

## Characterization of Polyphenol Oxidase from *Zyzyphus spina-christi* from Iraq

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**Abstract:** Polyphenol oxidase (PPO) from *Zyzyphus spina christi* found in central part of Iraq was extracted and purified by  $(\text{NH}_4)_2\text{SO}_4$  precipitation, ion-exchange chromatography and gel filtration chromatography. The biochemical characteristics reveal that the PPO from *Zyzyphus spina christi* has higher affinity towards catechol ( $K_M = 11.4\text{mM}$  and  $V_{max} = 16,400 \text{ U/ml min}^{-1}$ ) at an optimum pH of 5.8. The enzyme had an optimum temperature of  $37^\circ\text{C}$  and was relatively stable up to  $50^\circ\text{C}$  for a period of 60 minutes with almost 80% activity remaining. Among the various PPO inhibitors tested, the most effective inhibitor for the enzyme with 10mM catechol as substrate was ascorbic acid.

**Key words:** Polyphenol Oxidase • *Zyzyphus Spina Christi* • Catechol

### 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) in plants is not yet clear, but it has been proposed that it may be involved in necrosis development around damaged leaf surfaces and in defence mechanisms against insects and plant pathogen attack. It is also suggested that it may be involved in immunity reactions and in biosynthesis of plant components and it also may play the role of a scavenger of free radicals in photosynthesizing tissues [6-7].

The phenomenon of enzymatic browning often occurs in fruits and vegetables and is the cause of a decrease in their sensory properties and nutritional value [8]. The browning is principally initiated by the activity of

Polyphenol oxidase catalyses two distinct reactions: the *O*-hydroxylation of monophenols to *O*-diphenols and the oxidation of *O*-diphenols to *O*-quinones [9-10]. Polyphenol oxidase is frequently reported as a latent enzyme, which can be activated *in vitro* by a number of different factors and treatments such as detergents [10-14]. Proteases [11], low and high pH levels [12] and exposure to fatty acids in the incubation mixture [13].

Activity of PPO has been studied in apples (*Malus* sp.) [13], pears (*Pyrus* sp.) [14], peppermint (*Mentha piperita* L.) [15], coffee (*Coffea arabica* L.) [16]. However, no research has been reported on *Zyzyphus spina-christi* which is mainly grown in the central and southern part of Iraq and used as a medicinal and common fruit.

In the present study, PPO was extracted, partially purified and the characteristics of the enzyme were investigated.

### MATERIAL AND METHODS

**Material:** Ripe undamaged fruits were obtained from three locations in the central part of Iraq. Catechol, DEAE-Sephadex A-50, Sephadex G100, caffeic acid, chlorogenic acid, phloroglucinol, Polyvinylpyrrolidone

(PVP 40), ascorbic acid, tyrosine, thiourea, sodium metabisulphate and glutathione were obtained from Sigma-Aldrich/ USA and all other chemicals were of analytical grade.

**Enzyme Extraction and Purification:** Thirty grams of the ripe fruits were homogenized in 240ml of 0.1M sodium phosphate buffer (pH=6.8) containing 10mM ascorbic acid and 0.5% polyvinylpyrrolidone using Ultra homogenizer and extracted with the aid of magnetic stirrer for one hour. The crude extract samples were centrifuged at 32,000g for 30 min at 4°C. Solid  $(\text{NH}_4)_2\text{SO}_4$  was added to the supernatant to obtain 80% saturation. After an hour, the precipitated proteins were separated by centrifugation at 32,000g for 30 min. The precipitate was dissolved in a small amount of 5mM phosphate buffer (pH 6.8) and dialyzed in a cellulose bag (MW cut off >12,000) at 4°C in the same buffer for 24 hours with four changes of the buffer during the analysis. In order to conduct further purification, the dialysate was transferred to a column filled with DEAE-Sephadex A-50 gel, balanced with 5mM phosphate buffer (pH 6.8). The column was eluted with the same buffer at the flow rate of 25 ml/h keeping linear gradient of NaCl concentration from 0 to 1.0M. Three milliliter fractions were collected in which the protein level and PPO activity towards catechol as substrate were monitored. The fractions which showed PPO activity were collected, concentrated and then dissolved in 3 ml of phosphate buffer (pH 6.8). The combined fractions were transferred to a glass column filled with Sephadex G100 gel. The column was then eluted with the same buffer solution. Three milliliter fractions were collected and the protein content and the Polyphenol oxidase (PPO) activity towards catechol were monitored spectrophotometrically. The fractions showing PPO activity were combined and concentrated.

**PPO Activity Assay:** PPO activity assay was determined by measuring the initial rate of quinone formation as indicated by an increase in absorbance at 420 nm [17]. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of  $0.001 \text{ min}^{-1}$  [18]. The PPO activity was assayed in triplicate measurements. The sample cuvette contained 2.95 ml of 10mM catechol solution in 0.1 ml phosphate buffer (pH 6.8) and 0.05 ml of the enzyme solution. The blank sample contained only 3ml of the substrate solution.

**Protein Determination:** Protein content of the enzyme extract was determined using Bovine Serum Albumin (BSA) as standard according to Bradford [19].

### Characterization of PPO

**Effect of Ph on Enzyme Activity:** The PPO activity as a function of pH was determined under standard conditions using various buffers in the pH buffer range 2.0-12.0 using 0.02 M catechol as the substrate. The buffer solution was prepared according to Britton-Robinson [19]. PPO activity was assayed as described above with catechol as the substrate. The pH value corresponding to the highest enzyme activity was taken as the optimal pH.

**Kinetic Data Analysis and Substrate Specificity:** The specificity of PPO extracted from *Zyzyphus spina-christi* was investigated in five different substrates like catechol, caffeic acid, chlorogenic acid, phloroglucinol and tyrosine at a concentration of 10mM. The activity of PPO extract as function of the concentration of catechol was investigated. Michaelis constant ( $k_M$ ) of the PPO was determined by Lineweaver-Burk's method.

**Effect of Temperature on Enzyme Activity and Thermal Stability:** PPO activity as a function of temperature was determined under standard assay conditions using temperatures from 5 to 80°C. Thermal stability of PPO was determined by heating the enzyme solution at various temperatures between 20 to 80°C for 60 minutes at pH 5.8. Residual PPO activity was measured under standard 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 enzyme in the absence of the inhibitor.

## RESULTS AND DISCUSSION

**Extraction and Purification of PPO:** The elution profile of the PPO with DEAE Sephadex A-50 and Sephadex G100 is shown in Figs 1 and 2. A purification fold of 1.87 relative to a protein yield of 24.5% was achieved (Table 1).

The technique of gel filtration is widely used in enzyme separation. Xu *et al.* [20] purified PPO from Henry chestnuts (*Castanea henryi*) and achieved purification for 1 of 36.87 with protein recovery of 12.94%. Whereas, Selles-Marchart *et al.* [21] purified polyphenol oxidase obtained from *Eriobotrya japonica* Lindl. And achieved a purification fold of 39.9 with protein recovery of 15%.

Table 1: Purification of PPO from *Zyzyphus spina-christi*.....

Purification Steps	Protein conc.	Activity	Specific activity	Purification fold	Yield (%)
Crude extract	3.12	38.90	12.47	1.00	100.00
(NH <sub>4</sub> ) <sub>2</sub> SO	2.23	34.50	15.47	1.24	89.70
DEAE- Sephadex A-50	1.11	19.45	17.52	1.40	50.00
Sephadex G-100	0.41	9.54	23.27	1.87	24.50

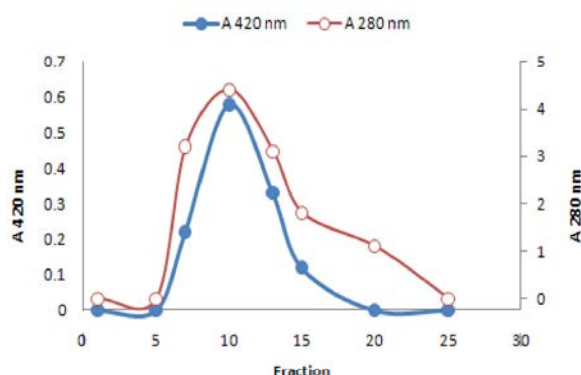


Fig. 1: Purification of PPO from *Zyzyphus spina-christi*.....

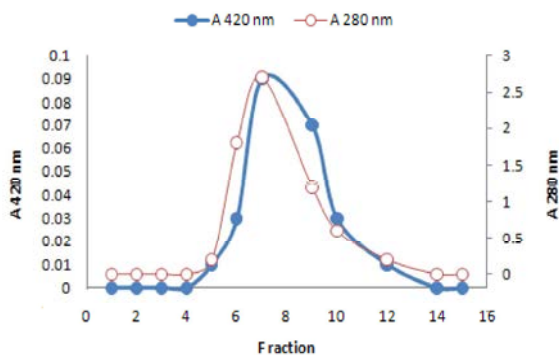


Fig 2: Chromatographic profile of purification of PPO from *Zyzyphus spina-christi* by gel chromatography on Sephadex G-100.....

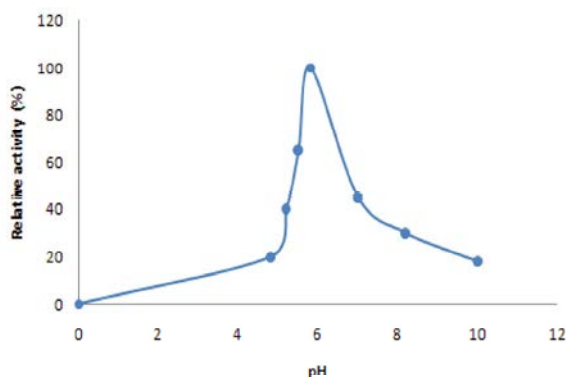


Fig. 3: Activity of *Zyzyphus spina-christi* as function of pH.....

Table 2: Relative enzyme activity at 10mM substrate concentration.....

Substrate (10 mM)	Relative activity (%)
Catechol	100.00 3.110
Caffeic acid	8.80 0.870
Chlorogenic acid	2.27 0.660
Phloroglucinol	0.88 0.007
Tyrosine	0.00

### Characterization of PPO

**Effect of Ph on Enzyme Activity:** The activity of PPO was measured at different pH using catechol. As seen in Fig 3, the optimum pH of the enzyme was found to be 5.8 using catechol as the substrate.

It is seen that in general most plants show PPO activity at or near neutral pH values. The result shown in this study corresponds well with the results obtained by Urszula *et al.* [5] for broccoli (*Brassica oleracea*) florets (pH 5.72). It also corresponds with the results obtained by Dogan and Dogan [22] for DeChaunac apple (pH 6.0).

**Enzyme Kinetics and Substrate Specificity:** Though there are several compound that are used as substrates for polyphenol oxidase [22], in this study we selected the most commonly used substrates such as catechol, caffeic acid chlorogenic acid phloroglucinol and tyrosine. As shown in Table 2, Polyphenol oxidase showed the highest activity towards catechol (dihydroxy phenols) and the lowest activity towards phloroglucinol (trihydroxy phenol), whereas, no activity was shown towards tyrosine (monophenols). Cho and Ahn [23] used catechol in the studies of kinetic properties of PPO from potatoes, whereas, Janovitz-Klapp *et al.* [24] compared the activity of PPO in apples against several substrates.

The Lineweaver-Burk plot analysis of polyphenols oxidase from *Zyzyphus spina-christi* showed that the Michaelis Menten constant ( $K_m$ ) and the maximum reaction velocity ( $V_{max}$ ) were 11.4mM and 16,400 U/ml  $\text{min}^{-1}$  respectively for catechol (Fig. 4). This value for catechol was similar to that of tea leaf (12.5 mM) [25] and also with field bean seed (10.5 mM) [26].

**Optimum Temperature and Thermal Stability:** The effect of temperatures between 5 and 80°C on PPO activity has shown that the optimum temperature for the PPO

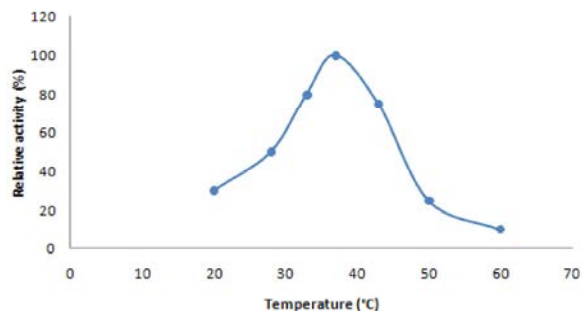


Fig. 4: Determination of optimum temperature of *Zyzyphus spina-christi* PPO activity.....

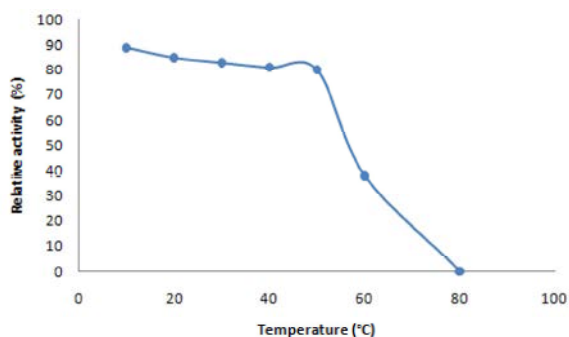


Fig 5: Thermal stability of PPO of *Zyzyphus spina-christi*.....

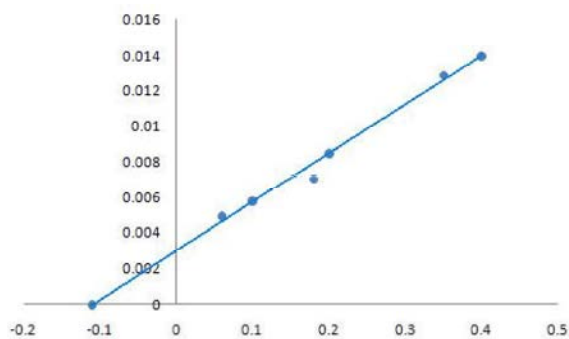


Fig. 6: Lineweaver-Burk plot from *Zyzyphus spina-christi* activity with catechole as substrate.....

enzyme from *Zyzyphus spina-christi* to be 37°C (Fig 5). This value was similar to that obtained by Dincer *et al.* [27] from plum (37°C). The value is also similar to that obtained by Urszula *et al.* [5] in investigations of PPO from butter lettuce.

The thermal stability profile of *Zyzyphus spina-christi* PPO, showed as residual activity after pre-incubation at the specified temperature in shown in Fig 6. This PPO showed thermal stability up to 50°C for a period of 60 minutes with almost 80% activity remaining. The enzyme from medlar fruits was stable for 30 minutes

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

Inhibitor	$K_i(M)$	Type of inhibition	Inhibition (%)
Ascorbic acid	$8.2 \times 10^{-5}$	Competitive	67
Thiourea	$3.2 \times 10^{-5}$	Non competitive	60
Sodium meta bisulphate	$3.8 \times 10^{-5}$	Non competitive	58
Glutathione	$5.1 \times 10^{-5}$	Non competitive	61

at 60°C [27]. It has been reported that *Allium sp.* PPO was stable at 40°C for 30 min [28]. PPO from latex of *Hevea brasiliensis* was stable up to 60°C for 60 minutes [29].

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

From the  $K_i$  constants, it is concluded that the inhibition modes for thiourea, sodium metabisulphate and glutathione are non competitive and ascorbic acid competitive. Enzymatic browning of plants and fruits may be delayed or eliminated by removing the reactants such as oxygen and phenolic compounds or by using PPO inhibitors. There are a number of inhibitors used by researchers to prevent enzymatic browning [30-32]. The inhibitory reaction mechanism differs and depends on the reducing agent that is employed.

It is seen that the compounds tested in the present study (ascorbic acid, thiourea, sodium metabisulphate and glutathione) also inhibited the action of the PPO enzyme isolated from artichoke (*Cynara scolymus L.*) heads [33].

Ascorbic acid acts more as an antioxidant than as an enzyme inhibitor because it reduces the initial quinone from the enzyme to the original diphenol before it under goes secondary reactions, which lead to browning [34]. Ascorbic acid has also been found to show competitive activity towards PPO isolated from peppermint [15] and potato [23].

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