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Partial purification and characterisation of polyphenol oxidase from tomatoes *(solanum lycopersicum)*

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ABSTRAT

Polyphenol oxidase (PPO) from tomatoes was extracted and partially purified through $(NH_4)_2SO_4$ precipitation, dialysis and ion exchange chromatography. The activity of polyphenol oxidase was investigated in solanum lycopersicum. Spectrophotometric method was used to assay the enzyme activity and the kinetic constants - maximum enzyme velocity (Vmax) and Michealis - Menten constant (Km). Of the substrates tested, pyrogallol was the best substrate for PPO with a K_m value of 1.5 mM. The optimum pH for PPO activity was found to be 6.8. 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 6.8 and 50-60 °C, respectively. km value for tomatoe PPO is calculated 25 mM for catechol and 1.5 mM for pyrogallol and 8.5 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 was sodium kojic acid.

Keywords: Inhibition, Kinetics, Purification, Polyphenol oxidase, Tomatoes, Thermal inactivation

INTRODUCTION

Many vegetables and fruits become discoloured during storage or processing, an action mediated by the enzyme polyphenol oxidase (PPO) (Broothaerts et al. 2000). 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 (Van Gelder et al. 1997). The enzyme is a copper protein widely distributed in a multitude of organisms, from bacteria to mammals (Robb et al. 1984). 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 (SanchezA-Amat et al. 1997). When cell membrane integrity is disrupted, phenolic substrates encounter the enzyme and are converted to o-quinones in a two-step process of hydroxylation of monophenols to diphenols (monophenolase activity), followed by the oxidation of diphenols to o-quinones (diphenolase activity) (Espin et al., 1998). These highly reactive quinones polymerize with other quinones, amino acids and proteins to produce coloured compounds, and nutrient quality and attractiveness is reduced

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(Matheis et al. 1984). 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 small cherry tomato cultivated in Kurdistan, Iran under different conditions. Substrate and temperature effects were also studied. One unusual characteristic of this enzyme is its ability to exist in an inactive or latent state (Gandia-Herrero et al. 2004). PPO can be released from latency or activated by various treatments or agents, including urea (Okot-Kotber et al. 2002), polyamines (Jimenezatienzar et al. 1991), anionic detergents such as SDS (Santosh et al. 2006), proteases (Laveda et al., 2001) and fatty acids **(**Golbeck et al. 1981**).**

MATERIAL AND METHODS

Materials and Reagents

The tomatoes used in this study were obtained from Kurdistan of Iran and frozen at -25 °C until used. Catechol, polyvinylpyrolidone (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 (76x49mm) and DEAE-cellulose were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were of analytical grade.

Enzyme Extraction

200 grams of tomatoes were homogenized in 150 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 4ºC. Solid ammonium sulphate (NH4)2SO4 was added to the supernatant to obtain30 and 80% (NH4)2SO4 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 4ºC against distilled water for 24 h with 4 changes of the water during dialysis.

Ion Exchange Chromatography

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. The fractions which showed PPO activity were combined and were used as enzyme source in the following experiments (Ding CK. et al; 1998.).

Protein Determination

Protein contents of the enzyme extracts were determined according to lowry method using bovine serum albumin as a standard (Lowry, O.H. et al; 1984).

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 lcatechol and 0.02 M pyrogallol separately) and different concentrations (0.1-0.3 mL) of the enzyme preparation (1mg/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.

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 8.0 was used at the assays. The pH stability was determined by incubating the enzyme in the above buffer (pH 3.0 to 8.0) 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.

Substrate Specificity

Enzyme Kinetics

For determination of Michaelis constant (*Km*) and maximum velocity (*Vmax*) 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.

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 6.8). 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.

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 6.8), 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 (%) = $[(A_O - A_i)/A_0)].100$, where, A_0 is the initial PPO activity (without inhibitor) and A_i is the PPO activity with inhibitor.

RESULTS

Extraction and Purification

PPO was purified from tomatoes 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 12.5 fold purification was achieved.

Table 1. Purification of PPO from tomatoes

Purification step	Volume (ml)	Total Protein (mg)	Total activity (µM/min)	Specific activity (µM/min . mg protein)	Purification (Fold)
Crude extract	180	9.5	165	0.096	1
$(NH_4)_2SO_4$ precipitation (30%)	110	7.0	280	0.363	3.7
$(NH_4)_2SO_4$ precipitation (80 %)	70	5.5	295	0.766	7.9
DEAE-cellulose	15	3	54	1.2	12.5

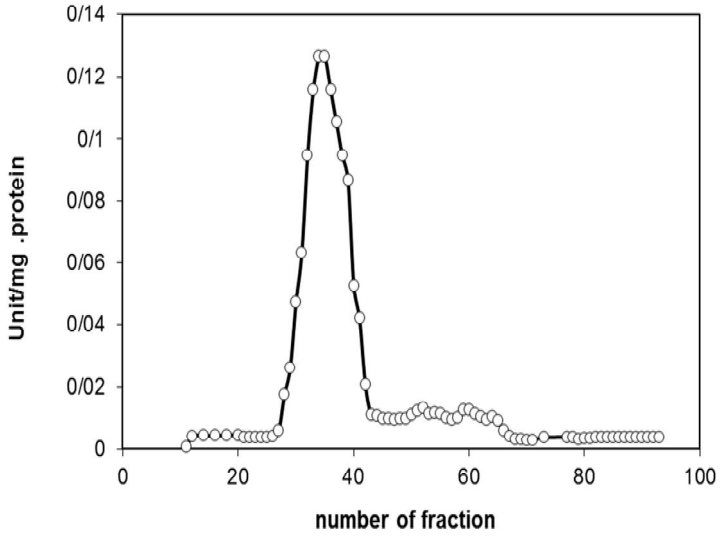

Figure 1. Elution pattern of tomatoe PPO on DEAE-cellulose. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to a 2.5 cm x 30 cm column, equilibrated and washed with 10 mM phosphate buffer, pH 6.8. Elution of adsorbed proteins was performed using a linear gradient of 10 to 200 mM phosphate buffer (pH 6.8) at a flow rate of 0.5 mL/min.

PH Optima

Optimum pH for PPO activity with catechol and pyrogallol as substrates was 6.8 (Fig 2). As the pH increased from 4 to 6.8, the enzyme activity increased, with maximal activity occurring at pH 6.8, 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 (Lourenço et al., 1990; Gonzalez et al., 2000; Kavrayan and Aydemir, 2001; Dogan et al., 2005; Rapeanu et al., 2006). However, pH optima for PPO activity in presence of catechol and pyrogallol in tomatoe is the same.

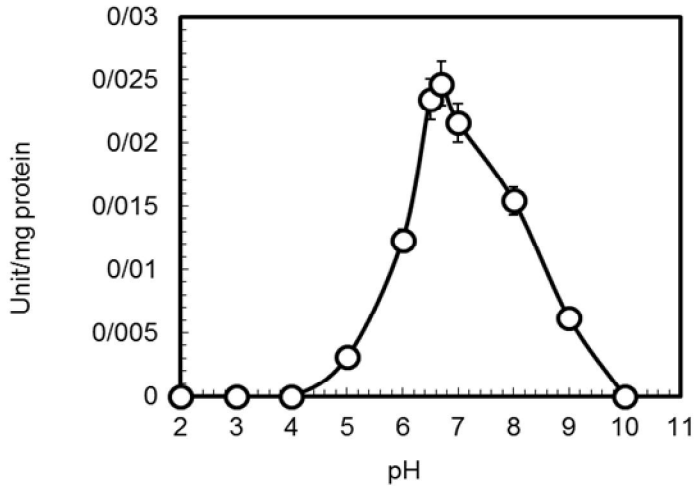

Figure 2. Activity of tomatoe PPO as a function of pH. Each data point is the mean of three determinations. The vertical bars represent standard deviations.

The effect of temperature on PPO activity

Optimum temperature for PPO activity with pyrogallol 50°C; however, when using cathecol as substrate, it was 60°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 55-60°C and, as expected, the rate of inactivation was higher with increasing temperature (Fig. 3). When enzyme exposed to 60°C, a 100 % residual activity was registered for 10 minute. The times required for 50% inactivation of PPO activity at 60°C was 30 minute (Fig. 4). Tomatoe - PPO is a heat-stable enzyme at 40 - 60°C; so is more resistant to heating than PPO from peppermint (Rapeanu et al., 2006, Lourenco et al., 1992, Dogan et al., 2005).

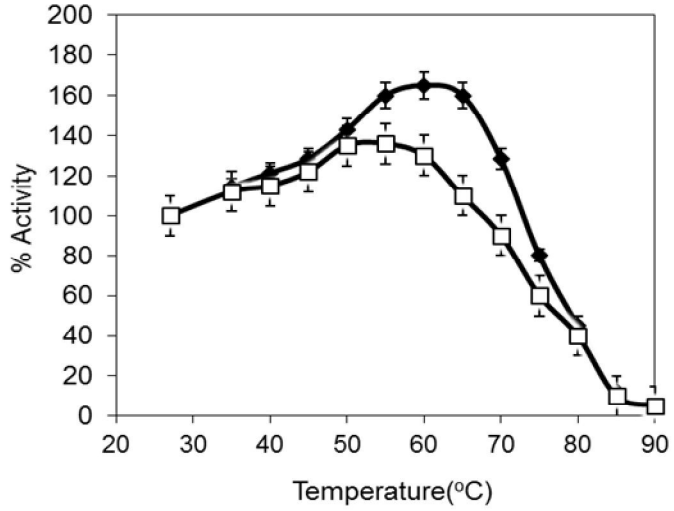

Figure 3. Activity of tomatoe PPO as a function of temperature. Each data point is the mean of three determinations. The vertical bars represent standard deviations. [(■) , catechol 20 mM]and [(□), pyrogallol 8 mM].

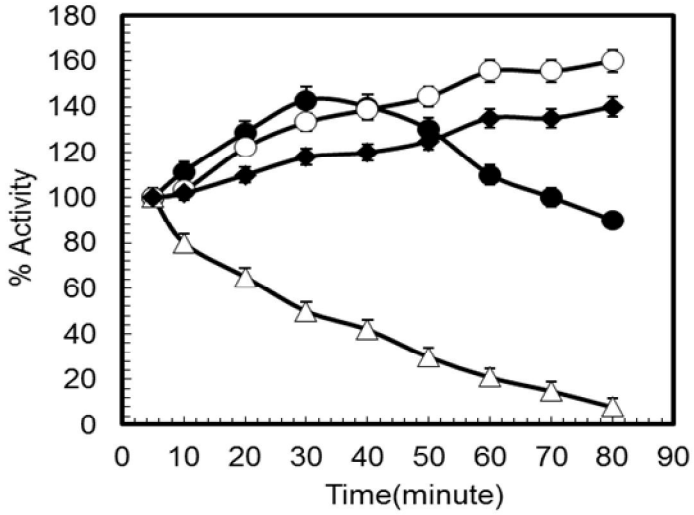

Figure 4 - Heat inactivation of PPO at different temperatures. The enzyme was incubated at the temperatures [27,(■); 40,(○); 50,(●) and 60(Δ) °C) and the remaining activity was determined with catechol as substrate.

Effect of Inhibitor

Effects of cysteine, glycine and kojic acid on tomatoes 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. Cysteine was the least potent inhibitor.

Table 2. Effect of inhibitors on tomatoes PPO activity

Inhibitor	Concentration (mM)	Inhibition* (%)
Cysteine		
	0.20	25 ± 1.2
	1.50	50 ± 2.5
Glycine	0.05	20 ± 1.2
	0.20	40 ± 4.5
	1.50	65 ± 1.2
Kojic acid	0.01	12.2 ± 1.2
	0.20	55 ± 3.0
	1.50	90 ± 1.5

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

Kinetic Parameters

K_m and V_m values for tomatoes PPO for different substrates are presented in Table 3. The affinity of the enzyme varied depending on the substrate used. Tomatoes 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 tomatoes PPO was pyrogallol. The enzyme showed no activity against tyrosine. In a study carried out by Gao jia et al. (2011) K_m value for PPO from sour cherry pulp was found to be 3.5 mM, using catechol. Duangmal and Apenten (1999) 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 km value for tomatoe PPO is calculated 25 mM for catechol and 1.5 mM for pyrogallol and 8.5 mM for L-dopa. As can be seen, affinity of PPOs from various sources for various substrates varies widely.

Table 3. Kinetic parameters of tomatoes PPO.

Substrate	Km (mM)	V_{max} (Unit/ mg.protein)	V_{max}/K_m Unit/mg protein. mM^{-1}
catechol	25	550	22
pyrogallol	1.5	87	58
Dopa	8.5	65	7.6

DISCUSSION

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 (Vamos-Vigyazo, L. 1981.). The pH optimum for PPO activity from tomatoe was found to be 6.8. 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 (Ünal MÜ. 2007), 4.20 for grape PPO (Ünal MÜ and fiener A. 2006), 5.70 for broccoli PPO (Gawlik-Dziki U, et al 2007) and 7.5 for avocado PPO (Gomez-Lopez VM. 2002). Halder et al. (1998) reported an optimum pH value of 5.0 for tea PPO. In a study carried out by Dogan et al. (2002) on different aubergine cultivars, the temperature optima varied between 20-30 °C using catechol and 4-methylcatechol as substrates. Ding et al. (1998) reported an optimum temperature of 30°C for loquat PPO using chlorogenic acid as substrate. Other reported values include 25 °C for grape PPO (Ünal MÜ and fiener A. 2006) and 30 °C for banana PPO (Yang C-P, et al. 2001). The optimum temperature obtained in this study is 50-60°C that is dependent on the substrate. An optimum temperature (50 °C) for strawberry PPO was reported by Serradell et al. (2000) that is the same of optimum temperature of tomatoe PPO. 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 (Robinson DS, et al. 1991). PPO from tomatoes 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 an unavailable for O2 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), it was found that the most effective inhibitor for avocado PPO was cysteine. Rapeanu et al. (2006) found that most potent inhibitors for grape PPO were ascorbic acid, cysteine and sodium metabisulfite. In conclusion, after the final purification step, a 12.5 fold purification. The optimal pH and temperature for enzyme activity were found to be 6.8 and 50-60 °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 sodium kojic acid.

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