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PURIFICATION AND KINETIC CHARACTERIZATION OF POLYPHENOL OXIDASE FROM PALMYRAH *(BORASSUS FLABELLIFER* L.)FRUIT

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Polyphenol oxidase(PPO) was extracted from the fruit mesocarp of Palmyrah palm and purified through ammonium sulfate precipitation, ion exchange chromatography, gel filtration and affinity chromatography. The purification factor for PPO was 328.48 with 10.58% yield. The enzyme was characterized for thermal stability, pH and kinetic parameters. The molecular mass of PPO was approximately 68kDa estimated by SDS-PAGE. The purity was confirmed by native PAGE, showing a single prominent band. The optimum pH was 6.5. The enzyme had a temperature optimum at 35°C and was relatively stable at 60°C. L-cysteine, EDTA, calcium chloride ($CaCl_2$), thiourea and ascorbic acid were significantly inhibited PPO activity. The K_m value and the maximum velocity V_{max} were determined by the Lineweaver Burk plot. The K_m value for the substrate Pyrocactechol was calculated as 9.09 with V_{max} / K_m ratio 15.18. Pyrocatechol was found to be an efficient diphenolic substrate for palmyrah PPO.

Keywords : Affinity choromatography; Ascorbic acid; Inhibition; Palmyrah palm; Polyphenol oxidase; Pyrocatechol.

Introduction

Polyphenol oxidase(PPO) , also known as tyrosinase (E.C.1.14.18.1), is a copper containing enzyme, widely distributed in plants and micro organisms. This enzyme is responsible for the deleterious effects of enzymatic browning reactions in fruits and other food materials[1]. PPO utilizes molecular oxygen to catalyse the oxidation of mono-, di- and polyhydric phenols to o-quinones. These o-quinones readily polymerise or react with endogenous amino acids and proteins to form complex brown pigments which leads to organoleptic and nutritional modifications, thus depreciating the food value. This has been well understood that browning reactions occur generally post-harvest when the tissues are exposed either to stress conditions or during storage and processing. PPO has been isolated from various sources such as banana,pear, apple, egg plant, peppermint, grapes and artichoke[2-9].

Palmyrah *(Borassus flabellifer* L.), also known as the jaggery palm or toddy palm is a tropical palm; flourish luxuriantly in the dry lands of many tropical countries including India. The large edible fleshy fruits have a very short shelf life under ambient conditions due to quality deterioration during storage. However no work has been carried out on Palmyrah fruit PPO. Therefore the objective of our study was to isolate, purify and characterize the PPO from Palmyrah fruits.

Material and Methods

Materials- Palmyrah fruits were harvested fresh from the field and stored at -20°C.

Chemicals used- DEAE- Cellulose, Sephadex G-200, Phenyl Sepharose and polyvinylpyrrolidone were obtained from Sigma Chemicals Co.,St. Louis, MO, USA. Acrylamide,bis acrylamide, coommassie brilliant blue R-250, Sodium dodecyl sulfate (SDS), TEMED, Pyrocatechol, L-dopa, L-cysteine were from SD Fine Chemicals, India. All chemicals used were of analytical grade.

Isolation and purification of PPO- The extraction of PPO was performed by the proceedure of Jiang[10].All the purification steps were carried out at 4°C. The tender fruit peel was homogenized with 0.1M sodium phosphate buffer (pH 6.8) with Poly-Clar AT (insoluble high molecular weight grade of polyvinylpyrrolidone; 1% of fruit weight). After filtration the filtrate was centrifuged at 15000g for 20 min and the supernatant was collected.The enzyme protein was fractionated with solid ammonium sulfate (50-80% saturation) and the precipitate was recovered by centrifugation at 15000g for 20 min, redissolved in 0.01M sodium phosphate buffer (pH 6.8) and dialized against the same buffer for 24 h. Following

Table 1. Purification profile of Palmyrah fruit Polyphenol oxidase.

Purification step	Total activity (μ/g tissue)	Total protein (μ/g tissue)	Specific activity (μ/g tissue)	Purification fold	Yield (%)
Crude	30612	248.42	123.22	1	100
80% Ammonium sulphate precipitation	15234	8.48	1796.46	14.58	49.76
Ion exchange chromatography (DEAE-Cellulose)	9640	1.6	6025	48.9	31.49
Gel filtration chromatography (Sephadex G-200)	6362	0.53	12003.77	97.42	20.78
Affinity chromatography	3238	0.08	40475	328.84	10.58

Table 2. Effect of inhibitors on Palmyrah PPO activity.

Inhibitors	Percentage of Inhibition	
	1mM	10mM
L Cystine	24	84
Ascorbic acid	47	91
EDTA	10	94
$CaCl_2$	-	2
Thiourea	7	65

dialysis, the extract was loaded onto a DEAE - Cellulose column pre equilibrated with 0.01M sodium phosphate buffer (pH 6.8). The enzyme solution was eluted with the same buffer and the fractions with highest enzymatic activity were pooled, lyophilized and redissolved in small volume of 0.01M sodium phosphate buffer (pH 6.8). The dialyzed fraction of the enzyme was loaded on a Sephadex G-200 column pre equilibrated with 0.01M sodium phosphate buffer (pH 6.8). Fractions with highest activity were collected , pooled and then dialyzed against the same buffer. After dialysis, the enzyme concentrate was loaded onto a Phenyl Sepharose column equilibrated with 0.01M sodium phosphate buffer (pH 6.8) containing 1M ammonium sulphate and 1M KCl.The purified fraction of PPO was eluted with a gradient of 100, 80, 60, 40, 20, 10 to 0% of the same equilibrium buffer (pH 6.8). The active fractions from the Phenyl Sepharose column were pooled, lyophilized and dissolved in a small volume of 0.01 M sodium phosphate buffer (pH 6.8). Following overnight dialysis, the dialysed solution was collected and used as the enzyme source.

Assay of PPO activity- PPO activity was determined by measuring the increase in absorbance at 420nm. The reaction mixture contained 0.2ml of enzyme solution and 2.8 ml of 100mM catechol solution in 0.1M Na-P buffer, pH 7.0, at 40°C. 3 ml of substrate solution was taken as blank. One unit of the enzyme activity was defined as the amount of enzyme that caused an increase in absorbance of $0.001min^{-1}$. The quantity of protein was determined by the method of Lowry *et al.*[11].

Effect of pH and temperature- The effect of pH on PPO activity was determined with two substrates(pyro catechol and L-dopa). Substrates were used at 10mM concentration with a pH range 4-10 in 0.2 M phosphate buffer to determine the optimum pH.The optimum pH values obtained from this assay were used in all other experiments.

The effect of temperature on PPO activity was measured within the range10°C to 75°C using the two substrates.

*SDS-PAGE-*The purified PPO after affinity chromatography, was run on SDS-PAGE, using 10% polyacrylamide gel[12].The enzyme was again subjected to native PAGE and the activity was localised by incumbating in 15mM catechol in 0.1mM phosphate buffer (pH 7.0) at 35°C for 1h, followed by 1mM ascorbic acid solution until bands appeared.

Effect of inhibitors- L-cisteine, L-ascorbate, EDTA, Calcium chloride ($CaCl_2$) and thiourea were examined for their effectiveness as inhibitors of PPO using pyrocatechol as the substrate. The rate of inhibition was measured as percent catechol inhibition. The compounds

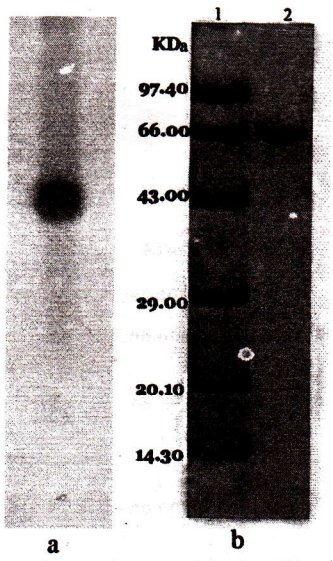

Fig.1.a. Native PAGE of PPO localized with Catechol from Palmyrah fruit; b. SDS-PAGE of the purified PPO protein. Line 1: Marker, Line 2: Purified protein

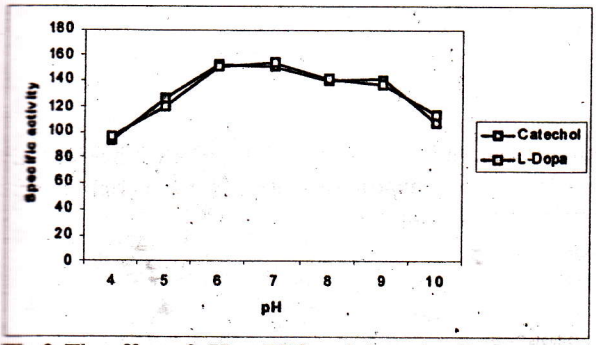

Fig.2. The effect of pH on PPO activity using Pyrocatechol and L-Dopa as substrates.

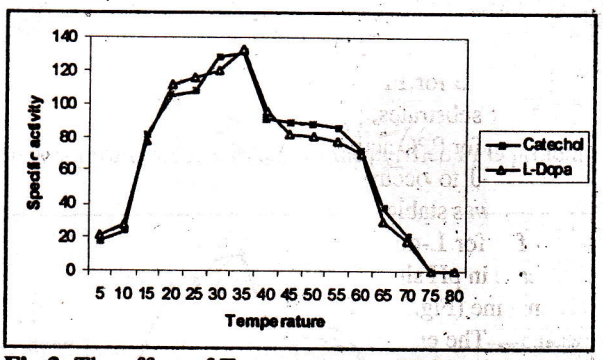

Fig.3. The effect of Temperature on PPO activity using Pyrocatechol and L-Dopa as substrates.

showing highest inhibition were further asssessed by studying the kinetics of interaction of PPO with inhibitors. In different experiments, inhibitors at various concentrations (1,2,3,5 and 10mM)were added and were monitored at time intervals of 2min(upto 10min).

Enzyme kinetics and substrate specificity- The activity of PPO was assayed using Pyro catechol and L-dopa in buffers at optimum pH value for each substrate.The K_m value and the maximum velocity V_{max} were determined by the Lineweaver Burk plot. Substrate specificity V_{max} / K_m was calculated by using the data obtained from the above plot.

Results and Discussion

Purification-PPO was purified from Palmyrah fruit using Phenyl Sepharose affinity chromatography[10]. Purification profile is summarized in Table 1. The crude extract of PPO showed a specific activity of 632.22U/mg protein and the profile of purification was further increased to near homogeneity by Phenyl Sepharose. The specific activity of the enzyme increased further to 40,475 U/mg protein after affinity chromatography, with a low protein content of 0.08mg. Overall, the specific activity increased about 64.02 with 10.58 yield of activity. This value was significantly higher than that obtained for pear[13] and guava[14]. The activity was checked by gel assay which showed a thick band, indicating the localization site of the PPO activity, as well as homogeneity of enzyme protein (Fig.1.a). This was matched with that of Barbados cherry[15] but different from those of pear[5] . The electrophoretic pattern of Palmyrah PPO in SDS-PAGE revealed a single prominent band[16,17] with molecular mass of 68 kDa (Fig.1.b). Thus this enzyme differs from PPO isolated from Barbados cherry[15] which was reported to be a dimer but, was similar to those enzymes (monomers)

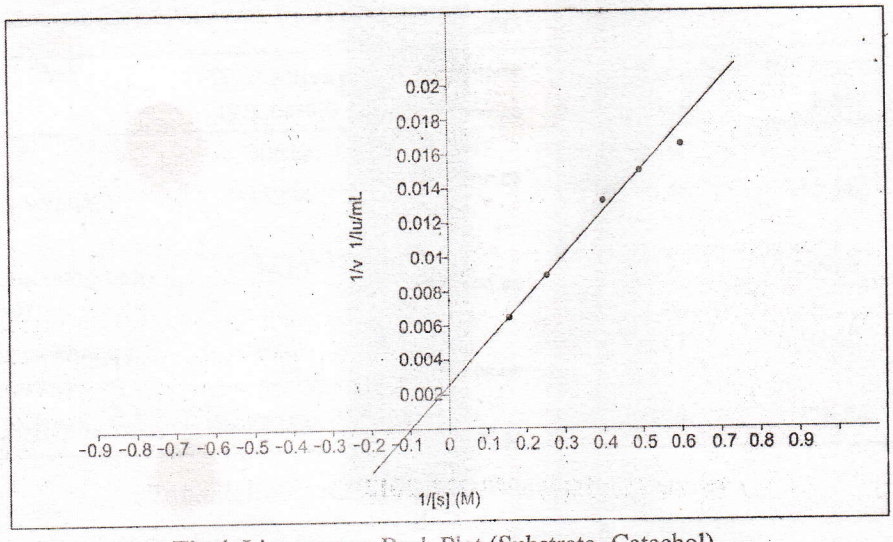

Fig.4. Line weaver Burk Plot (Substrate- Catechol)

isolated from pear[5] and Chinese cabbage[18].

Effect of pH and temperature-The optimum pH and temperature for PPO activity were calculated with two different substrates, pyro catechol and L-dopa. The pH optimum for PPO activity with L- dopa and Pyrocatechol was found to occur at pH 6 and 6.5 respectively. The enzyme was stable within pH range 5 to 8 with optimum pH of 6 for L-dopa and 6.5 for pyro catechol. Further increase in pH showed profound decrease in the activity of enzyme (Fig.2).

The enzyme activity was measured at different temperatures using pyro catechol and L-dopa as substrates under optimum pH. PPO showed maximum activity at 35°C with both substrates.Above 40°C the activity decreased as the temperature increased(Fig. 3) , but the enzyme was not inactivated even at 60°C[19]. This value was slightly different from that of Barbados cherry[15].

Effect of inhibitors on PPO activity : Table 2 shows the effect of various compounds on the purified PPO at a concentration range 1mM to 10mM. The activity was progressively inhibited by various compounds. Ascorbic acid was the strongest inhibitor of the enzyme at 1mM concentration followed by, EDTA and thiourea[20]. EDTA showed marked inhibition at 10mM concentration. Only $CaCl_2$ showed minimum inhibition at 10mM cocentration and did not affect the enzyme at 1mM. L-cysteine was previously reported to be a strong inhibitor of apple PPO[21]and barbados cherry[15]. Ascorbic was also known to be an effective inhibitor on PPO activity in dog rose[12] and barbados cherry[15]. Since cysteine and ascorbate are naturally occurring substances and non-toxic, they may be useful for preventing the enzymatic browning of Palmyrah Palm fruit. The inhibitors are copper- chelating agents and they suppress browning activities in which copper is directly involved in the oxidation of phenolic compounds.

Substrate specificity-K_m and V_{max} value of Palmyrah fruit PPO for the substrate Catechol were determined by the Lineweaver burk Plot and the result is shown in Fig.4. V_{max}/K_m ratio clearly showed that Pyrocatechol was effective substrate for PPO activity. The result was consistant with the previous report on plant PPOs[15-22]

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