

Kinetics properties of polyphenol oxidase in hawthorn (*Crataegus spp*)

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ABSTRACT

Polyphenol oxidase (PPO) from hawthorn was extracted and partially purified through $(\text{NH}_4)_2\text{SO}_4$ precipitation, dialysis and ion exchange chromatography. The activity of polyphenol oxidase was investigated in *Crataegus spp*. Spectrophotometric method was used to assay the enzyme activity and the kinetic constants - maximum enzyme velocity (V_{max}) and Michealis - Menten constant (K_m). Of the substrates tested, catechol was the best substrate for PPO with a K_m value of 2.2 mM. The optimum pH for PPO activity was found to be 7. The enzyme showed high activity over a broad pH range of 4 - 8. The optimal pH and temperature for enzyme activity were found to be 7 and 40-45 °C, respectively. k_m value for hawthorn PPO is calculated 22 mM for catechol and 6.7 mM for pyrogallol and 9.7 mM for L-dopa. As can be seen, affinity of PPOs for various substrates varies widely. The enzyme showed a broad activity over a broad pH and temperature range. The thermal inactivation studies showed that the enzyme is heat resistant. The enzyme showed the highest activity toward pyrogallol and no activity toward tyrosine. Of the inhibitors tested, the most potent inhibitors were kojic acid, cysteine and glycine, respectively.

Keywords: Inhibition; Kinetics; Purification; Polyphenol oxidase; Hawthorns' Thermal inactivation

1. INTRODUCTION

Hawthorn, a common name of all plant species in the genus *Crataegus*, is a thorny shrub or small tree that normally has bright green leaves, white flowers, and bright red berries, each containing one to three or five seeds, depending on the species [1]. Hawthorn is a member of the Rosaceae family and is recognized to have approximately 280 species primarily from northern temperate zones in East Asia, Europe, and eastern North America [2,3]. It is a widely used herb for preventing and treating cardiovascular diseases.

Hawthorn fruits are used for stimulating digestion and promoting the function of the stomach, improving blood circulation, and removing blood stasis. In Europe, hawthorn fruits, leaves, flowers, or a combination there of have been traditionally used as an astringent, antispasmodic, cardiotoxic, diuretic, hypotensive, and antiatherosclerotic agent [3].

Many vegetables and fruits become discoloured during storage or processing, an action mediated by the enzyme polyphenol oxidase (PPO) [4]. PPO (tyrosinase, EC 1.14.18.1) is a copper-containing enzyme that is widespread in plants, and synthesised early in tissue development and stored in chloroplasts [5]. The enzyme is a copper protein widely distributed in a multitude of organisms, from bacteria to mammals [6]. Enzymatic browning is the main function of PPOs in fruits and vegetables but is often undesirable and responsible for

unpleasant sensory qualities as well as losses in nutrient quality [7]. These highly reactive quinones polymerize with other quinones, amino acids and proteins to produce coloured compounds, and nutrient quality and attractiveness is reduced [8]. PPO from different plant tissues shows different substrate specificities and degrees of inhibition.

Therefore, characterisation of the enzyme could enable the development of more effective methods for controlling browning in plants and plant products. Our objective was to characterise PPO from hawthorn cultivated in Kurdistan, Iran under different conditions. Substrate and temperature effects were also studied.

2. MATERIALS AND METHODS

2. 1. Materials and Reagents

The hawthorns used in this study were obtained from baneh in Kurdistan of Iran and frozen at -25 °C until used. Catechol, polyvinylpyrrolidone (PVPP), pyrogallol, tyrosine were purchased from Merck (Darmstadt, Germany). Acetone, ammonium sulphate, L-cysteine, kojic acid, L-glycine, polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), cellulose membrane (76 x 49 mm) and DEAE-cellulose were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were of analytical grade.

2. 2. Enzyme Extraction

500 grams of fruit of hawthorns were homogenized in 500 mL of 0.1M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid and 0.5% polyvinylpyrrolidone with the aid of a magnetic stirrer for 1h. The crude extract samples were centrifuged at 30000 g for 20 min at 4 °C. Solid ammonium sulphate $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant to obtain 30 and 80% $(\text{NH}_4)_2\text{SO}_4$ saturation, respectively.

After 1 h, the precipitated proteins for each stage were separated by centrifugation at 30000 g for 30 min. The precipitate was redissolved in a small volume of distilled water and dialyzed at 4 °C against distilled water for 24 h with 4 changes of the water during dialysis. The dialysate was applied to a column (2.5 cm x 30 cm) filled with DEAE-cellulose, balanced with 10 mM phosphate buffer, pH 6.8. In order to remove non adsorbed fractions the column was washed with 200 mL of the same buffer at the flow rate of 0.5 mL/min.

Then, a linear gradient of phosphate buffer concentration from 20 to 180 mM was applied. 5 mL fractions were collected in which the protein level and PPO activity towards catechol as substrate were monitored.

2. 3. Protein Determination

Protein contents of the enzyme extracts were determined according to lowry method using bovine serum albumin as a standard [9].

2. 4. Assay of Enzyme Activity

PPO activity was determined by measuring the absorbance at 420 nm using a spectrophotometer (6305 JENWAY). To determine the best concentration of enzyme preparation corresponding to the highest enzyme activity, the activity was assayed in 3 mL of reaction mixture consisting of 2.5 mL substrate (0.02 M catechol and 0.02 M pyrogallol separately) and different concentrations (0.1-0.3 mL) of the enzyme preparation (1 mg/mL). This mixture was topped-up to 3.0 mL with the phosphate buffer

(pH 6.8) in a 1 cm light path quartz cuvette. The blank consisted of 3.0 mL 0.1 M phosphate buffer (pH 6.8). Two controls were prepared: the cuvette of the first control contained 2.5 mL substrate and 0.5 mL buffer solution, whereas the second control cuvette contained 2.9 mL buffer and 0.1 mL enzyme preparation. Absorbance values of these controls were subtracted from that of the sample. PPO activity was calculated from the linear portion of the curve. The initial rate of PPO catalyzed oxidation reaction was calculated from the slope of the absorbance-time curve. An enzyme preparation of 0.2 mL showed the highest activity using catechol as a substrate which was used in all other experiments. One unit of PPO activity was defined as the amount of enzyme that produces 1 micromole of quinone per minute. Assays were carried out at room temperature and results are the averages of at least three assays and the mean and standard deviations were plotted.

2. 5. pH optimum and stability

PPO activity as a function of pH was determined using catechol and pyrogallol as substrates. Phosphate and phosphate-citrate buffer, ranging from pH 3.0 to 9.0 was used at the assays. The pH stability was determined by incubating the enzyme in the above buffer (pH 3.0 to 9.0) for 30 min and at the end of the incubation period, samples were taken and assayed under standard conditions as described above. All of the assays were performed in triplicate. PPO activity was calculated in the form of unit per mg protein at the optimum pH. The optimum pH value obtained from this assay was used in all the other experiments.

2. 6. Substrate Specificity

2. 6. 1. Enzyme Kinetics

For determination of Michaelis constant (K_m) and maximum velocity (V_{max}) values of the enzyme, PPO activities were measured with two substrates at various concentrations. $1/V$ and $1/S$ values, obtained from these activity measurements, were used for drawing Lineweaver – Burk graphs. In order to determine Michaelis constant (K_m) and maximum velocity (V_m), PPO activities were measured using catechol (0-50 mM), pyrogallol (0-50 mM) as substrates. K_m and V_m values of the enzyme were calculated from a plot of $1/V$ vs. $1/S$ by the method of Lineweaver and Burk.

2. 6. 2. Effect of Temperature on PPO Activity

To determine the optimum temperature for PPO, the activity of the enzyme was measured at different temperatures (25-80 °C) using 0.2 mL enzyme, 2.7 mL of 20 mM catechol as substrate and completed to 3 mL with 0.1 M sodium phosphate buffer (pH 7). The blank consisted of 3.0 mL of 0.1 M phosphate buffer. Controls were run under the same tested temperature. The tubes were pre-heated to the selected temperature to prevent temperature lag before the addition of a 0.2 mL aliquot of enzyme solution. The enzyme samples were removed from water bath after pre-set times and were immediately transferred to ice bath to stop thermal inactivation. After the sample was cooled in ice bath, the residual activity was determined spectrophotometrically using the standard reaction mixture. A non-heated enzyme sample was used as blank. The percentage residual activity was calculated by comparison with the unheated sample.

2. 7. Effects of Inhibitors

The inhibitors examined were L-glycine, L-cysteine and kojic acid. The reaction mixture contained 2.7 mL of catechol at a final concentration of 20 mM in 0.1M phosphate buffer (pH 7), 0.1 mL inhibitor at a final concentration of 0.2, 1 or 1.5 mM and 0.2 mL enzyme solution. The change in absorbance was measured spectrophotometrically at 420 nm. Control tests for inhibitors plus substrate plus buffer were also run at the same time. Percentage inhibition as calculated using the following equation: Inhibition (%) = $[(A_0 - A_i)/A_0].100$, where, A_0 is the initial PPO activity (without inhibitor) and A_i is the PPO activity with inhibitor.

3. RESULTS

3. 1. Extraction and Purification

PPO was purified from hawthorns using a DEAE- cellulose column. A summary of extraction and purification is given in Table 1. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to DEAE-cellulose column, yielding one peak with PPO activity (Fig. 1). A 15.7 fold purification was achieved.

Table 1. Purification of PPO from hawthorns.

Purification step	Volume (ml)	Total Protein (mg)	Total activity (μ M/min)	Specific activity (μ M/min . mg protein)	Purification (Fold)
Crude extract	150	12	255	0.14	1
(NH ₄) ₂ SO ₄ precipitation (30%)	105	10	335	0.32	2.3
(NH ₄) ₂ SO ₄ precipitation (80 %)	60	8	380	0.79	5.6
DEAE-cellulose	8	4.5	78	2.2	15.7

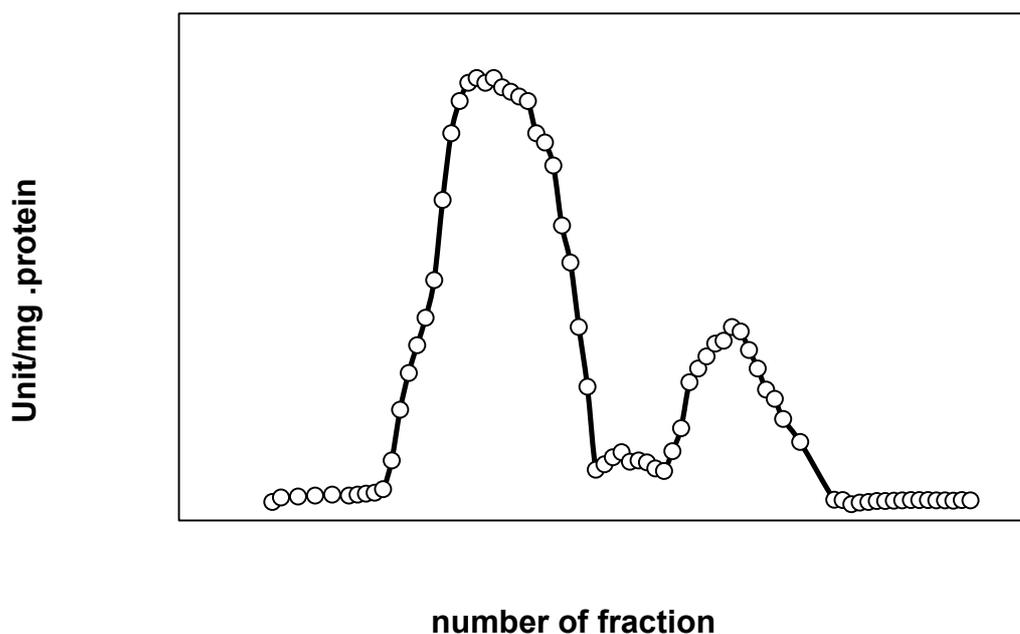


Figure 1. Elution pattern of hawthorn PPO on DEAE-cellulose. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to a 2.5 cm x 30 cm column, equilibrated and washed with 10 mM phosphate buffer, pH 7. Elution of adsorbed proteins was performed using a linear gradient of 10 to 200 mM phosphate buffer (pH 7) at a flow rate of 0.5 mL/min.

3. 2. pH Optima

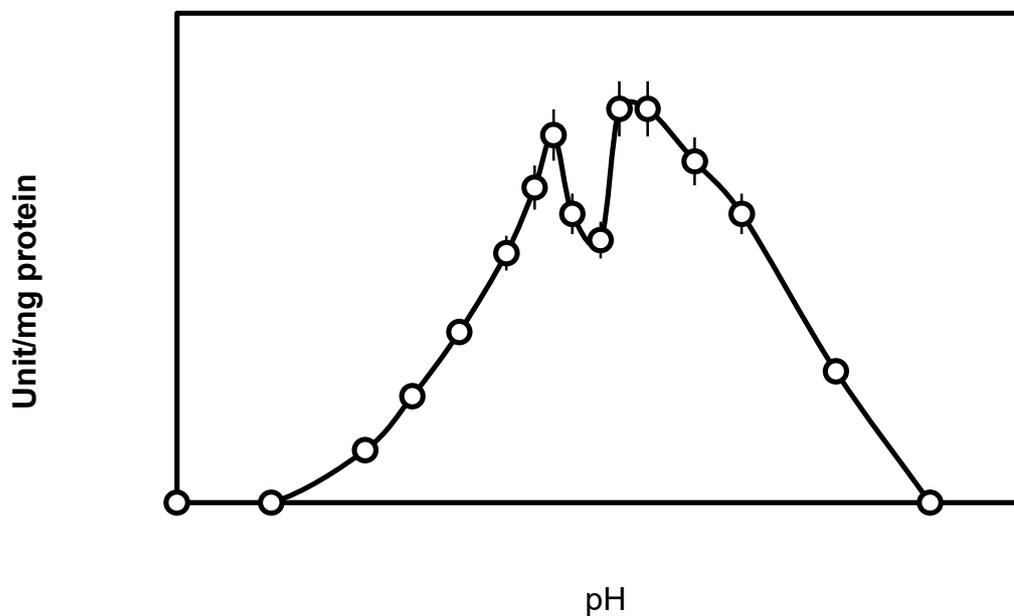


Figure 2. Activity of hawthorn PPO as a function of pH. Each data point is the mean of three determinations. The vertical bars represent standard deviations.

Optimum pH for PPO activity with catechol and pyrogallol as substrates was 7 (Fig. 2). As the pH increased from 4 to 10, the enzyme activity increased in two step at pH 6 and 7,

with maximal activity occurring at pH 7, after which the activity started to decline. Differences in optimum pH for PPO with distinct substrates have been reported for the enzyme from various sources [10-13]. However, pH optima for PPO activity in presence of catechol and pyrogallol in hawthorn is the same.

3. 3. The effect of temperature on PPO activity

Optimum temperature for PPO activity with pyrogallol 45 °C; however, when using catechol as substrate, it was 50 °C (Fig. 3). This behavior of the PPO enzyme with these substrates was confirmed after several repetitions. Heating for 80 min at 27 and 40 °C for pyrogallol and catechol increases the activity; however, at the higher temperatures, the enzyme after 30 minute incubation was rapidly inactivated. Optimum temperatures for PPO activity in others sources were reported to be between 20 and 40 °C. The enzyme was reasonably stable at 50 °C and, as expected, the rate of inactivation was higher with increasing temperature (Fig. 3). When enzyme exposed to 60 °C, a decrease in activity earned so activity reached to 80% after 10 minute and zero after 60 minute of incubation. The times required for 50% inactivation of PPO activity at 60 °C was 15 minute (Fig. 4). Hawthorn - PPO is a heat-stable enzyme at 40-50 °C; so is more resistant to heating than PPO from *solanum lycopersicum* [14].

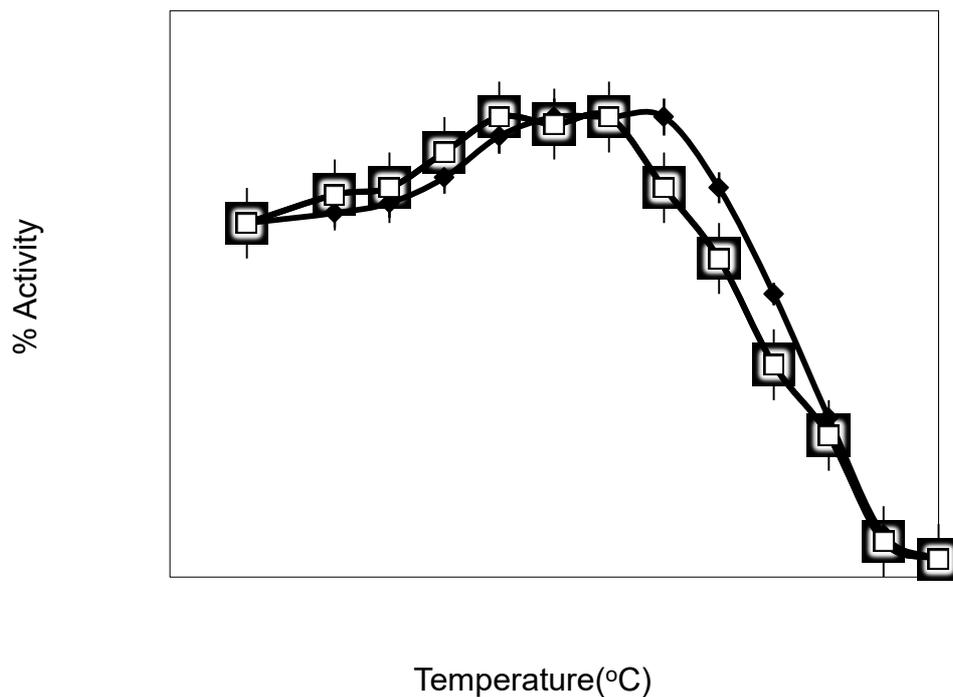


Figure 3. Activity of hawthorn PPO as a function of temperature. Each data point is the mean of three determinations. The vertical bars represent standard deviations. [(■) , catechol 20 mM] and [(□), pyrogallol 8 mM].

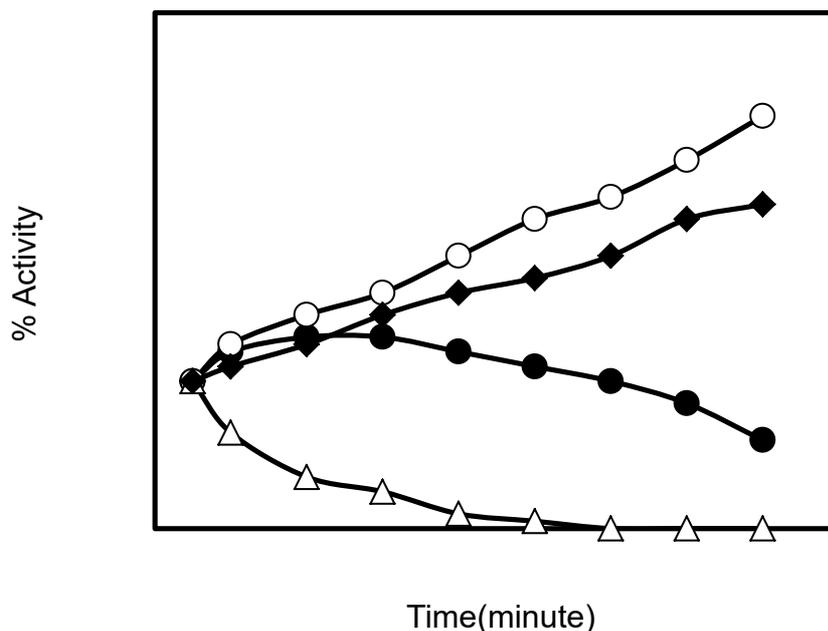


Figure 4. Heat inactivation of PPO at different temperatures. The enzyme was incubated at the temperatures [27,(■); 40,(○); 50,(●)] and [60(Δ) °C] and the remaining activity was determined with catechol as substrate.

3. 4. Effect of Inhibitor

Effects of cysteine, glycine and kojic acid on hawthorns PPO activity were studied at various concentrations using catechol as the substrate and the results were reported as percentage inhibition in Table 2. The inhibition degree varied in dose dependent manner. From the results, it can be concluded that the most potent inhibitors was kojic acid, because a higher degree of inhibition was achieved. Glycine was the least potent inhibitor, so in presence of 1.5 mM kojic acid, activity of PPO in hawthorn reached to 15% , but in the same concentration of cysteine and glycine, activity reached to 35% and 37%, respectively.

Table 2. Effect of inhibitors on hawthorns PPO activity.

Inhibitor	Concentration (mM)	Inhibition* (%)
Cysteine	0.20	45 ± 2
	1.50	65 ± 1.8
Glycine	0.05	30 ± 1.2
	0.20	55 ± 3
	1.50	63 ± 1.5
Kojic acid	0.01	22 ± 1.5
	0.20	68 ± 2.5
	1.50	85 ± 2.5

*Each value is the mean of three determinations ± standard deviations

3. 5. Kinetic Parameters

K_m and V_m values for hawthorns PPO for different substrates are presented in Table 3. The affinity of the enzyme varied depending on the substrate used. Hawthorns PPO had a higher affinity for pyrogallol, as evidenced by lower K_m value. The criterion for the best substrate is the V_m/K_m ratio. Of the substrates tested, the best substrate for hawthorns PPO was catechol. The enzyme showed no activity against tyrosine. Duangmal and Apenten (1999) [15] reported the following K_m values for taro PPO: 9.0 mM for 4-methylcatechol, 67.9 mM for catechol and 89.9 mM for pyrogallol. The same investigators reported the following K_m values for potato PPO: 1.1 mM for 4-methylcatechol, 6.8 mM for catechol and 1.5 mM for pyrogallol, but K_m value for hawthorn PPO is calculated 22 mM for catechol and 6.7 mM for pyrogallol and 9.7 mM for L-dopa. As can be seen, affinity of PPOs from various sources for various substrates varies widely.

Table 3. Kinetic parameters of hawthorns PPO.

Substrate	K_m (mM)	V_{max} (Unit/ mg. protein)	V_{max}/K_m Unit/mg protein. mM^{-1}
catechol	22	445	20.2
pyrogallol	6.7	112	16.7
Dopa	9.7	110	11.3

4. DISCUSSION AND CONCLUSIONS

It has been reported that some plant PPOs are membrane-bound. Therefore, use of detergents is required to solubilize the enzyme. Phenol compounds interfere with purification of proteins from plants. They cross-link proteins by hydrogen bonds and covalent interactions. Furthermore, homogenization of the plant tissues initiates enzymatic browning which results in the formation of quinones. The quinones may also form covalent linkages that may not be reversible. Use of phenol-absorbing polymers, such as polyethylene glycol (PEG) or PVPP and use of reducing agents such as ascorbic acid are commonly applied in order to overcome these problems [16]. The pH optimum for PPO activity from hawthorn was found to be 7. It is noteworthy to mention that the pH optimum for PPO is found to be dependent on the enzyme source, substrate and extraction methods used. Other reported values include 6.50 for banana peel PPO [17], 4.20 for grape PPO [18], 5.70 for broccoli PPO [19] and 7.5 for avocado PPO [20]. Halder et al. (1998) [21] reported an optimum pH value of 5.0 for tea PPO. In a study carried out by Dogan et al. (2002) [22] on different aubergine cultivars, the temperature optima varied between 20-30 °C using catechol and 4-methylcatechol as substrates. Ding et al. (1998) [23] reported an optimum temperature of 30°C for loquat PPO using chlorogenic acid as substrate. Other reported values include 25 °C for grape PPO (18) and 30 °C for banana PPO [24]. The optimum temperature obtained in this study is 40-50 °C that is dependent on the substrate. PPO is generally considered as an enzyme of low thermostability. Heat stability was reported to differ among cultivars and multiple forms of PPO from the same source as well as between fruit tissue homogenates and their respective juices (25). PPO from hawthorns showed high thermal stability at the temperatures studied. The mode of action

of inhibitors differs from each other. The mode of inhibition of kojic acid is by reducing the enzyme Cu^{2+} to Cu^+ rendering the enzyme inactive and unavailable for O_2 binding and by complexing with quinone compounds to prevent melanin formation via polymerization. L-cysteine is a thiol compound, which is a strong nucleophile and suppresses enzymatic browning mainly via formation of colourless addition products with *o*-quinones. At the concentrations tested, the inhibition degrees of the inhibitors were very different. L-cysteine being the least potent inhibitor and kojic acid showed a higher degree of inhibition. In a study carried out by Gomez-Lopez (2002) [20], it was found that the most effective inhibitor for avocado PPO was cysteine. Rapeanu et al. (2006) [13] found that most potent inhibitors for grape PPO were ascorbic acid, cysteine and sodium metabisulfite. In conclusion, after the final purification step, a 15.7 fold purification. The optimal pH and temperature for enzyme activity were found to be 7 and 40-50 °C, respectively. The enzyme showed a broad activity over a broad pH and temperature range. The thermal inactivation studies showed that the enzyme is heat resistant. The enzyme showed the highest activity toward pyrogallol and no activity toward tyrosine. Of the inhibitors tested, the most potent inhibitors was kojic acid.

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Biography

Shahryar Saeidian is Associate Professor of Payame Noor University. He teaches biochemistry, molecular biology, enzymology and advanced biochemistry of proteins and nucleic acids. saeidian's research focuses on the investigation of activity of enzymes, ligand binding of proteins and purification of enzyme. One strand of research examines the activity of polyphenol oxidase in plants. A current project of research relates to purify polyphenol oxidase and peroxidase and determine peroxidase activity of fruits and plants in Kurdistan (Iran). Saeidian received his Ph.D. degree in 2006 from Institute of Biochemistry and Biophysics (IBB) of Tehran university and has a BA and MA in biology and biochemistry at Shiraz and Tehran university (Iran). Shahryar is from Iran and was born in Kurdistan (Baneh). Dr. Saeidian has published over 15 articles in the areas of enzymology and ligand binding. He is a member of the biochemistry society and Asia-Pacific Chemical, Biological & Environmental Engineering Society (APCBEEES). He has been as a referee and a member of technical committee of many of national and international congress.

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