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Purification and Characterization of Polyphenol Oxidase from *Zyzyphus spina-christi* from Telangana Region

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Abstract: ***Polyphenol oxidase (PPO) from Zyzyphus spina-christi from Telangana region was extracted and purified by $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography and gel filtration chromatography. The biochemical characteristics of reveals that this PPO has higher affinity towards catechol (K_m = 12.2mM and V_{max} = 17, 200 U/ml min^{-1}) at an optimum pH of 5.75. The enzyme has an optimum temperature of 37.5^{0}C and was relatively stable up to 48^{0}C for a period of 60 minutes with almost 78% of activity remaining. Among the various polyphenol oxidase inhibitors tested, the most effective inhibitor for the enzyme with 10mM catechol as substrate was ascorbic acid.***

Key words: Polyphenol Oxidase, *Zyzyphus spina-christi*, Telangana, Catechol, inhibitor, Optimum

1. Introduction

Polyphenol Oxidase (E.C. 1.41.18.1) is a common copper containing enzyme responsible for melanization in Animals and browning in plants also known and reported under various names (tyrosinase, phenolase, catechol oxidase, monophenol oxidase, O-diphenol oxidase and ortho-phenolase) based on substrate specificity [1-3]. These are widely distributed in plants and fungi [4].

In higher plants the enzyme has been localized to the thylakoid membranes of chloroplasts and other plastid organelles [5]. The role of polyphenol oxidase (PPO) is not yet clear but it is conceived that might be involved in necrosis development around the damaged leaf and in defense mechanism against insects and plant pathogens. It is also involved in biosynthesis in plants, in immunity reactions and also as a scavenger of free radicals.

The phenomenon of enzymatic browning often occurring in plants and vegetables leads to decrease in their nutritional value and the loss of quality which is undesirable in the Food industry. Polyphenol oxidase (PPO) catalyses two distinct reactions namely the O-hydroxylation of monophenols to O-diphenols and the oxidation of O-diphenols to O-quinones [8-10]. Polyphenol oxidase is frequently reported as a latent enzyme, which can be activated *in vitro* by a number of different factors as low and high pH levels [12] proteases [11] and exposure to fatty acids in the incubation mixture [7].

Activity of Polyphenol oxidase has been studied in apples (*Malus sp*) [13], pears (*Pyrus sp*) [14], coffee (*Coffee arabica* L) [15], tea leaf [1] and Victoria grape [32]. However, no research investigations have been reported on *Zyzyphus spina-christi* found in the Telangana region of India which is cherished as a common fruit.

In the present study Polyphenol oxidase was extracted, partially purified and some of its characteristics were investigated.

2. Materials and Methods

Materials: Ripe and undamaged fruits were collected from different trees in the Telangana region. Analytical grade chemicals were used in this study.

Enzyme Extraction and partial purification: A total of 100gms of the ripe and undamaged fruits collected were homogenized in 250ml of 0.1M sodium phosphate buffer (pH 6.9) containing 10mM ascorbic acid and 0.5% polyvinylpyrolidine using ultra homogenizer and extracted using a magnetic stirrer for an hour. The crude extract was then centrifuged at 30, 000g for 25 min at 4^{0}C followed by addition of solid $(NH_4)_2SO_4$ to the supernatant for obtaining maximum saturation. After one hour the precipitated proteins were separated by centrifugation at 40, 000g for 30 min. The precipitate is then dissolved in 5mM phosphate buffer (pH 6.9) and subjected to dialysis in a cellulose bag (MW cut off >12, 000) at 4^{0}C in the same buffer with five changes of buffer during the dialysis. For further purification, the dialysate was transferred into a column filled with DEAE-Sephadex A-50 gel saturated with 5mM phosphate buffer (pH 6.9). The column was eluted with the same buffer at a flow rate of 25ml/h keeping linear gradient of NaCl concentration0 to 1.0M. Fractions of 4ml were collected and the protein levels as well as the PPO activity towards catechol as substrate were monitored spectrophotometrically. Fractions showing PPO activity were combined and transferred to a glass column filled with

Sephadex G100 gel. The column was then eluted with the same buffer solution. Three milliliter fractions were then collected and the protein content as well as the Polyphenol Oxidase (PPO) activity towards catechol were monitored spectrophotometrically. The fractions containing PPO activity were combined and concentrated.

PPO Activity Assay: PPO activity was determined by measuring the initial rate of quinine formation as indicated by increase in absorbance at 420nm and one unit of enzyme activity is defined as the amount of enzyme that caused a change in absorbance of 0.001 min^{-1} [16]. The activity of PPO was performed in triplicate and the cuvette with sample contains 2.85ml of 10mM catechol solution 0.1ml phosphate buffer (pH 6.9) and 0.05ml of the enzymatic solution. The blank contained only 3ml of the substrate solution.

Protein Determination: Protein content of the separated extract was determined using Bovine Serum Albumin (BSA) as standard at 595nm spectrophotometrically as explained by Bradford [17].

Effect of pH on Enzyme Activity: The Polyphenol oxidase activity as a function of pH was determined under standard laboratory conditions using different pH buffer range 3.0 to 12.0 using 0.02 M catechol as the substrate. The PPO activity was analysed as described earlier and the pH value corresponding to the highest enzyme activity was taken as the optimal pH.

Substrate Specificityand enzyme kinetics: The specificity of PPO extracted was investigated over four different substrates like catechol, tyrosine, phloroglucinol and caffeic acid at a concentration of 10mM. The highest enzymatic activity was obtained with 10mM of catechol, and therefore, Michaelis constant (K_M) and maximum velocity (V_{max}) of the PPO extract as a function of the concentration of catechol was determined using Lineweaver-Burk's graph method.

Effect of Temperature on Enzyme Activity and Thermal Stability: The PPO activity as a function of temperature was determined under standard conditions at temperatures ranging from 20 to 90^0C. by heating the enzyme solution for 45 minutes at the optimal pH. Residual PPO activity was measured under standards assay conditions.

Effect of Inhibitors on PPO: The inhibitory effects of ascorbic acid, thiourea, sodium metabisulphate and glutathione on PPO activity were determined. 5mM concentrations of the above compound were tested using 10mM of catechol as substrate. The corresponding control contained the same concentration of the enzyme without the inhibitor.

3. Results and Discussion

The elution profile of Polyphenol Oxisade (PPO) with DEAE Sephadex A-50 and Sephadex G100 showed a purification fold of 2.16 relative to a protein yield of 21.32 (Table 1). The technique of gel filtration is widely used in enzyme separation. Bahar Aydin *et. al* [33] purified polyphenol oxidase from Hemsin Apple (*Malus communis* L.) and achieved a purification fold of 28.80 with a protein yield of 19.75%. Whereas, Selles-Marchart *et. al [*19] purified polyphenol oxidase and obtained a purification fold of 39.9 with protein recovery of 15%.

Table 1: Purification of PPO from Zyzyphus spina - *Christi* from Telangana region

Purification steps	Protein Conc (mg/ml)	Activity (u/ml)	Specific Activity (u/mg)	Purification fold	Yield (%)
Crude extract	3.24	36.68	12.43	1.00	100
$(NH_4)_2SO_4$	2.29	34.53	15.46	1.24	89.62
DEAE Sephadex A-50	1.19	19.57	17.54	1.43	52.34
Sephadex G-100	0.43	9.56	23.46	2.16	21.32

Characterization of PPO

Effect of pH on Enzyme Activity: The activity of polyphenol oxidase was measured at different pH using catechol as the substrate and the optimum pH was found to be 5.75 as shown in Fig.1. In general, it is seen that, most plants and its components show PPO activity at or near neutral pH values. The results shown in this study corresponds well with the results of Broccoli (*Brassica oleracea*) florets where the pH is 5.7 as reported by Urszula*et. al [*29]. The value is also similar to the one reported for PPO from strawberry (pH 5.5) by Wesche-Ebling. P *et. al* [20].

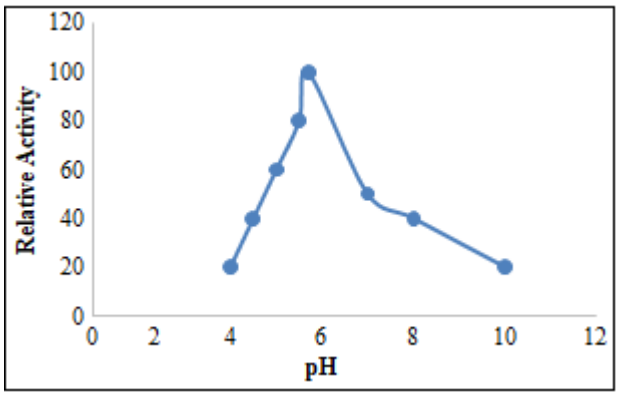


Figure 1: Effect of pH on PPO activity

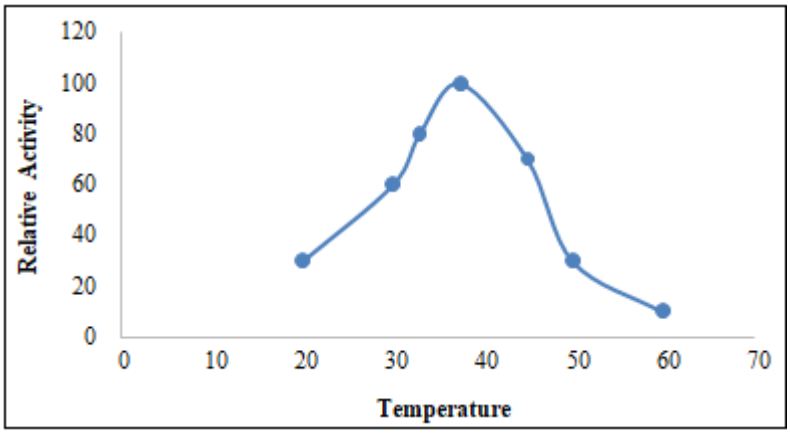
Substrate Specificity and Enzyme Kinetics: Though there are several compounds that are used as substrates for polyphenol oxidase, in the present study we have selected the most commonly employed substrates like catechol, caffeic acid, chlorogenic acid, phloroglucinol and tyrosine. As shown in Table 2, polyphenol oxidase showed the highest activity towards catechol (dihydroxy phenols) and no activity was shown against tyrosine (mono phenols). Cho and Ahn [21] used catechol in the studies of kinetic properties of PPO from potatoes. Whereas, Janovitz-Klapp *et. al* [22] compare the activity of PPO against several substrates.

Table 2: Relative Enzyme Activity of PPO at 10mM substrate concentration

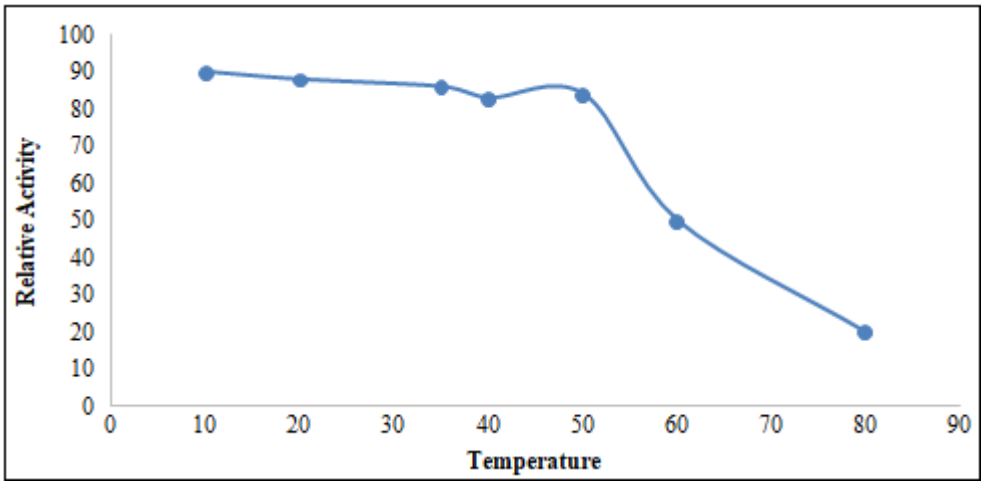
Substrate (10mM)	Relative Activity (%)
Catechol	100.00 ± 3.101
Caffeic acid	8.89 ± 0.821
Chlorogenic acid	3.82 ± 0.746
Phloroglucinol	0.85 ± 0.006

The Line weaver-Burk plot analysis of polyphenol oxidase from zyzyphus spina christi of Telangana region reveals that the Michaelis Menten constant (K_m) and the maximum reaction velocity (V_{max}) were 12.2mM and 17, 200 U/ml min^{-1} respectively for catechol as substrate. This value of K_m with catechol as substrate was similar to that of Tea leaf (12.5mM) [23] and also of field been seeds (10.5mM) [24].

Optimum Temperature and Thermal Stability: The effect of temperature on PPO activity between 10^0C and 80^0C showed that the optimum temperature of PPO from *Zyzyphus spina-christi* from Telanagana Region to be 37.5^0C (Fig 2). This value was similar to that obtained by Heimdal [6] for butter lettuce (37.5^0C) and for plum (37^0C) by Dincer *et. al* [25].



The thermal stability profile of *Zyzyphus spinap-christi* PPO from Telengana region showed a residual activity after pre-incubation at a specified temperature as shown in Figure 3. The PPO showed thermal stability up to 48^0C for almost a period of 60 minutes with around 78% activity remaining. It has been reported that the PPO of *Allium* Sp. was stable up to 40^0C [26] and that for the PPO from the latex of *Heveabrasiliensis* the stability was up to 60^0C [27].



Effect of Inhibitors: The effect of various inhibitors like ascorbic acid, thiourea, sodium metabisulphate and glutathione on *Zyzyphus spina Christi* PPO from Telangana region with catechol as the substrate is shown in Table 3.

Table 3: K_i values and inhibition types of PPO with different inhibitors

Inhibitor	K_i (M)	Type of Inhibition	Inhibition (%)
Ascorbic acid	$8.1x10^{-5}$	Competitive	68
Thiourea Sodium	$3.1x10^{-5}$	Non-competitive	61
Meta Bisulphate	$3.7x10^{-5}$	Non-competitive	59
Glutathione	$4.9x10^{-5}$	Non-competitive	58

From the K_i values it is concluded that the inhibition modes of thiourea, sodium meta bisulphate and glutathione are non-competitive and competitive for ascorbic acid. Enzymatic browning of plants and fruits can be delayed or prevented by removing the reactants such as oxygen, phenolic compounds or by PPO inhibitors. There are a number of inhibitors used by researchers to prevent enzymatic browning and the inhibitory reaction depends upon the reducing agent that is employed [28].

It is seen that the compounds tested in the present study i.e., ascorbic acid, thiourea, sodium metabisulphate and glutathione also inhibited the action of the PPO enzyme isolated from artichoke (*CyanaraScolymus*L) heads [31]. Ascorbic acid acts more like an antioxidant than as an enzyme inhibitor because it reduces the initial quinine from the enzyme to the original diphenol before it undergoes secondary reactions, which lead to browning. Ascorbic acid has also been found to show competitive activity towards PPO isolated from peppermint [30] and potatoes [18]. Innovative treatments using non-thermal technologies and natural inhibitors as eco-friendly technologies seems to effectively reduce the PPO activity thus preventing the

enzymatic browning consequently improving the sensory, antioxidant and nutritional properties of fruit and vegetable products.

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