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EXTRACTION, PARTIAL PURIFICATION AND CHARACTERISATION OF POLYPHENOL OXIDASE FROM TEA LEAF *(Camellia sinensis)*

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Abstract

Polyphenol oxidase (PPO) from tea leaves was extracted and partially purified through $(NH_4)_2SO_4$ precipitation, dialysis and ion exchange chromatography. Of the substrates tested, 4-methylcatechol was the best substrate for PPO with a K_m value of 127.8 mM. The optimum pH for PPO activity was found to be 6.02. The enzyme showed high activity over a broad pH range of 4.03-7.00. The optimum temperature for PPO activity was 30 °C. The enzyme had more than 70% of the maximum activity between 20-80 °C. Energy of activation (Ea) and Z values were found to be 58.301 kJ/mol (r^2= 0.961) and 39.68°C (r^2= 0.965), respectively. Of the inhibitors tested, L-cysteine was the least potent inhibitor.

Keywords: Inhibition, kinetics, purification, polyphenol oxidase, tea, thermal inactivation

POLİFENOL OKSİDAZIN ÇAY *(Camellia sinensis)* YAPRAĞINDAN EKSTRAKSİYONU, KISMİ SAFLAŞTIRILMASI VE KARAKTERİZASYONU

Özet

Polifenol oksidaz (PFO) çay yapraklarından ekstrakte edilip $(NH_4)_2SO_4$ çökeltmesi, diyaliz ve iyon değişim kromatografisi ile kısmen saflaştırılmıştır. Test edilen substratlardan, 127.8 mM K_m değeri ile 4-metilkateşol PFO için en iyi substrat olarak belirlenmiştir. PFO aktivitesi için optimum pH 6.02 olarak saptanmıştır. Enzim, 4.03-7.00 gibi geniş bir pH aralığında aktivite göstermiştir. PFO aktivitesi için optimum sıcaklık 30°C olarak bulunmuştur. 20-80 °C'ler arasında enzim maksimum aktivitesinin %70'den fazlasını korumuştur. Aktivasyon enerjisi (Ea) ve Z değerleri, sırasıyla, 58.301 kJ/mol (r^2= 0.961) ve 39.68 °C (r^2= 0.965) olarak bulunmuştur. Test edilen inhibitörlerden L-sisteinin en düşük inhibisyon derecesine sahip olduğu belirlenmiştir.

Anahtar kelimeler: Çay, inhibisyon, kinetik, polifenol oksidaz, saflaştırma, termal inaktivasyon,

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INTRODUCTION

Tea is one of the world's popular beverages and is produced from the tender, rapidly growing shoots of the tea plant (tea flush) or tender young leaves. It is classified according to the process of manufacture as fermented tea (black tea) and non fermented tea (green tea) (1). It is estimated that about 2.5 million metric tons of dried tea are manufactured annually, of which about 78% is black tea. The oxidative and hydrolytic enzymes endogenous to tea shoots are crucial for generation of various characteristic quality attributes of black tea. During black tea manufacture, the mechanical maceration of green tea shoots triggers the enzyme catalyzed oxidative reactions involving the tea catechins as the substrates, leading to the formation of theaflavin and thearubigin pigments which affect the desirable colour and briskness of the made tea. The theaflavins are formed by the enzymatic oxidation and condensation of catechins with di- and trihydroxylated B rings. The reaction involves the oxidation of the B rings to the quinones followed by a Michael addition of the gallocatechin quinone to the catechin quinone, prior to carbonyl addition across the ring and subsequent decarboxylation (2, 3).

Polyphenol oxidase (PPO) plays a key role in the oxidation of flavanols to black tea components such as theaflavins and thearubigins. It catalyses the crucial initial reaction during tea fermentation, the oxidation of *o*-diphenols to their corresponding quinones, which are then spontaneously transformed to more complex fermentation products (4).

Only the tender shoots of the plant are processed. The shoots are a rich source of polyphenols and PPO. The enzyme is found in all parts of the plant, and tea quality is positively correlated with its content in the shoots (5).

Turkey with an annual tea production of 200100 tonnes which constitutes 6% of world tea production is an important tea producer along with are India, China, Sri Lanka, Kenya. Turkey with 2.3 kg tea consumption per capita is the 4th biggest tea consumer in the world (6). To the best of our knowledge, this is the first report on PPO from Turkish tea leaves. The present study was aimed at purification and characterization of PPO from tea leaves.

MATERIAL AND METHODS

Materials and Reagents

The tea leaves used in this study were obtained Black sea region of Turkey and frozen at -25 °C until used. Ascorbic acid, catechol, polyvinylpyrolidone (PVPP), pyrogallol, sodium metabisulfite, triton X-100 were purchased from Merck (Darmstadt, Germany). Acetone, ammonium sulphate, L-cysteine, citric acid, gallic acid, caffeic acid 4-methylcatechol, 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

150 g of frozen tea leaves 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 1 L 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 1 h at 4 °C, it was centrifuged at 10000 x *g* for 30 min at 4 °C. The resulting supernatant was subjected to ammonium sulphate precipitation. The fraction precipitated between 30-90% saturation was separated by centrifugation at 10000 x g for 30 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

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 nonadsorbed fractions the column was washed with 130 mL of the same buffer at the flow rate of 0.5 mL/min. Then, a linear gradient

of phosphate buffer concentration from 10 to 200 mM was applied. 4 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 (9).

Protein Determination

Protein contents of the enzyme extracts were determined according to Bradford method using bovine serum albumin as a standard (10).

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 catechol in 200 mM phosphate buffer (pH 6.02). 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. Enzyme activity for PPO was performed in duplicate and the results are expressed as mean. 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 (11).

pH Optima

PPO activity was determined in a pH range of 4.03-5.49 in 200 mM citric acid buffer and 6.02-7.00 in 200 mM phosphate buffer. PPO activity was assayed, using the standard reaction mixture but changing 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 80 °C. 0.9 mL of 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.

Substrate Specificity

In order to determine Michaelis constant (K_m) and maximum velocity (V_m), PPO activities were measured using catechol (25-200 mM), 4-methylcatechol (6.25-100.00 mM), pyrogallol (50-200 mM), gallic acid (50-200 mM) and caffeic acid (0.75-3.00 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.

Thermal Inactivation Kinetics

Thermal inactivations of PPO was studied at the selected temperatures for various times (5, 10, 15 and 20 min at 70 °C; 5, 10 and 15 min at 75 °C and 2, 5 and 10 min at 80 °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 0.3 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 (A) was determined spectrophotometrically using the standard reaction mixture. A non-heated enzyme sample was used as blank (A_o). 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 ($t_{1/2}$) were calculated by using the following equation: $t1/2 = 0.693/ k_D$.

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-log10 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) (12).

Effects of Inhibitors

The inhibitors examined were L-cysteine, ascorbic acid and sodium metabisulfite. The reaction mixture contained 0.8 mL of catechol at a final concentration of 100 mM in 200 mM phosphate buffer (pH 6.02), 0.1 mL inhibitor at a final concentration of 0.01, 0.1 or 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

Extraction and Purification

PPO was purified from tea leaves 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 3.32 fold purification with a recovery of 5.11% was achieved. Halder et al. (2) who studied purification and characterization of PPO from tea leaves obtained three fractions on DEAE cellulose column, of which two of them were absorbed and one was unabsorbed.

pH Optima

PPO activity as a function of pH was determined in a pH range of 4.03-7.00, and the results are depicted in Fig. 2. As the pH increased from 4.03 to 6.02, the enzyme activity increased, with maximal activity occurring at pH 6.02, after which the activity started to decline. The enzyme

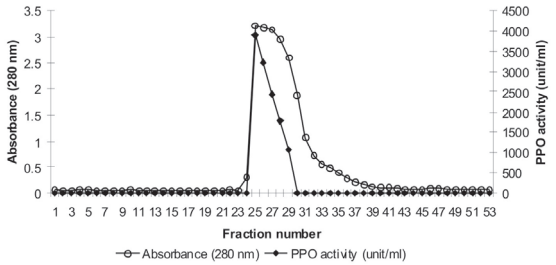

Figure 1. Elution pattern of tea PPO on DEAE-cellulose. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to a 2.5 cm x 30 cm column, equilibrated and washed with 10 mM phosphate buffer, pH 6.8. Elution of adsorbed proteins was performed using a linear gradient of 10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

exhibited high activity in a broad pH range. In the pH range of 4.5-7.0 the enzyme activity was more than 63%.

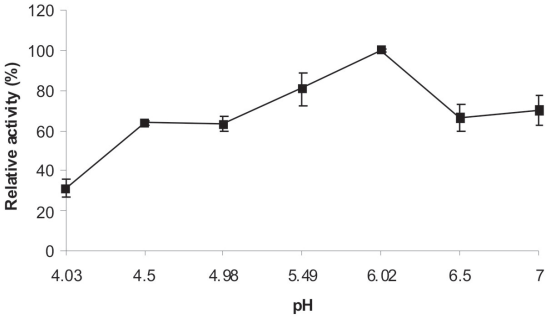

Figure 2. Activity of tea PPO as a function of pH. Each data point is the mean of two determinations. The vertical bars represent standard deviations

Temperature Optima

Effect of temperature on PPO activity was investigated in the range 20-80°C and the results are depicted in Fig. 3. As seen in Fig. 3, the optimum temperature for PPO activity was found to be at 30°C. After 30°C, the activity started to decrease gradually. However, the enzyme had a very high activity at a broad temperature range. The enzyme had 72% of the maximal activity even at 80 °C.

Table 1. Purification of PPO from tea leaves

Purification step	Volume (ml)	Total Protein (mg)	Total activity (units)	Specific activity (unit/mg)	Purification (Fold)	Recovery (%)
Crude extract	936	130.38	3232008	24789	1.00	100.00
$(NH_4)_2SO_4$ precipitation and dialysis	40	7.70	200067	25983	1.05	6.19
DEAE-cellulose	36	2.01	165288	82233	3.32	5.11

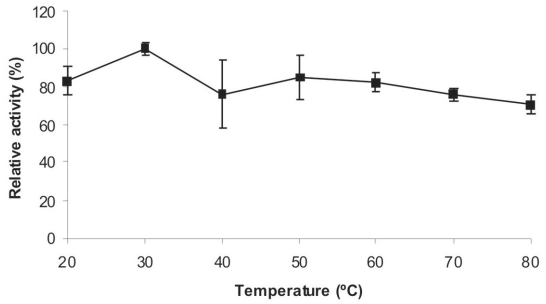

Figure 3. Activity of tea PPO as a function of temperature. Each data point is the mean of two determinations. The vertical bars represent standard deviations

Thermal Inactivation Kinetics

The thermal inactivation parameters of tea PPO 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. An increase in temperature resulted in a decrease in $t_{1/2}$ values (Table 2). Some of the reported PPO half-life values include 18.8 min at 60 °C and 8.5 min at 70 °C for mango kernel PPO (13); 4.5 and 31.6 min at 75 °C for Ravat and Niagara grapes (14), respectively. The decimal reduction time (D value) is the time, at a given temperature and pressure, needed for 90% reduction of the initial activity. D values obtained in this study ranged between 98 and 176 min at the temperatures studied (Table 2). Rapeanu et al. (15) reported D values for Victoria grape PPO between 133-11.5 min between the temperatures of 55-65°C. The temperature dependence of the decimal reduction time is characterized by Z value, which is the temperature increase needed for a one $\log_{10}$ reduction (90% decrease) in the D value. The Z and E_a values obtained in this study were 39.68°C (r^2= 0.9645) and 58.301 kJ/mol (r^2= 0.9614), respectively. Some of the reported E_a values for PPOs include 219 kJ/mol for Dechaunac grape (16), 208.5 kJ/mol Sultaniye grapes (17), 154.7 kJ/mol for banana (12).

Table 2. Thermal inactivation parameters of tea PPO

Temperature (°C)	k (1/min)	r2	t1/2 (min)	D (min)
70	0.0131	0.74	53	176
75	0.0159	0.92	44	145
80	0.0234	0.97	30	98

Effect of Inhibitors

Effects of cysteine, ascorbic acid and sodium metabisulfite on tea PPO activity were studied at various concentrations using catechol as the substrate and the results were reported as percentage inhibition in Table 3. The inhibition degree varied in dose dependent manner. From the results, it can be concluded that the most potent inhibitors were sodium metabisulfite and ascorbic acid, because a higher degree of inhibition was achieved. Cysteine was the least potent inhibitor.

Table 3. Effect of inhibitors on tea PPO activity

Inhibitor	Concentration (mM)	Inhibition* (%)
Cysteine	0.01	No inhibition
	0.10	3.3 ± 1.7
	1.00	6.6± 2.1
Ascorbic acid	0.01	12.7 ± 1.5
	0.10	14.5 ± 4.1
	1.00	15.5± 0.9
Sodium metabisulfite	0.01	6.4 ± 0.2
	0.10	7.9 ± 2.0
	1.00	16.4± 0.5

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

Kinetic Parameters

K_m and V_m values for tea PPO for different substrates are presented in Table 4. The affinity of the enzyme varied depending on the substrate used. Tea PPO had a higher affinity for 4-methylcatechol, as evidenced by lower K_m value. The criterion for the best substrate is the V_m/K_m ratio (18). Of the substrates tested, the best substrate for tea PPO was 4-methylcatechol. The enzyme showed no activity against caffeic acid and gallic acid. In a study carried out by

Table 4. Kinetic parameters of tea PPO

Substrate	K_m (mM)	V_m (U/min/mL)	V_m/K_m
Catechol	243.2	28835	118.6
4-Methylcatechol	127.8	54140	423.6
Pyrogallol	3113.4	78988	25.4
Caffeic acid	-	-	-
Gallic acid	-	-	-

Orenes-Pinero et al. (19) K_m value for latent PPO from quince was found to be 1.2 mM, using 4-*tert*-butylcatechol. Duangmal and Apenten (20) 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. As can be seen, affinity of PPOs from various sources for various substrates varies widely. Affinity of plant PPO for the phenolic substrates is relatively low, i.e. the K_m is high, usually around 1 mM (1).

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 (21).

The changes in ionization of prototropic groups in the active site of an enzyme at lower acid an higher alkali pH values may prevent proper conformation of the active site, binding of substrates, and/or catalysis of the reaction (22). The pH optimum for PPO activity from tea leaf was found to be 6.02. Halder et al. (2) reported an optimum pH value of 5.0 for tea PPO, which is different than the one obtained in this study. 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 (23), 4.20 for grape PPO (11), 5.70 for broccoli PPO (24) and 7.5 for avocado PPO (25).

Temperature significantly influences the catalytic activity of the enzymes. It is well known that a decrease in the kinetic energy of the reactant molecules at low temperatures corresponds to a slower reaction. In addition, integrity of the delicate three-dimensional structure of the enzyme is subjected to disruption and denaturation at high temperatures (22). In a study carried out by Dogan et al. (26) on different aubergine cultivars, the temperature optima varied between 20-30 °C using catechol and 4-methylcatechol as substrates. Ding et al. (9) reported an optimum temperature of 30°C for loquat PPO using chlorogenic acid as substrate. Other reported values include 25 °C for grape PPO (11) and 30 °C for banana PPO (14). The optimum temperature obtained in this study compares well with the reported values. A higher optimum temperature (50 °C) for strawberry PPO was reported by Serradell et al. (8).

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 (1). PPO from tea leaf showed high thermal stability at the temperatures studied. The half lives values varied between 30-53 min at 80-70 °C (Table 2).

The mode of action of inhibitors differs from each other. Ascorbic acid and metabisulfite are reducing agents, which can either reduce *o*-qionones to colourless diphenols, or react irreversibly with *o*-qionones to form stable colourless products. 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. The inhibitory effect of citric acid is attributed to pH lowering effect and chelate formation with copper (1). At the concentrations tested, the inhibition degrees of the inhibitors were very low, with L-cysteine being the least potent inhibitor. In a study carried out by Gomez-Lopez (25), it was found that the most effective inhibitor for avocado PPO was cysteine. Rapeanu et al. (15) found that most potent inhibitors for grape PPO were ascorbic acid, cysteine and sodium metabisulfite.

In conclusion, after the final purification step, a 3.32 fold purification with a recovery of 5.11% was achieved. The optimal pH and temperature for enzyme activity were found to be 6.02 and 30 °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 4-methylcatechol and no activity toward caffeic acid and gallic acid. Of the inhibitors tested, the most potent inhibitors were sodium metabisulfite and ascorbic acid.

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REFERENCES

1. Robinson DS, Eskin NAM (eds), Zawistowski J, Biliaderis CG, Eskin NAM. 1991. *Oxidative Enzymes in Foods*. Elsevier, New York, pp. 217-273.

2. Halder J, Tamuli P, Bhaduri AN. 1998. Isolation and characterization of polyphenol oxidase from Indian tea leaf *(Camellia sinensis). J Nutr Biochem,* 9:75-80.

3. Subramanian N, Venkatesh P, Ganguli S, Sinkar VP. 1999. Role of polyphenol oxidase and peroxidase in the generation of black tea theaflavins. *J Agric Food Chem,* 47:2571-2578.

4. Finger A. 1994. In-Vitro studies on the effect of polyphenol oxidase and peroxidase on the formation of polyphenolic black tea constituents. *J Sci Food Agric*, 66:293-305.

5. Singh HP, Ravindranath SD. 1994. Occurrence and distribution of PPO activity in floral organs of some standard and local cultivars of tea. *J Sci Food Agric,* 64:117- 120.

6. Demir A. 2002. *Çay,* TEAE (Tarımsal Ekonomi Araştırma Enstitüsü) 1:1-4 (In Turkish).

7. Coseteng MY, Lee CY. 1987. Changes in apple polyphenol oxidase and polyphenol concentrations in relation to degree of browning. *J Food Sci,* 52:985-989.

8. Serradell MA, Rozenfeld PA, Martinez GA, Civello PM, Chaves AR, Anon MC. 2000. Polyphenoloxidase activity from strawberry fruit (Fragaria x ananassa, Duch., cv Selva): characterization and partial purification. *J Sci Food Agric,* 80:1421-1427.

9. Ding CK, Chachin K, Ueda Y, Imahori Y. 1998. Purification and properties of polyphenol oxidase from loquat fruit. *J Agric Food Chem,* 46:4144-4149.

10. Bradford MM. 1976. A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal Biochem,* 72:248-254.

11. Ünal MÜ, Şener A. 2006. Determination of biochemical properties of polyphenol oxidase from Emir grape (*Vitis vinifera* L. cv. Emir). *J Sci Food Agric,* 86:2374-2379.

12. Ünal MÜ. 2007. Properties of polyphenol oxidase from Anamur banana (*Musa cavendishii*). *Food Chem,* 100:909-913.

13. Arogba SK, Ajiboye OL, Ugboko LA, Esienette SY, Afolabi PO. 1998. Properties of polyphenol oxidase in mango (*Mangifera indica*) kernel. *J Sci Food Agric,* 77:459-462.

14. Wissemann KW, Lee CY. 1981. Characterization of polyphenoloxidase from Ravat 51 and Niagara grapes. *J Food Sci,* 46:506-508.

15. Rapeanu G, Loey A.V, Smout C, Hendrickx M. 2005. Effect of pH on thermal and/or pressure inactivation of Victoria grape (*Vitis vinifera sativa*) polyphenol oxidase: A kinetic study. *J Food Sci,* 70:301-307.

16. Lee CY, Smith NL, Pennesi AP. 1983. Polyphenol oxidase from Dechaunac grapes. *J Sci Food Agric,* 34:987-991.

17. Ünal MÜ, Şener A, Şen K. 2007. Characterization of polyphenol oxidase from Sultaniye grapes (*Vitis vinifera cv Sultana*). *Int J Food Sci Technol,* 42:1123-1127.

18. Palmer, JK. 1995. *Understanding Enzymes.* Prentice Hall/Ellis Horwood, Herdfordshire.

19. Orenes-Pinero E, Garcia-Caromana F, Sanchez-Ferrer A. 2006. Latent polyphenol oxidase from quince fruit pulp (*Cydonia oblonga*): purification, activation and some properties. *J Sci Food Agric,* 86:2172-2178.

20. Duangmal K, Apenten RKO. 1999. A comparative study of polyphenoloxidases from taro (*Colocasia esculenta*) and potato (*Solanum tuberosum* var. *Romano*). *Food Chem,* 64:351-359.

21. Vamos-Vigyazo, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. *Crit Rev Food Sci,* 15:49-127.

22. Yoruk R, Marshall MR. 2003. Physicochemical properties and function of plant polyphenol oxidase: a review. *J Food Biochem,* 27:361-422.

23. Yang C-P, Fujita S, Kohno K, Kusubayashi A, Ashrafuzzaman MD, Hayashi N. 2001. Partial purification and characterization of polyphenol oxidase from banana (*Musa sapientum* L) peel. *J Sci Food Agric,* 49:1446-1449.

24. Gawlik-Dziki U, Szymanowska U, Baraniak B. 2007. Characterization of polyphenol oxidase from broccoli (*Brassica oleracea* var. *botrytis italica*) florets. *Food Chem,* 105:1047-1053.

25. Gomez-Lopez VM. 2002. Some biochemical properties of polyphenol oxidase from two varieties of avocado. *Food Chem,* 77:163-169.

26. Dogan M., Arslan O., Dogan S. 2002. Substrate specificity, heat inactivation and inhibition of polyphenol oxidase from different aubergine cultivars. *Int J Food Sci Technol,* 37:415-423.