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DETERMINATION OF BIOCHEMICAL PROPERTIES OF POLYPHENOL OXIDASE FROM DOMAT OLIVES

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Abstract
Enzymatic browning of damaged tissues of fruits and vegetables occurs from the oxidation of phenolic compounds and contributes significantly to quality loss. The primary enzim responsible for the browning reaction is polyphenol oxidase (PPO). PPO from Domat olives was extracted and purified through $(NH_4)_2SO_4$ precipitation, dialysis and ion exchange chromatography. Optimum pH and temperature for olive PPO were found to be 4.5 and 30 °C, respectively, using 4-methyl catechol as substrate. K_m and V_{max} values for olive PPO using 4-methyl catechol as substrate were found to be 14.52 mM (r^2= 0.985) and 1.78 Abs/min, respectively. Of the inhibitors tested, the most potent inhibitors were L-cysteine and sodium disulfite. The Z and Ea values obtained in this study were 18.6 °C (r2 = 0.953) and 121.0 kj/mol (r^2= 0.950), respectively.

Keywords: Olive, polyphenol oxidase, enzymatic browning, kinetics, thermal inactivation

DOMAT ZEYTİNİ POLİFENOL OKSİDAZININ BİYOKİMYASAL ÖZELLİKLERİNİN BELİRLENMESİ

Özet
Zarar görmüş meye ve sebzelerdeki enzimatik esmerleşme fenolik bileşiklerin oksidasyonu sonucu meydana gelir ve önemli kalite kayıplarına neden olur. Esmerleşme reaksiyonuna neden olan en önemli enzim polifenol oksidaz (PFO)'dır. Domat zeytininden polifenol oksidaz enzimi ekstrakte edilmiş ve amonyum sülfat çökeltmesi, dializ ve iyon değişim kromatografisi ile saflaştırılmıştır. Zeytin PFO'sunun optimum pH ve sıcaklığı sırasıyla 4-metil kateşol substratı kullanılarak 4.5 ve 30 °C bulunmuştur. Zeytin PFO'su K_m ve V_{max} değerleri sırasıyla 4-metil kateşol substratı kullanılarak 14.52 mM (r^2= 0.985) ve 1.78 Abs/dak bulunmuştur. Test edilmiş inhibitörlerin en etkili olanları sistein ve sodyum disülfittir. Bu çalışmada elde edilmiş Z ve Ea değerleri sırasıyla 18.6 °C (r^2 = 0.953) ve 121.0 kj/mol (r^2= 0.950) bulunmuştur.

Anahtar kelimeler: Zeytin, polifenol oksidaz, enzimatik esmerleşme, kinetik, termal inaktivasyon

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INTRODUCTION

Enzymatic browning of foods occurs during handling, storage and processing of fruits and vegetables, which impairs the sensory properties and marketability of the product and also lowers the nutritional value due to associated changes in colour, flavour and softening (due probably to the action of pectic enzymes) (1-2). It is estimated that over 50% losses in fruit occur as a result of enzymatic browning (3). Oxidative browning is catalysed by polyphenol oxidase (PPO) (EC 1.14.18.1). PPO, which is widely distributed in the plant and animal kingdoms, is a copper-containing enzyme. In the presence of molecular oxygen, PPO catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity) (4). Quinones formed by the action of PPO are highly reactive and can react with amino and sulfhydryl groups of proteins and enzymes as well as with other substrates, such as cholorogenic acid derivatives and flavonoids. Quinones also contribute to the formation of brown pigments by participating in polymerisation and condensation reactions with proteins (5).

Mechanical harvesting can cause brown spots in sensitive olive cultivars because of the blows that fruits receive during operation. Brown spots remain after the fermentation, which reduces the quality and marketability of the product. The browning reaction, which results from mechanical injury during postharvest storage or processing of fruits and vegetables, is a widespread phenomenon catalyzed mainly by polyphenol oxidase (PPO) (6).

Varietal differences in enzyme activity and browning susceptibility are well-known phenomena. Prevention of enzymatic browning requires a detailed study of the PPO's biochemical properties. No research has been conducted on PPOs from Domat olives grown in Turkey. The present work was undertaken to study some of the biochemical properties of PPO from Domat olives.

MATERIAL AND METHODS

Materials

Domat olives used in this study were obtained from the orchard of Agricultural Faculty of University of Cukurova, Adana, Turkey. They were frozen at -25 °C until used. Triton X-100, sodium metabisulfite, polyvinylpyrolidone (PVPP), ascorbic acid and commasie brillant blue (CBB) were purchased from Merck (Darmstadt, Germany). Polyethylene glycol (PEG), phenylmethylsulfonyl fluoride (PMSF), L-cysteine, citric acid, acetone, 4-methyl catechol, and cellulose membrane (76 x 49 mm) were purchased from Sigma-Aldrich (St. Louis, USA). Toyopearl DEAE-650 M was purchased from Supelco (Montgomeryville, USA). All chemicals were of analytical grade.

Enzyme Extraction

The deseeded olives (150 g) were homogenized in 225 mL of cold acetone (-25 °C) containing 1.875 g of polyethylene glycol, using a pre-chilled Waring blender (Model HGBTWTS3, Torrington, Connecticut, USA) for 2 min at low speed. The slurry was vacuum filtered through filter paper. The residue was re-extracted with 150 mL of cold acetone. This procedure was repeated until a white powder was obtained. The resultant acetone powder was dried overnight at room temperature and stored at -25 °C (7).

10 g of acetone powder was homogenized for 40 sec in 300 mL of 0.1 M phosphate buffer (pH 6.8) containing 10 mM ascorbic acid, 0.1% polyvinylpyrolidene, 0.5% Triton X-100 and 1 mM PMSF, using Waring blender. After the homogenate was magnetically stirred for 3 h at 4 °C, it was centrifuged at 10000 x g for 45 min at 4 °C. The resulting supernatant was subjected to ammonium sulphate precipitation. The fraction precipitated at 90% saturation was separated by centrifugation at 10000 x g for 45 min at 4 °C. The precipitate was dissolved in a small amount of 10 mM phosphate buffer, pH 6.8, and dialyzed overnight at 4 °C in the same buffer (8).

Ion Exchange Chromatography

For ion exchange chromatography, the dialysate was applied to a column (2.5x30 cm) filled with DEAE-Toyopearl 650 M, balanced with 10 mM

phosphate buffer, pH 6.8. The column was eluted with same buffer at a flow rate of 0.5 mL/min until the 60th fraction after which a linear gradient of phosphate buffer from 10 to 200 mM was applied. Fractions in 4 mL were collected in which the protein level and PPO activity towards 4-methyl catechol as substrate were monitored. The fractions which showed PPO activity were combined and were used as enzyme source in the following experiments.

Protein Determination

Protein contents of the enzyme extracts were determined according to Bradford method using bovine serum albumin as a standard (9).

Assay of Enzyme Activity

PPO activity was determined in 1.0 mL assay mixtures in a spectrophotometer (Shimadzu UV-1700, Kyoto, Japan) fitted with a thermostatted cuvette by measuring the increase in absorbance at 410 nm at 30 °C. The initial rate was calculated from the slope of the linear part of the absorbance -time curve. Unless otherwise stated, the standard reaction mixture consisted of 0.1 mL of enzyme solution and 0.9 mL of 4-methyl catechol in 0.2 M citric acid buffer (pH 4.50). In all experiments, control experiments without enzyme were conducted and no significant oxidation of substrate was observed during the short period employed to measure PPO activity. One unit of enzyme activity was defined as the amount of enzyme that caused an increase of 0.001 in the absorbance value per min under the assay conditions (10).

pH Optima

PPO activity was determined in a pH range of 3.04-5.80 in 0.2 M citric acid buffer and 6.30-6.70 in 0.2 M phosphate buffer. PPO activity was assayed, using the standard reaction mixture but changing the pH of the buffer. PPO activity was calculated in the form of percent residual activity at the optimum pH. The optimum pH value obtained from this assay was used in all the other experiments.

Temperature Optima

The activity of PPO was determined at temperatures ranging from 20 °C to 70 °C. 0.9 mL of 4-methyl catechol solution in buffer was heated to the appropriate temperature in a water bath. After equilibration of the reaction mixture at the selected temperature, 0.1 mL of the enzyme solution was added and the enzyme activity was measured. PPO activity was calculated in the form of percent residual PPO activity at the optimum temperature.

Kinetic Parameters

In order to determine Michaelis constant (Km) and maximum velocity (Vm), PPO activities were measured using 4-methyl catechol as substrate at various concentrations (3.125-50 mM). Km and Vm values of the enzyme were calculated from a plot of 1/V vs. 1/S by the method of Lineweaver and Burk.

Thermal Inactivation Kinetics

Thermal inactivations of PPOs were studied at the selected temperatures for various times (5, 10 and 15 min at 65, 70 and 75 °C) using screw-cap tubes. The screw-cap tubes were pre-heated to the selected temperature to prevent temperature lag before the addition of a 0.5 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 and brought to room temperature, 0.1 mL of the heated enzyme solution was mixed with 0.9 mL of 4-methyl catechol at a final concentration of 50 mM in 0.2 M citric acid buffer (pH 4.50 for isoenzyme A and pH 4.98 for isoenzyme B) and the residual activity (A) was determined spectrophotometrically. A non-heated enzyme sample was used as blank (Ao). The percentage residual activity was calculated by comparison with the unheated sample. First order inactivation constant (k_D) was calculated from the slope of the natural logarithm (ln) of A/A_o vs. time graph. Half-lives of the enzymes (t1/2) were calculated by using the following equation: $t_{1/2} = 0.693/ k_D$.

Decimal reduction time (D value) was estimated from the relationship between k_D and D value: $D = \ln (10)/k_D$. The Z value, which is the temperature increase required for a one-$\log_{10}$ reduction (90% decrease) in D value was determined from a plot of $\log_{10}$D versus temperature. The slope of the graph is equal to 1/Z value. The energy of activation of denaturation (E_a) was calculated by multiplying the slope of Arrhenius plot (i.e. natural logarithm of k_D values vs. reciprocal of absolute temperatures ($1/T$)) with universal gas constant, R (kJ/mol.K) (10).

Effects of Inhibitors

Inhibitors examined were ascorbic acid, L-cysteine and sodium metabislufite. The reaction mixture contained 0.8 mL of 4-mehtyl catechol at a final concentration of 50 mM in 0.2 M citric acid buffer (pH 4.50), 0.1 mL inhibitor at a final concentration of 0.01, 0.1, 1.0 mM and 0.1 mL enzyme solution. Percentage inhibition was calculated using the following equation: Inhibition (%) = $[(A_o - A_i) /A_o)].100$, where, A_o is the initial PPO activity (without inhibitor) and A_i is the PPO activity with inhibitor.

RESULTS AND DISCUSSION

Extraction and Purification

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 (11-12).

PPO was purified from olives using a DEAE-Toyopearl 650M column. A summary of extraction and purification is given in Table 1. Following ammonium sulfate precipitation, the dialyzed enzyme extract was applied to Toyopearl 650 M column, yielding two protein peaks of which only one had PPO activity (Fig. 1). The total activity of PPO was reduced at the ammonium sulfate precipitation. However, the total enzyme activity of the ammonium sulfate fraction was increased during the Toyopearl 650 M column chromatography (Table 1). Total enzyme activity is expected to decrease during each step of purification, which is not always the case as in this study. This can attributed to removal of the inhibitory factors during the purification, which was reported by other researchers. Nakao et al. (13) reported that total enzyme activity was remerkably increased during the DEAE-Sepharose CL-6B purification step. The increase in the total activity suggests that an enzyme inhibitor(s) may have been removed in the ammonium sulphate fraction. Similar results were also obtained by Nakao et al. (14), Shiiba et al. (15), Lourenço et al. (16) and Gawlik-Dziki at al. (17). Furthermore, the total enzyme activity was reduced at the ammonium sulphate step due to the presence of proteins that interfered with quinononid metabolism and high protein concentrations. However, such interferences were removed during the Toyopearl 650 M column chromatography step and the total enzyme activity was restored (18).

PPO was purified 11.7 fold with a recovery of 24.5% (Table 1). Ion-exchange chromatography is widely used in enzyme preparation. Gülçin et al. (19) purified polyphenol oxidase obtained from nettle using CM-Sephadex and achieved a 17.8 fold purification with a recovery of 12.4%. Gawlik-Dziki et al. (20) employed DEAE-Sephadex A-50 to purify PPO from butter lettuce and they

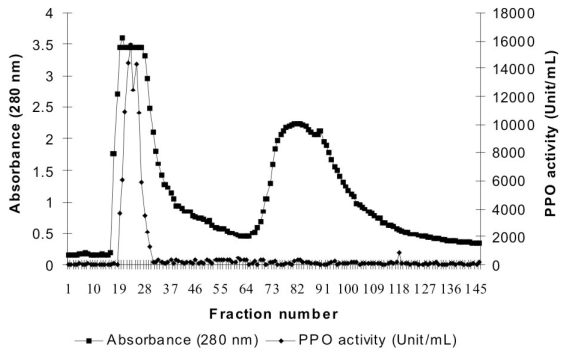

Figure 1. Elution pattern of Domat olive PPO on Toyopearl 650 M. In order to remove unadsorbed proteins, the column was washed with 10 mM pH, 6.8 phosphate buffer until the 60th fraction after which gradient elution was started.

Table 1. Purification of PPO from olive

Purification step	Volume (mL)	Total protein (mg)	Total activity (units)	Specific activity (unit/mg protein)	Purification (fold)	Recovery (%)
Crude extract	270	67.4	3759210	55737	1.00	100.0
$(NH_4)_2SO_4$ precipitation and dialysis	40	20.6	494280	23994	0.43	13.1
DEAE-Toyopearl 650M	24	1.41	922320	654128	11.7	24.5

achieved a purification of 8.0 fold with a recovery of 20.9%. Shi (21) who studied PPO from tobacco leaves achieved a purification level of 39 fold using DEAE-Sephadex A-50 chromatogtaphy.

pH Optima

Enzyme activity is strongly affected by the pH of the medium because amino acid side chains in the catalytic centre may act as weak acids and bases with critical functions that depend on their maintaining a certain state of ionization, and ionized side chains may play an essential role in maintaining protein structure (22). PPO activity as a function of pH was determined in a pH range of 3.04–6.70. The results are depicted in Figure 2. As can be seen from the graph, the enzyme activity increased with increasing pH from 3.04 to 4.5 with maximal activity occurring at pH 4.5. Thereafter, there was a sharp drop in enzyme activity.

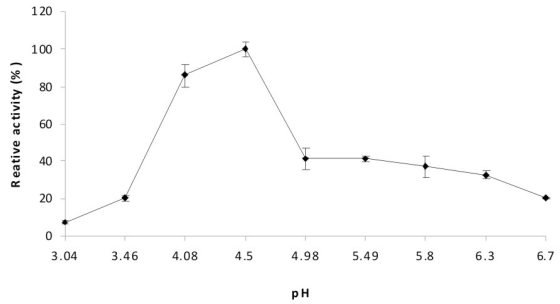

Figure 2. Activity of olive PPO as a function of pH. Each data point is the mean of two determinations. The vertical bars represent standard deviations.

The pH optimum for PPO is found to be dependent on the enzyme source, substrate and extraction methods used. Segovia-Bravo et al. (6) who studied olive PPO reported an optimum pH value of 6.0 using 4-methylcatechol as substrate. Gawlik-Dziki et al. (17) reported an optimum pH of 5.50 for butter lettuce PPO using 4-methylcatechol as substrate. Erat et al. (22) who studied ferula PPO reported optimum pH values of 7.0, using catechol as substrate. Yang et al. (23) reported an optimum pH value of 6.5 for banana peel PPO, using dopamine as substrate. Other reported values include 5.0 for loquat PPO (24), 4.20 for grape PPO (25), 5.3-6.0 for strawberry PPO (26) and 4.5-8.0 for mulberry PPO (27).

Temperature Optima

Effect of temperature on PPO activity was investigated in the range 20-70 °C and the results are depicted in Figure 3. Temperature optimum of olive PPO is found to be 30 °C, after which the enzyme activity declined rapidly. The enzyme retained only 21.75% of the maximum activity at 70 °C.

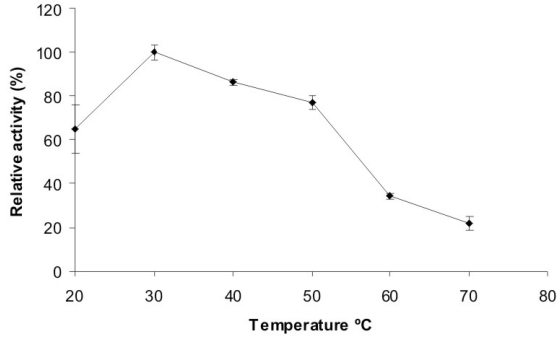

Figure 3. Activity of olive PPO as a function of temperature. Each data point is the mean of two determinations. The vertical bars represent standard deviations.

Different optimum temperatures for PPO obtained from various sources have been reported in the literature. Yemenicioğlu and Cemeroğlu (28) reported an optimum temperature of 30-35 °C for apricot. Yang et al. (23) reported an optimum temperature of 30 °C for banana peel PPO using dopamine as substrate. Other reported values include 25 °C for grape PPO (25) and 35 °C for butter lettuce PPO (20). The optimum temperature obtained in this study compares will with the reported values.

Thermal Inactivation

Detailed kinetic study of thermal inactivation of olive PPO was performed in the range 65-75 °C and the results are presented in Table 2. The first order inactivation constants (k_D) increased with increasing temperature, indicating that the enzyme was less thermostable at higher temperatures. The half-life ($t_{1/2}$) is another important parameter used in the characterization of enzyme stability. An increase in temperature resulted in a decrease in $t_{1/2}$ values (Table 2). PPO is generally considered as an enzyme of low thermostability. Some of the reported PPO half-life values include 79 min at 55 °C, 63 min at 60 °C, 31 min at 65 °C, 21 min at 70 °C for Eşme quince PPO and 462 min at 55 °C,

119 min at 60 ºC, 80 min at 65 °C, 34 min at 70 °C for Kalecik quince PPO (8). Decimal reduction time (D value) is a common parameter used in the characterization of enzyme stability and is defined as the time needed for a 90% reduction of the initial activity. D values obtained in this study ranged between 30.7 and 106.1 min at the temperatures studied (Table 2).

Table 2. Thermal inactivation parameters of olive PPO

Temperature (°C)	k (1/min)	r^2	$t_{1/2}$ (min)	D (min)
65	0.0217	0.956	31.9	106.1
70	0.0318	0.998	21.8	72.4
75	0.0750	0.981	9.2	30.7

The Z and Ea values obtained in this study were 18.6 °C (r^2 = 0.953) and 121.0 kj/mol (r^2= 0.950), respectively. Some of the reported Ea values are 47.07 kJ/mol at pH 4.0, 25.10 kj/mol at pH 5.0, 13.73 kj/mol at pH 6.0 for olive PPO (6), 155 kJ/mol for banana PPO (10).

Kinetic Parameters

K_m and V_{max} values for olive PPO using 4-methyl catechol as substrate were found to be 14.52 mM (r^2= 0.985) and 1.78 Abs/min, respectively. K_m value is a measure of affinity of the enzyme for the substrate, with smaller values representing greater affinity. Affinity of plant PPO for the phenolic substrates is relatively low. The Km is high, usually around 1 mM (11). The literature provides information on a number of different Km values, e.g. 8.6 mM for catechol, 3.5 mM for 4-methycatechol, 0.3 mM for catechin, 10.4 mM for DL-DOPA and 3.6 mM for chlorogenic acid for PPO from olive fruits and 5.2 mM for catechol, 13.6 mM for 4-methycatechol, 10.9 mM for catechin, 9.9 mM for DL-DOPA and 12.4 mM for chlorogenic acid for olive leaves PPO (29); 1.0 mM for 4-methylcatechol for butter lettuce PPO (20); 20.5 mM for catechol, 9.9 mM for 4-methycatechol and 7.6 mM for pyrogallol for Eşme quince PPO and 4.9 mM for catechol, 3.6 mM for 4-methycatechol and 1.9 mM for pyrogallol for Kalecik quince PPO (8); 6.6 mM for apricot PPO (27); 25.1 mM for grape PPO (25).

Effect of Inhibitors

Inhibitory effects of cysteine, ascorbic acid and sodium metabisulfite on olive PPO activity were studied at various concentrations using catechol as substrate and the results were reported as percentage inhibition in Table 3. These inhibitors are reducing agents, which play a role in preventing enzymatic browning either by reducing o-quinones (which are the products of diphenol oxidation) to colorless diphenols or by reacting irreversibly with *o*-quinones to form stable colorless products (30). None of the inhibitors tested caused 100% inhibition at the concentrations studied. L-cysteine and sodium disulfite were the most potent inhibitors. Arslan et al. (27) reported that apricot PPO activity was markedly inhibited by ascorbic acid, 2-mercaptoethanol, sodium metabisulfite and thiourea. In a study carried out by Gawlik-Dziki et al. (17) it was found that the most effective inhibitor for broccoli PPO was sodium sulphate. Ding et al. (24) found that most potent inhibitors for loquat PPO were sodium ascorbate, metabisulfide, L-cysteine and reduced glutathione.

Table 3. Effect of inhibitors on olive PPO activity

Inhibitor	Concentration (mM)	Inhibition* (%)
Ascorbic asid	0.01	13.20 ± 2.09
	0.10	23.33 ± 6.53
	1.00	31.65 ± 1.24
Sodyum disülfit	0.01	33.58 ± 0.58
	0.10	59.66 ± 5.33
	1.00	69.42 ± 2.78
L-cysteine	0.01	23.71 ± 0.23
	0.10	48.48 ± 8.4
	1.00	69.63 ± 3.01

*Each value is the mean of two determinations ± standard deviations.

CONCLUSION

PPO from Domat olive was partially purified through ammonium sulfate precipitation and ion exchange chromatography (DEAE-Toyopearl 650 M) which yielded two peaks of which only one had PPO activity. The optimal pH and temperature for enzyme activity were found to be 4.5 and 30 °C, respectively. The enzyme showed a high activity over a narrow pH and temperature range. Of the inhibitors tested, the most potent inhibitors were L-cysteine and sodium disulfite.

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