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Biochemical Studies on Peroxidase from the Seeds of Macrotyloma uniflorum

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ABSTRACT

Peroxidases are the heme containing enzymes that catalyse oxidation of substrates like phenolic compounds by reducing hydrogen peroxide concurrently. They play a key role in a number of metabolic processes such as regulation of cell elongation, lignification of cell wall, phenolic oxidation and defence against stress. Peroxidase was isolated from 4 days old seedlings of Macrotyloma uniflorum followed by germination study. The enzyme showed optimum pH 6 and optimum temperature 30°C. Peroxidase showed a stability over a broad pH range (4 to 9). Enzyme also was found to be stable up to 70°C. Kinetic studies were also performed.

Keywords : Peroxidase, Macrotyloma Uniflorum, Seedlings, Hydrogen Peroxide.

I. INTRODUCTION

Peroxidase (E.C. 1.11.1.7) is an oxidoreductase enzyme which reduces H2O2 to water and oxidizing variety of phenolic compound like diphenol, polyphenol, aminophenol and much more which act as proton donor in the reaction.(1) The majority of reactions catalysed by the peroxidase can be express as follows:

$$H2O2 + 2RH \xrightarrow{\text{Peroxidase}} 2H2O + 2R^{\bullet}$$

Peroxidase is a heme containing glycoprotein which is widely distributed in living organism including microorganism, plants, and animals (2). In plant kingdom it is widely distributed. Extraxction of peroxidase from several plants also reported like Horseradish (Armoracia rusticana), peach (Prunus persica), turnip (Brassica campestre rapifera), Caribbean plant (Euphorbia continifolia), Date palm leaves (Phoenix dactylifera L.), sweet potato (Ipomoea batatas ex L., Lam) etc (3).

Plant peroxidases play various physiological roles in plant growth and devolopment. It is mainly involved in rigidification and lignification of cell walls, synthesis of indole acetic acid (IAA), organogenesis, phenol oxidation, plant defence system during pathogenic attacks , wound healing , leaf senescence, ethylene biosynthesis , scavenging of peroxides and various environmental stress conditions etc. (4)

Peroxidases are having a prominent position in biochemical research and biotechnology (enzymology, biochemistry, physiology, histo chemistry, medicine and genetics) (5). Commercially peroxidases are used for production of secondary antibody in different process. Horseradish peroxidase (HRP) has been used in ELISA experiment as an important reagent. It is also used in various diagnostic kits. (8) Because of its wide substrate specificity peroxidase is used in waste water treatment, such as removal of phenolic compounds and in dye degradation process. (9)

Construction of biosensor using immobilised enzyme on different matrices is also an important emerging application of peroxidase.(10-14) The ability of enzyme to perform oxidation of H_2O_2 is used for developing reliable methods for detection of hydrogen peroxide generated in various biological as well as industrial processes.(15,16)

Although presence of peroxidase has been reported in a variety of plant sources, purification of peroxidase from seedlings of Macrotyloma uniflurom is not reported. The main purpose of this study is to determine different biochemical properties such as temperature and pH optima, stability of enzyme at various temperature and pH, kinetic studies and its purification.

II. METHODS AND MATERIAL

Chemicals:

Guaiacol, Sodium Chloride, Sodium Phosphate monobasic, Sodium Phosphate dibasic, Ammonium sulphate, hydrogen Peroxide from SRL.

Purification of peroxidase:

Peroxidase was isolated from 4 days old seedlings of Macrotyloma uniflurоum. The seedlings were grown from 100gm of seeds under controlled conditions which was followed by checking enzyme activity after different time intervals. The 4 days old seedlings were washed with distilled water and homogenised with 500 ml of physiological saline (0.145M, 0.85% NaCl) for 4 h at 4°C. The extract was filtered through cheese cloth and supernatant was clarified by centrifugation at 4°C (8000 rpm 10 min) and subjected to ammonium sulphate fractional precipitation (60-80%). The obtained pellet from 60-80% saturation was dissolved in minimum amount of distilled water and dialysed extensively against distilled water and finally against phosphate buffer (pH 7, 10mM). The dialyzed protein (Fraction A) solution clarified by centrifugation and applied on an ion exchange (UNO Sphere) column equilibrated with phosphate buffer (pH 7, 10mM) followed by washing of the column with equilibrating buffer till protein absorbance at 280nm was ≤ 0.02. The adsorbed proteins were eluted with a discontinuous gradient of NaCl in the same buffer. Fraction of 5ml each were collected at flow rate of 15ml/h and monitored for its protein content by taking the absorbance at 280nm and peroxidase activity were checked.

Enzyme assay:

Peroxidase activity was estimated by George's method at room temperature as follows (17). The 1.5 ml reaction mixture contain 0.5 ml of phosphate buffer pH 6.0(10mM); 0.5 ml of 20 mM guaiacol (2-methoxyphenol); 0.250 ml of 10mM H_2O_2; and 0.250 ml of enzyme extract. The reaction was carried out at room temperature and time required for change in optical density bye 0.1 observed at 470nm (ϵ = 26.6 cm. $^{-1}$ mM $^{-1}$). The amount of enzyme catalysing the oxidation of 1 mmol of guaiacol in 1 min represents one units of peroxidase activity (18).

Biochemical characterisation

Effect of pH

Optimum pH of the enzyme was determined as follows: The 0.250 ml peroxidase was incubated at 30 °C with 0.5 ml of different buffers in the presence of 0.250 ml guaiacoal (20 mM). Buffers used were sodium citrate buffer (20 mM, pH 3.0), sodium acetate buffer (20 mM, pH 4.0 -5.0), phosphate buffer (pH 6.0 – 8.0), Tris-HCl buffer (20 mM, pH 9.0), Glycine-NaOH buffer (20 mM, pH 10.0). The peroxidase activity was determined as described earlier.

pH stability of peroxidase:

The pH stability of peroxidase was determined by incubating 0.250 mL of enzyme with different buffers (0.5 mL) for 1 h at 30 °C. After incubation, pH was adjusted to 7.0 using acid or alkali and the peroxidase activity was determined using 0.5 mL guaiacoal in presence of 0.250ml H_2O_2 (10 mM) as described above.

Effect of temperature on Peroxidase activity

The temperature optima of the peroxidase were determine as follows : The 0.250 ml of enzyme were pre-incubate with 0.5ml of phosphate buffer (20 mM, pH 7) and 0.250ml guaiacol (20 mM) for 10 min for different temperatures . The reaction was carried out at temperatures ranging from 10 – 80 °C followed by addition of H_2O_2 (10 mM) for same temperature. The peroxidase activity was determined as described earlier.

Temperature stability of peroxidase:

The effect of temperature on peroxidase stability was determined by incubating 0.250 ml of peroxidase and 0.5 ml of buffer at varying temperatures (range 10 – 80 °C) each for 1 h. After pre-incubation with 0.250 ml Guaiacol the residual peroxidase activity was determined by addition of 0.250 ml of H_2O_2 (10 mM) at 30 °C as described above.

Kinetics studies:

Effect of substrate concentration on enzyme activity:

The rate of peroxidase activity was determined by varying concentrations of H_2O_2 (10, 20, 30, 40, 50, 60, 70, 80, 90 and100 mM/ml) at fix saturating concentration of Guaiacol. The same method was used to determine kinetic behavior of enzyme with keeping saturating concentration of H_2O_2 constant and varying concentration of Guaiacol (10,20, 30, 40, 50, 60, 70, 80, 90 and100mM/ml). The reaction was carried out as descried earlier. The peroxidase activity was determined and a double reciprocal plot was drawn according to the method of Lineweaver and Burke. The K_m and V_{max} of the enzyme were determined.

III. RESULTS AND DISCUSSION

The peroxidase isolated from seedlings of Macrotyloma uniflorum was purified with ammonium sulphate fractional precipitation (60-80%) followed by ion exchange chromatography. The column was pre equilibrate with phosphate buffer (pH 6, 10 mM). The dialysed clear protein solution applied on Cation exchanger column Uno-Sphere. The adsorbed proteins were eluted with a discontinuous gradient of sodium chloride (0.1, 0.2, 0.3, 0.5 and 1 M) in same buffer. Fractions of 3 mL each were collected at a flow rate of 20 mL/h and monitored for protein content by spectrophotometer at 280 nm. As seen in the elution profile (Fig 1), peroxidase was eluted as a major peak at 0.1 M NaCl concentration. These peroxidase rich fractions were used for further studies.

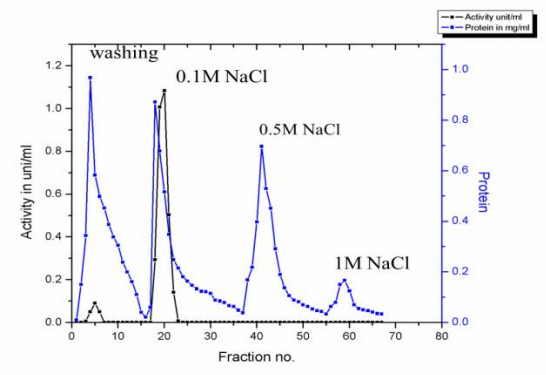

Figure 1. Elution profile of UNOsphere ion exchange chromatography

Table 1. Purification Table

Purification steps	Protein conc. mg/ml	Enzyme activity (u/ml)	Specific activity (u/mg)	Fold purification
Saline Extract	29.56	1.8	0.060	1
Ammonium Sulphate precipitation	22.7	2.811	0.19	2.08
Ion exchange chromatography	1.09	3.484	3.19	53.61

Effect of pH on peroxidase activity:

As shown in the figure 2, purified peroxidase enzyme showed optimum activity at pH 6 and purified peroxidase was found to be stable over a broad pH

range i.e. 3.0- 9.0 same results also reported in Caribbean plant: Euphorbia cotinifolia (4)

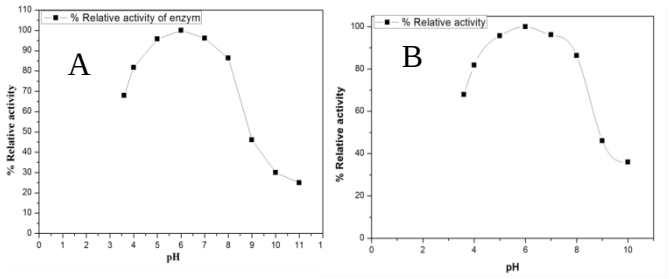

Figure 2. A) pH optima B) pH stability of peroxidase

Effect of temprature on peroxidase activity:

Purified peroxidase showed maximum activity temperature at 30⁰C and it was found to be stable in the range of 10–70°C. The maximum activity of enyzme betwwen 25-30°C also reported for the same soure in the tissue culture study. (19) Where as in leaves of Copaifera langsdorffii it was found 35°C. (**3)**

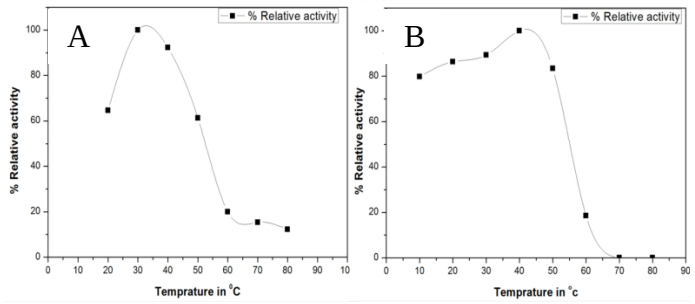

Figure 3. A) Temperature optima B) Temperature stability of peroxidase

Kinetic studies

In the figure4 (A) shows Michalies –Menten curves using guaiacol as variable concentration at saturating substrate concentration of H_2O_2 (10mM). While fig (B) represented Lineweaver Burk plot with km value of guaiacol is 40mM. In case of Daucus carota it was found 1300µM. (20)

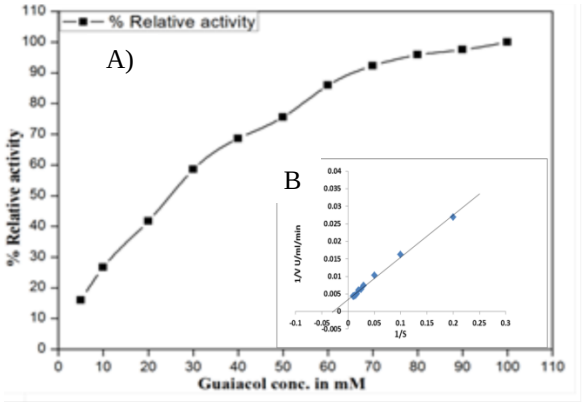

Figure 4. A) Michalies –Menten curves using guaiacol as variable concentration at saturating substrate concentration of H_2O_2. B) Lineweaver Burk plot

Same process was applied to found Km value of H_2O_2 by Michalies –Menten equation using H_2O_2 as variable concentration at saturating substrate concentration of guaiacol (20 mm). Fig5 (A) represented Michalies –Menten curves Fig5 (B) shows Lineweaver Burk plot with km value of H_2O_2 is 2mM. In case of Daucus carota it was found Km valu for guaiacol 1300µMand for H_2O_2 50µM (20).

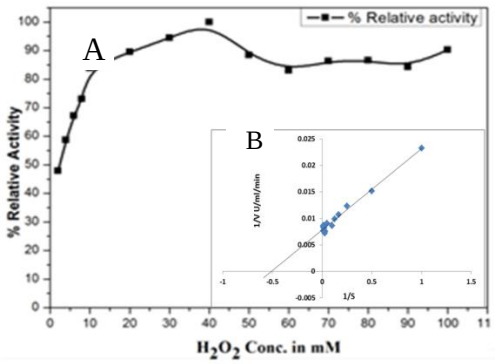

Figure 5. A) Michalies –Menten curves using as H_2O_2 variable concentration at saturating substrate concentration of guaiacol. B) Lineweaver Burk plot

IV. CONCLUSION

Peroxidase isolated from Macrotyloma uniflorum purified by cation-exchange chromatography successively with fold purification 53. The purified peroxidase shows broad pH and temperature range respectively 3-9 , 10-70 ° C. the optimum pH of

enzyme found at pH 6 and temperature optima observed at 30°C. In the kinetic study the Km value for guaiacol was found 40mM and for H_2O_2 2mM.

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