoglobulin (18.4 KDa), Lane 2- Trypsin Soyabean Inhibitor (20.1 KDa), Lane 3- Ovalbumin (43 KDa), Lane 4- Partially purified enzyme and Lane 5- Crude enzyme.

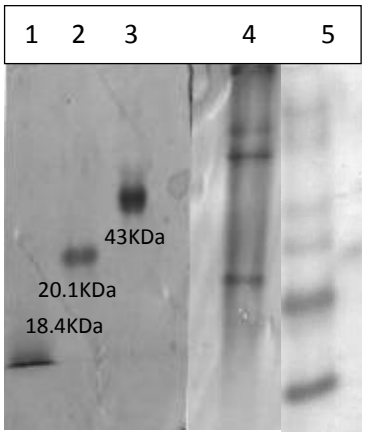


Figure: 2. Molecular weight determination from AlphaEaseFC software.

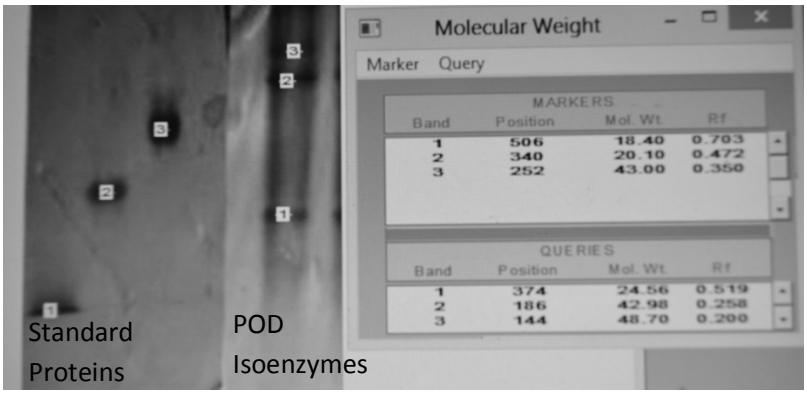


Table 2 Molecular weight of all the three POD isoenzymes obtained from the above method and from the AlphaEaseFC software

Isoenzymes	Molecular Weight(KDa)	
	Graphical method	AlphaEaseFC software
Band1	25	24.56
Band 2	44	42.98
Band 3	49	49.70

Kinetic studies

The optimum pH found by assaying enzyme activity at different pH levels was 6.0 in 0.1M Sodium Phosphate buffer. The activity measured was 607.411 µmoles/min/ml **(Figure 3 and Table 3).** POD was found more stable between the range of pH 6 to pH 9 in 0.1 M sodium phosphate buffer after incubating for 8 days **(Figure 4a, 4b and Table 3).** The optimum temperature measured by assaying enzyme activity at various temperatures was 50° C. The activity obtained was 1616.26µmoles/min/ml **(Figure 5 and Table 3).** The POD enzyme was more stable between the range of 25° C to 50° C **(Figure 6a, 6b, 6c** and **Table 3).** The optimum molarity for POD assayed was 0.1 M Sodium Phosphate buffer (pH 6.0). The activity measured was 304.76 µmoles/min/ml **(Figure 7 and Table 3).** Km and Vmax values from Lineweaver-Burk plots were calculated for o-dianisidine (0.0005 µM and 90.9 U/min/ml), pyrogallol (0.00487 µM and 0.00326 U/min/ml) and TMB (0.1 µM and 10 U/min/ml) substrates respectively. Also, Vmax/Km values for all the three substrates were calculated which were, 181800, 0.006 and 100 respectively **(Figure 8a, 8b, 8c and Table 3).** Vmax/Km values (181800) calculated for o-dianisidine was higher than other two substrate indicate that POD enzyme partially purified from *Lycopersicon esculentum* Mill is most specific for o-dianisidine substrate and shows highest rate of reaction with the same.

Figure: 3. Optimum pH profile for POD from *Lycopersicon esculentum* Mill

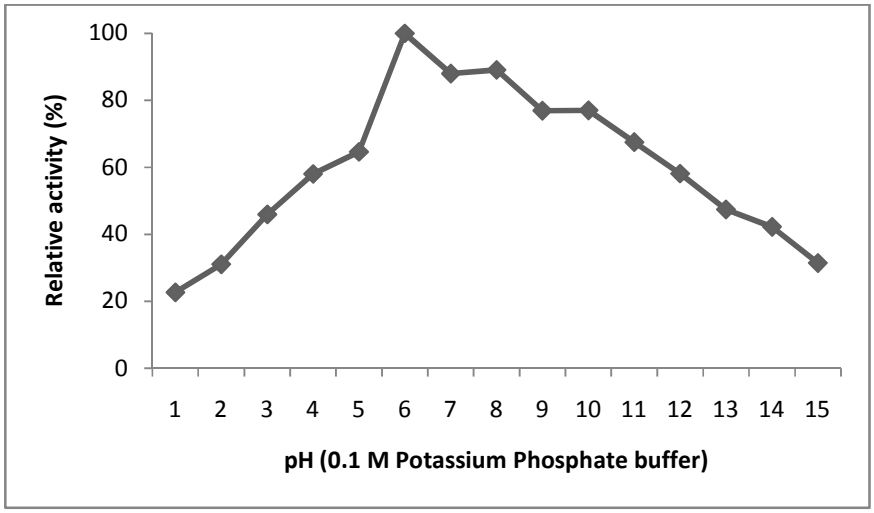


Table 3 Kinetic properties of partially purified POD from *Lycopersicon esculentum* Mill

Kinetic properties	o-dianisidine
Optimum pH	6.0
Stable pH	9.0
Optimum temperature	50^{o} C
Stable temperature range	25^{o} C to 50^{o} C
Optimum Molarity of Potassium phosphate buffer (pH 6.0)	0.1
Km(µM)	0.0005
Vmax(U/min/ml)	90.9
Vmax/Km	181800

Figure. 4a: pH stability profile for POD from *Lycopersicon esculentum* Mill (pH 2.8 to 6.8).

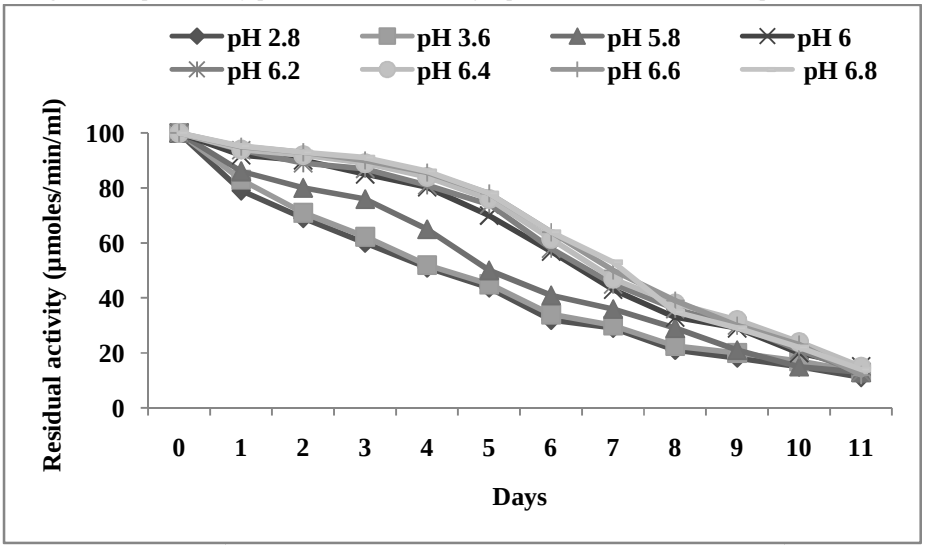


Figure. 4b: pH stability profile for POD from *Lycopersicon esculentum* Mill (pH 7 to 10)

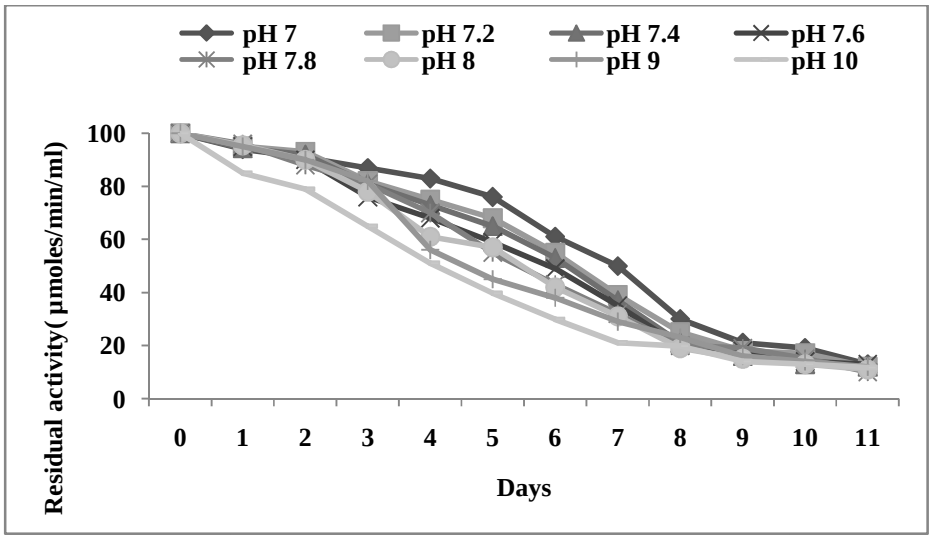


Figure. 5: Optimum temperature profile for POD from *Lycopersicon esculentum* Mill

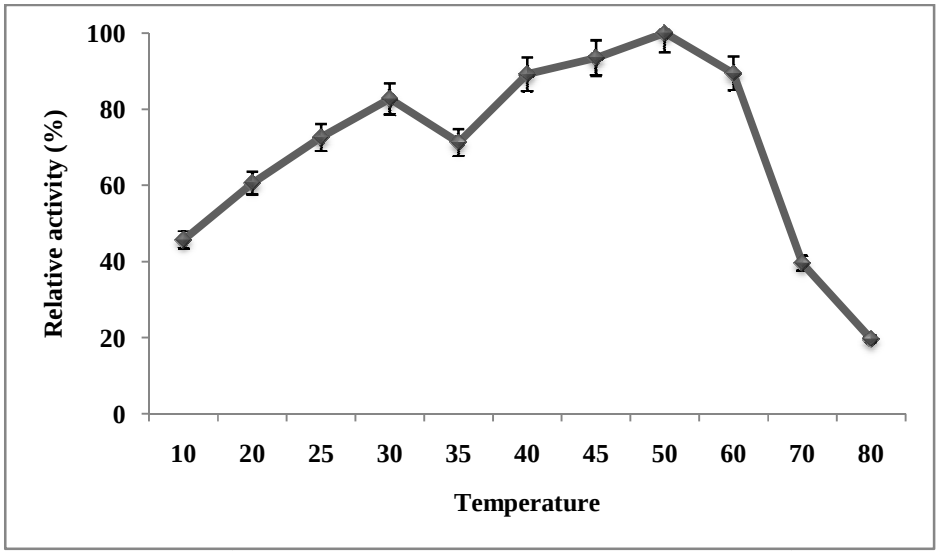


Figure. 6a: Temperature stability profile for POD from *Lycopersicon esculentum* Mill

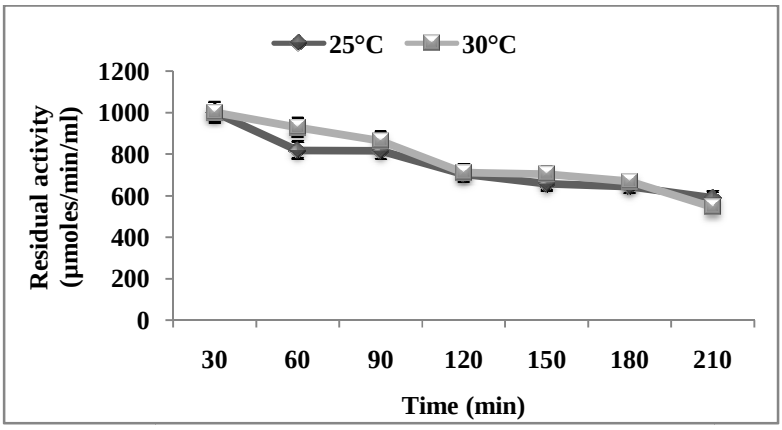


Figure. 6b: Temperature stability profile for POD from *Lycopersicon esculentum* Mill

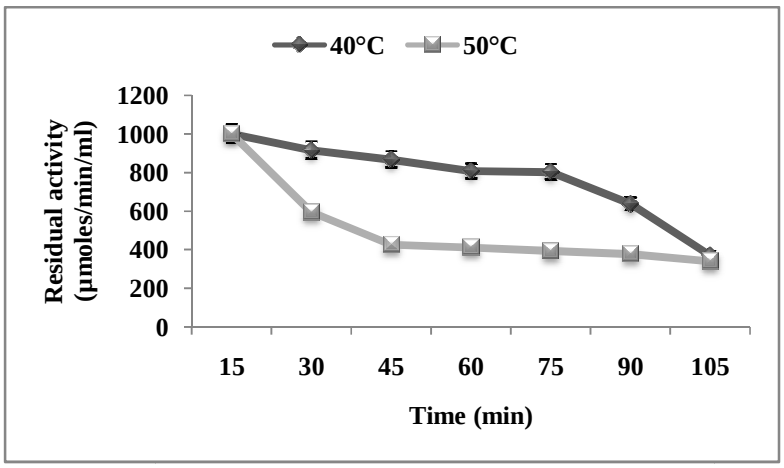


Figure. 6c: Temperature stability profile for POD from *Lycopersicon esculentum* Mill

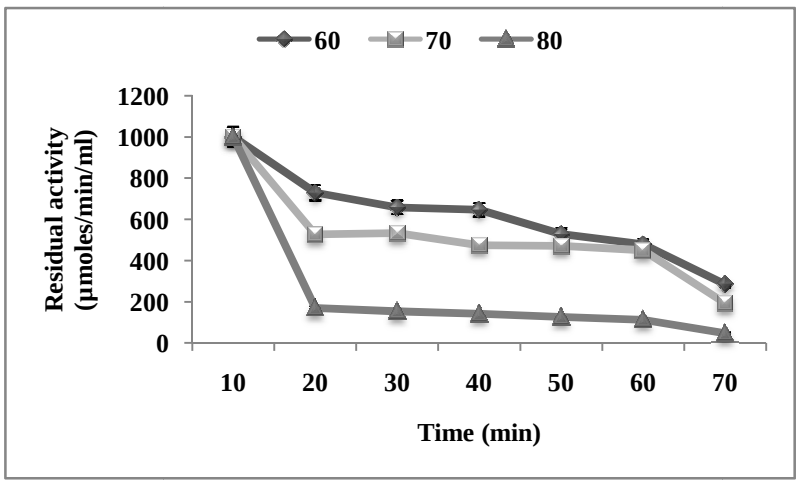


Figure. 7: Molarity optima profile for POD from *Lycopersicon esculentum* Mill

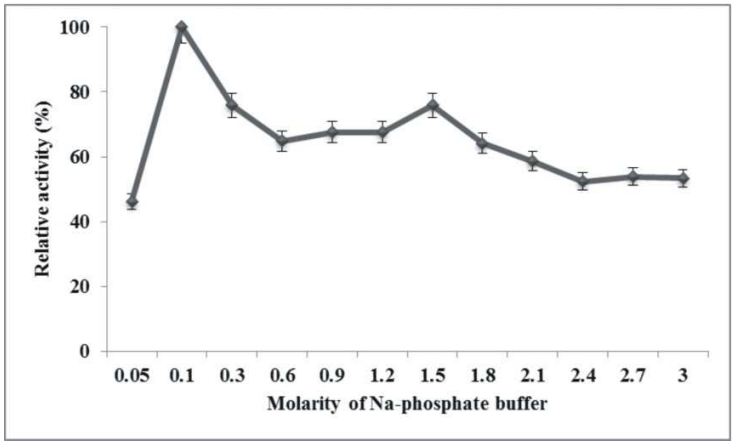


Figure. 8a: Double reciprocal plot for pyrogallol oxidation by the POD from *Lycopersicon esculentum* Mill

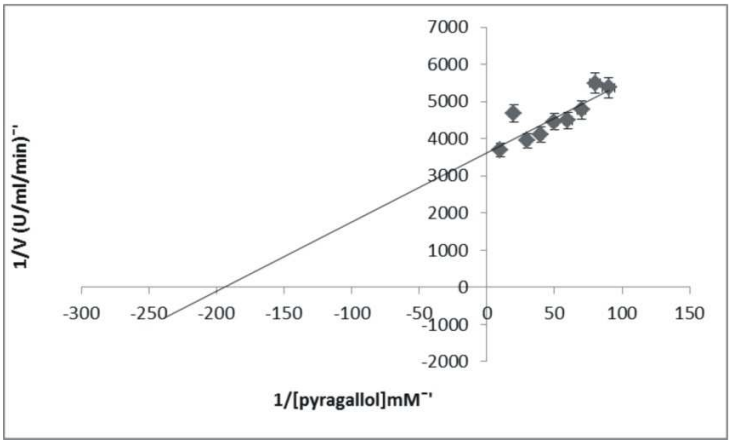


Figure. 8b: Double reciprocal plot for o-dianisidine oxidation by the POD from *Lycopersicon esculentum* Mill

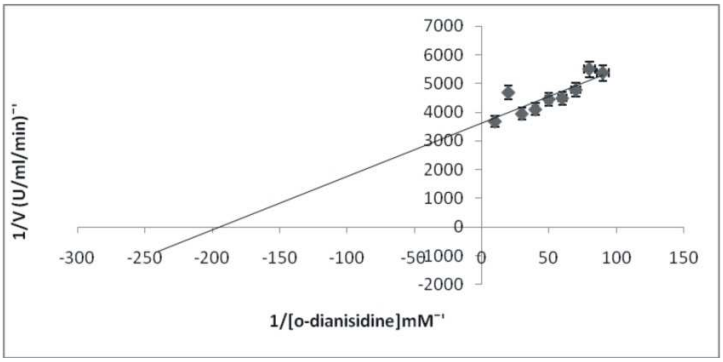
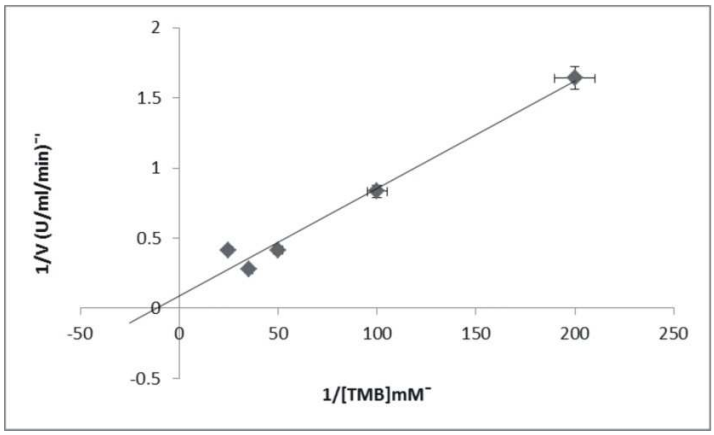


Figure. 8c: Double reciprocal plot for TMB oxidation by the POD from *Lycopersicon esculentum* Mill



DISCUSSION

Linear plots of Rf values calculated from Polyacrylamide Gel against Log MW of standard markers and AlphaeaseFC software are viable methods to characterize molecular weight within a system. Wide range of molecular weights of POD (36 to 120 KDa) from various plant sources have been reported[43]. It has also been reported that molecular weights of POD isoenzymes are in range of 35 to 105 KDa for plants of Solanaceae family[43-45]. In the present study molecular weight of three isoenzymes obtained from partial purification of POD from tomato seedlings was determined. Partial purification was carried out by 70% ammonium sulphate precipitation and then the extract was dialyzed against Sodium Phosphate buffer (pH 7.0, 0.1 M). The purification obtained was 1.42 fold with 1.3% yield (Table 1). POD has been purified from tomato fruit skin with 1 fold purification after dialysis[16] and with 2.2 fold purification using ion exchange chromatography from tomato[46]. The molecular weight was determined by Native PAGE according to modified Laemmli's method[37]. As can be seen in Figure 1, a R_f - Log MW graph was obtained using Lactoglobulin (18.4 KDa), Trypsin Soyabean Inhibitor (20.1 KDa) and Ovalbumin (43 KDa) as standard proteins. Molecular weights calculated by graphical method (25 KDa, 44 KDa and 49 KDa) and molecular weights determined using AlphaEaseFC software (24.56 KDa, 42.98 KDa and 49.70 KDa) corresponded with each other. POD isoenzymes are purified from tomato exocarp in range of 43 to 58 KDa [16]. Two isoenzymes from tobacco leaves each in form of single subunit with molecular weight of 35 KDa had also been purifired[44]. Also, four isoenzymes of 105 KDa, 94 KDa, 56.5 KDa and 48.5 KDa have been isolated from potato[45]. This similarity among the range of molecular weights may be due to same family of plants. Although, it is also found that POD purified from fresh cauliflower (*Brassica oleracea* L.) buds had molecular weight of 44 KDa though it belongs to a different family[7]

It is known that optimum pH of POD enzyme depends on the substrate used. In the present study, optimum pH obtained with oxidation of o-dianisidine and hydrogen peroxide as substrates was 6.0. It has been found that optimum pH of POD purified from cauliflower (*Brassica oleracea* L.) was between 4 to 9 with ABTS (2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and 2,6-DMP(2,6-Dimethoxyphenol) as substrates[47]. For guaiacol oxidation, optimum pH of POD purified from *R. sativus* is 6.0[41]. In present study enzyme was stable between the ranges of pH 6.0 to 9.0. It has been shown that POD purified from Turkish black radish (*R. sativus* L) was stable between the ranges of pH 4.0 and 9.0[41]. The optimum temperature for POD in present study with o-dianisidine oxidation was 50° C. POD obtained from Lettuce (*Lactuca sativa L*) had highest activity at 45° C[48]. In our study thermal stability of POD from tomato seedlings was determined wherein the enzyme was stable in the range of 25° C to 50° C. Purified POD from *R. sativus* L was stable in the range of 20° C to 40° C with guaiacol as substrate [41]. The optimum ionic strength of POD observed in this study is 0.1 M sodium phosphate buffer. POD from *Copaifera langsdorffii* leaves was purified in 0.05 M phosphate buffer[49] with guaiacol as substrate, while optimum ionic strength was 0.1 M of phosphate buffer for purified POD from Turkish Black Radish[41]. Km and Vmax values for pyrogallol/ H_2O_2, o-dianisidine/ H_2O_2, TMB/H2O2 substrate pair were determined. The enzyme was most specific with o-dianisidine/ H_2O_2 substrate pair (Km = 0.0005 µM) with highest rate of reaction (90.9 U/min/ml) among the three substrate pairs considered. Vmax and Km values calculated from tomato fruits were 2.86 mM and 0.971 mM respectively using guaiacol/ H_2O_2 substrate pair [15], however, from peach fruit using o-dianisidine /H_2O_2 was 9.35 mM and 15.38 mM respectively[50].

CONCLUSION

Present study concludes that *Lycopersicon esculentum* Mill seedlings produces substantial amount of POD with high specificity for o-dianisidine substrate. The partially purified substrate also showed better thermal stability indicating its extensive application in fields like chemical diagnostics and peroxide removal. Also, PODs behave kinetically dissimilar with distinct substrate indicating their different physiological functions.

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