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NOTE

Purification of a peroxidase from *Solanum melongena* fruit juice

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Solanum melongena fruit juice contains peroxidase activity of the order of 0·125 IU/mL. A method for the 11-fold purification of the enzyme was developed. The K_m values of the peroxidase for the substrates guaiacol and hydrogen peroxide were 6·5 m*M* and 0·33 m*M*, respectively. The *p*H and temperature optima were 5·5 and 84°C, respectively using guaiacol as the substrate. Sodium azide and phenyl hydrazine inhibited the enzyme competitively.

Keywords: Peroxidase, *Solanum melongena*, fruit

Peroxidases [E.C.1.11.1.7] are heme-containing enzymes that use H_2O_2 to oxidize a large variety of hydrogen donors such as phenols[1,2], aromatic amines[3], ascorbic acid, indole and certain inorganic ions. These enzymes are widely distributed in the plant kingdom[4-8] in a variety of tissues and are also found in some animal tissues and microorganisms[9-11]. They perform a variety of physiological functions like lignification of cell wall and in defense mechanism against pathogenic attacks[12]. Some peroxidases play a crucial role in delignification of lignocellulosic materials[13] and in degradation of recalcitrant organic pollutants[14]. Peroxidases have received extensive attention in the recent years as biocatalysts for synthetic applications in biotransformations[15-19]. They catalyze H_2O_2-dependent oxidation of aromatic compounds, oxidation of heteroatoms, epoxidation, and enantioselective reduction of racemic hydroperoxides.

In view of the biocatalytic potential[15-18] of peroxidases of different sources, in the present study, we have analyzed the *Solanum melongena* fruit juice for peroxidase activity. Purification of the enzyme from this source and some kinetic properties have been reported.

Materials

Guaiacol was from Sigma Chemical Co., St. Louis, USA and veratryl alcohol was from Aldrich Chemical Co., Wisconsin, USA. All other chemicals were from S. D. Fine Chemicals Ltd., Mumbai and were used without further purification.

Methods

The enzyme was isolated by cutting the *Solanum melongena* fruit into small pieces, crushing them in mortar with pestle and filtering the juice through four layers of cheese cloth. The filtered juice was then saturated upto 60% with ammonium sulphate and centrifuged using refrigerated centrifuge (model 3K 30, Sigma, Germany) at 4000 *g* for 20 min at 4°C. The precipitate was discarded and the supernatant was saturated up to 90% by further addition of ammonium sulphate. The resulting suspension was centrifuged, by repeating the same process of centrifugation and the supernatant was discarded. The precipitate was dissolved in 0·2 *M* sodium acetate/acetic acid buffer (*p*H 4·5) and dialyzed against 10 m*M* NaH_2PO_4/Na_2HPO_4 buffer (*p*H 7·0), with three changes at the intervals of 6 h.

The dialyzed enzyme (5 mL) containing 6 mg/mL protein was loaded to a Sephadex G-100 column (size 2·6 cm × 60 cm) equilibrated with 100 m*M* phosphate buffer (*p*H 7·0). The enzyme activity was eluted as the first peak and impurities were eluted after that. The specific activity of the enzyme which was 0·28 IU/mg, before loading on the column, reached up to 3·13 IU/mg after elution. The total enzymes units loaded on the column were 8·4 IU, of which 5·2 IU was recovered in 30 mL of the most active pooled fractions, showing a recovery of 62%. The homogeneity of the purified enzyme was checked using SDS-PAGE, but tight protein band could not be obtained and instead, continuous streaking in the gel lanes was obtained, making the results inconclusive. The native-PAGE was also performed, but conclusive results could not be obtained. Purity of the enzyme was checked, by passing through a second Sephadex G-100 column. The elution profile showed a single protein and activity peak, having the same specific activity, as the loaded protein showing that the enzyme was pure.

Peroxidase activity of the enzyme was measured in 50 m*M* NaH_2PO_4/Na_2HPO_4 buffer (*p*H 7·0) at 30°C

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using guaiacol (5 m*M*) and H_2O_2 (0·6 m*M*) as substrates and by monitoring the absorbance changes at 470 nm using molar extinction coefficient value of $2{\cdot}66 \times 10^4$ M^{-1} cm^{-1} for the product tetraguaiacol formed by the enzymatic reaction[19,20]. All spectrophotometric measurements were done with UV/vis Spectrophotometer (Hitachi, Japan, model U-2000), which was fitted with electronic control unit for the variation of temperature in the cuvettes. The least count of the absorbance measurement was 0·001 and one enzyme unit was defined as the amount of enzyme that produced 1 µ mole/min of the product. Protein estimation was done by Lowry method[21].

The *p*H optimum was determined by measuring the relative activity of the enzyme in the *p*H range 2.5-8.5 using buffers prepared with phosphoric acid/ NaH_2PO_4/Na_2HPO_4. Each point on the curve was an average of triplicate measurements and the percentage standard deviation was 2·1. The temperature optimum was determined by measuring the relative activity of the enzyme in the temperature range 20-90°C. Before each measurement, the reaction mixture in the spectrophotometer cuvette was allowed for 10 min for temperature equilibration and the reaction was initiated by the addition of 20 µL of concentrated enzyme stock having 0·125 enzyme unit/mL.

The effect of inhibitors on the activity of the enzyme was studied by monitoring the steady-state velocity of the enzyme-catalyzed reaction in the presence of varying concentrations of sodium azide (0-0.2 m*M*) using 5 m*M* guaiacol and 0·6 m*M* H_2O_2 in 50 m*M* NaH_2PO_4/Na_2HPO_4 (*p*H 5·5) at 30°C. The inhibition constant was determined by drawing double reciprocal plots at different concentrations of the inhibitors and then drawing secondary plots of the slopes vs the concentration of the inhibitors.

Ligninperoxidase activity of the enzyme was tested using veratryl alcohol (2 m*M*) and H_2O_2 (0·4 m*M*) in 50 m*M* sodium phosphate buffer (*p*H 2·0) at 30°C by monitoring the absorbance changes at 310 nm, due to the formation of the product veratraldehyde and using molar extinction coefficient value of $9{\cdot}3 \times 10^3$ M^{-1} cm^{-1} [22]. H_2O_2 used in the case of peroxidase and ligninperoxidase activity was freshly prepared each time by measuring absorbance at 240 nm of 100 dilution solution of 30% H_2O_2 stock using molar extinction coefficient value of 39·4 M^{-1} cm^{-1} and suitably diluting the solution.

Results and Discussion

Fig. 1a shows the elution profile of crude enzyme preparation, loaded to the first Sephadex G-100 gel filtration column. It is obvious from the figure that the enzyme activity came as a first peak, separated from the bulk impure protein. Fig. 1b shows the elution profile of purified enzyme preparation, loaded to a second Sephadex G-100 column. The presence of a single activity and protein peak was taken as the evidence of relative purity of the protein, because the results of SDS-PAGE and native-PAGE were inconclusive.

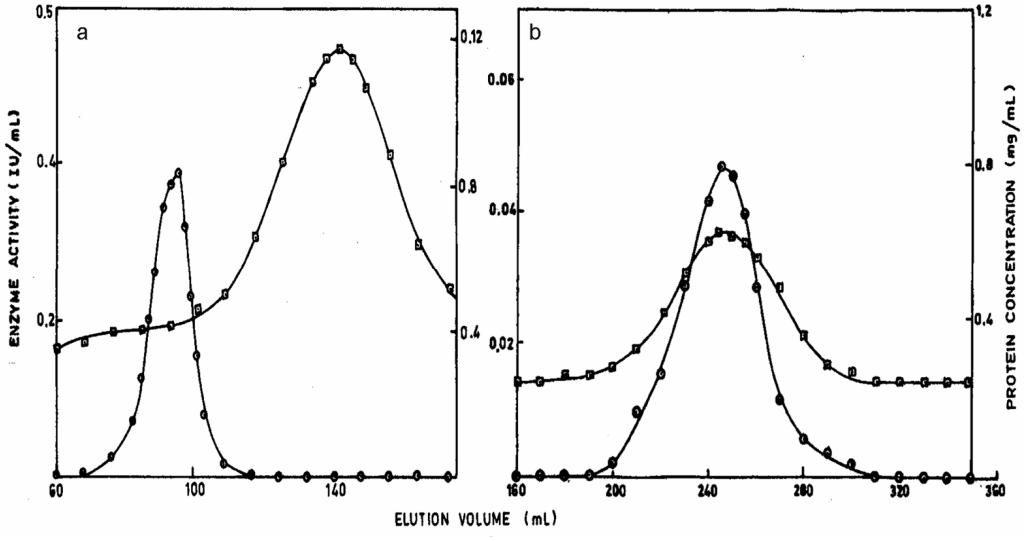

Fig. 1—Elution profiles of *Solanum melongena* fruit juice peroxidase from Sephadex G-100 gel-filtration column (2·6 cm × 60 cm) [(a): Elution profile for crude peroxidase; and (b): purified peroxidase; (O) activity profile; and (□) protein profile]

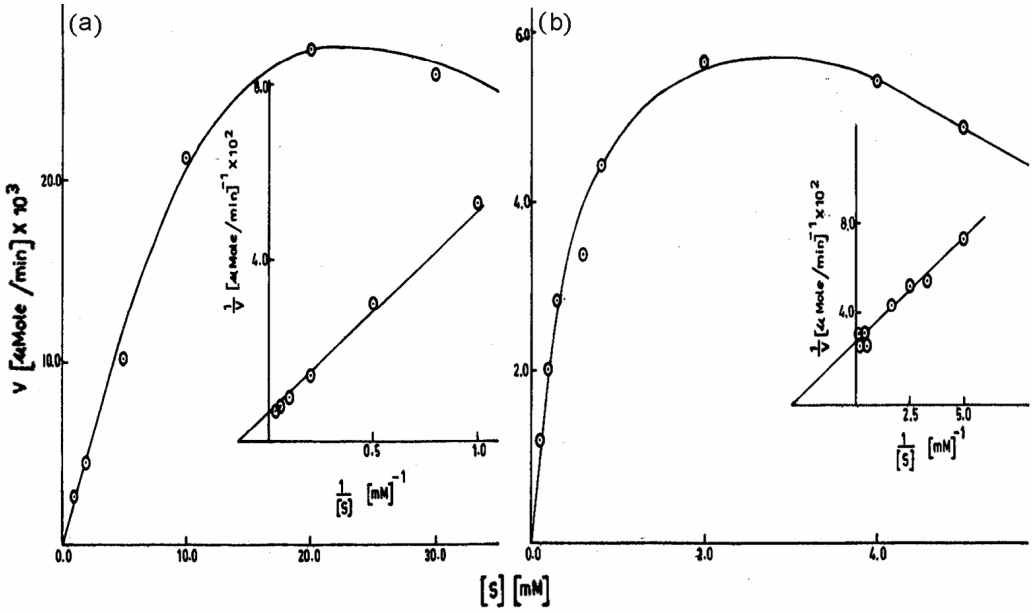

Fig. 2—Michaelis-Menten and double-reciprocal plots for the peroxidase [(a) H_2O_2 fixed at 0.6 m*M* and guaiacol varied; and (b) guaiacol fixed at 20 m*M* and the H_2O_2 varied. Assay solutions contained guaiacol and H_2O_2 at appropriate concentrations in 50 m*M* sodium phosphate buffer (*p*H 5·5) at 30°C and 20 µL of concentrated enzyme stock having 0·125 enzyme unit/mL was added; (a) Michaelis-Menten plot; and (b) double-reciprocal plot]

Figs 2a and b show the Michaelis-Menten curves using guaiacol and H_2O_2 as the variable substrates, respectively at the saturating concentration of other substrates (H_2O_2 0·6 m*M* and guaiacol 20 m*M*) for the determination of K_m values. The double-reciprocal plots shown as insets in both the cases were linear, showing that the enzyme obeyed Michaelis-Menten kinetics[23]. The calculated K_m values for guaiacol and H_2O_2 were 6·5 m*M* and 0·33 m*M*, respectively at 30°C. The double-reciprocal plots obtained by varying the concentration of guaiacol at three different fixed concentrations of H_2O_2 and by varying the concentration of H_2O_2 at different fixed concentrations of guaiacol were parallel lines, showing that peroxidase followed double displacement-type kinetics[22], which is usual for peroxidases.

Figs 3 and 4 show the variation of the enzymatic activity with *p*H and temperature, respectively. The enzyme had a *p*H optimum at 5·5 and showed more than half of the maximum activity in the *p*H range 3·5 to 8·5. The temperature optimum of the enzyme was 84°C (Fig. 4) and hence the enzyme could be used at relatively higher temperatures.

The effect of sodium azide and phenyl hydrazine, which are known to inhibit peroxidase activity[16] was

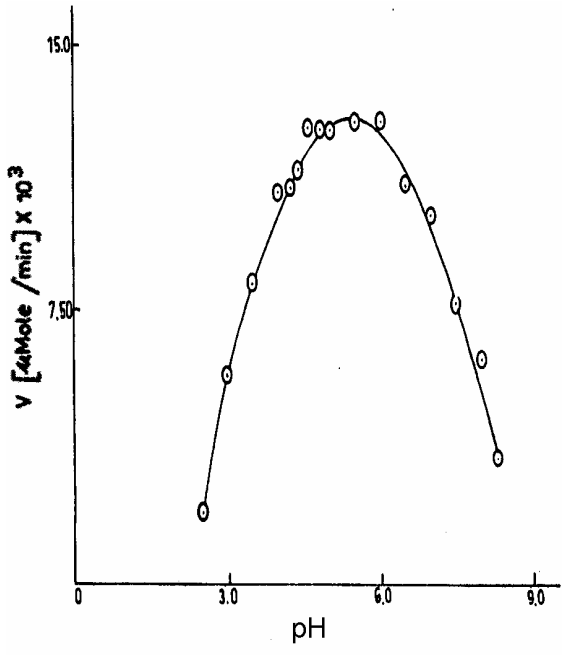

Fig. 3—Dependence of the enzyme activity on *p*H of the assay solution [Assay solution contained 5 m*M* guaiacol, 0·6 m*M* H_2O_2 at 30°C and 20 µL of concentrated enzyme stock having 0·125 enzyme unit/mL was added; *p*H of the reaction medium varied]

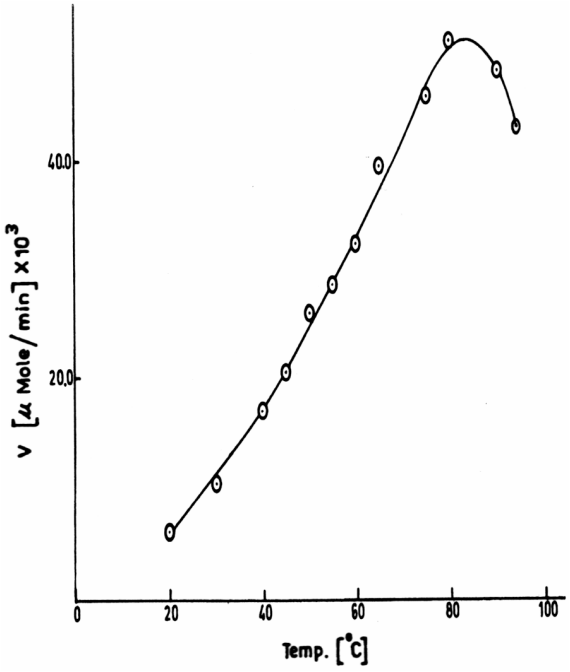

Fig. 4—Dependence of the enzyme activity on temperature of the assay solution. [Assay solution contained 5 m*M* guaiacol, 0·6 m*M* H_2O_2 in 50 m*M* phosphate buffer (*p*H 5·5) and 20 μL of concentrated enzyme stock having 0·125 enzyme unit/mL was added; temperature of the reaction medium varied]

also studied on the activity of *S. melongena* fruit peroxidase. The concentration of sodium azide and phenyl hydrazine needed to reduce the relative activities of the enzyme to half of its initial values were 20·3 m*M* and 0·2 m*M*, respectively. In order to decide the nature of inhibition, double- reciprocal plots were drawn in the presence of different fixed concentrations of inhibitors and varying the concentration of guaiacol and the results are shown in Figs 5a and b in both cases. The nature of inhibition was competitive, because the intercepts on y-axis were the same. The determined K_i values for sodium azide and phenyl hydrazine were 9·06 m*M* and 0·45 m*M*, respectively. The competitive inhibition suggests that the inhibitors compete for the binding of guaiacol substrate to the enzyme.

Since ligninperoxidases are biotechnologically important enzymes, and lignin peroxidase type activities were reported in soybean[24], tobacco[12] and *Musa paradisiaca* stem peroxidases[8], this peroxidase was analyzed for the ligninperoxidase activity using veratryl alcohol as the substrate and monitoring the formation of veratraldehyde at λ = 314 nm at low *p*H range (1·5 to 3·0). The *p*H range (1·5 to 3·0) was selected because the ligninperoxidase activity was

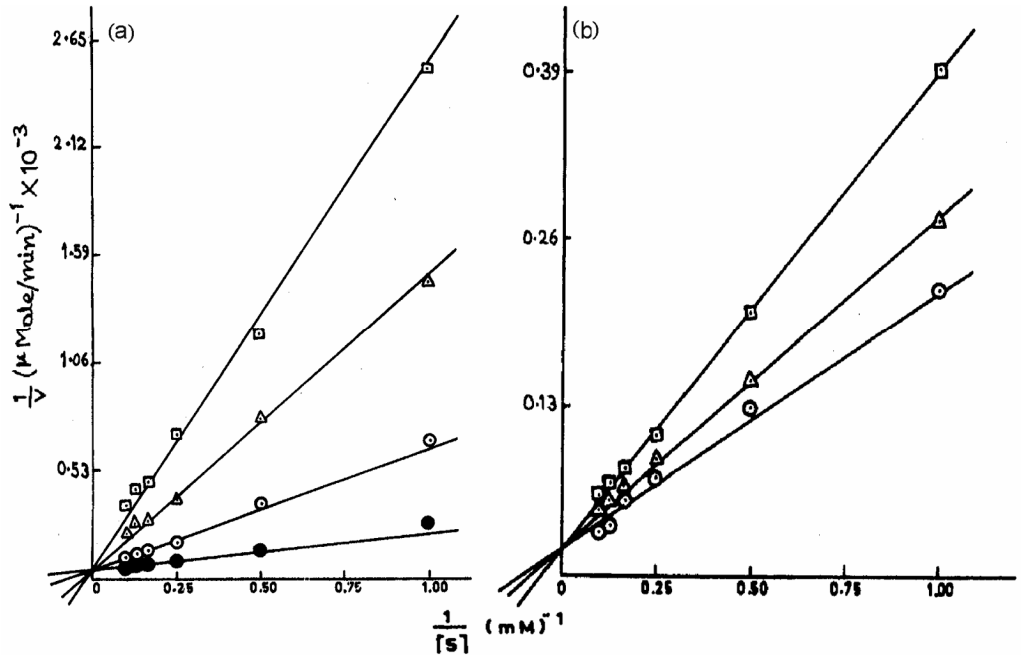

Fig. 5—Double-reciprocal plots for peroxidase using guaiacol as the variable substrate at different concentrations of inhibitors [(a): (•) 0·0 m*M*; (o) 20 m*M*; (Δ) 50 m*M*; and (□) 80 m*M* NaN_3; and (b): (o) 0·0 m*M*; (Δ) 0·1 m*M*; and (□) 0·2 m*M* phenyl hydrazine [Assay solution contained 0·6 m*M* H_2O_2 in 50 m*M* phosphate buffer (*p*H 5·5) at 30°C and 20 μL of concentrated enzyme stock having 0·125 enzyme unit/mL was added; concentration of guaiacol varied]

observed in this region. *S. melongena* peroxidase did not show ligninperoxidase activity.

In conclusion, we report here a convenient and rich source of peroxidase, which can be purified using a simple procedure and may find application for the enzymatic transformations in organic synthesis[15-18].

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