International Proceedings of Chemical, Biological and Environmental Engineering, Vol.95 (2016)
DOI:10.7763/IPCBEE.2016.V95.2

Biochemical Properties of Emir Grape Polyphenol Oxidase as Affected by Harvest Year

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Abstract. Effect of harvest year on biochemical properties of polyphenol oxidase (PPO) from Narince grapes was studied in 2006 and 2007.The optimum pH of the enzyme in both years were found to be 5.49 whereas optimum temperature for enzyme activity was 30 ℃ in the year 2006 and 35 ℃ in the year 2007. The apparent substrate specificities were established from V_{max}/K_m as: caffeic acid > 4-methylcatechol >catechol >pyrogallol for two enzymes in both years. There were marked differences between the two enzymes in terms of thermal stability. The effects of inhibitors varied in a dose dependent manner and according to the harvest year.

Keywords: enzymatic browning, polyphenol oxidase, Emir grape, wine, harvest, kinetics.

1. Introduction

Enzymatic browning occurring in fruits and vegetables is catalyzed by polyphenol oxidase (PPO, EC 1.14.18.1). The o-quinones formed by the action PPO interact with other molecules resulting in the formation of brown pigments. The reaction is detrimental to food quality and causes economic losses due to impairment in color, taste, flavor, and nutritional value [1]. The levels of PPO are linked to species, cultivar, maturity and age [2]. It has long been known that grape juice and wine will brown on contact with air because of enzymatic and non-enzymatic oxidation of phenols, quinones, amino acids, and other oxidizable substances.

Many constituents of wine, including phenolic compounds, certain metals, tyrosine and aldehydes, are susceptible to oxidation during the winemaking process and lead to browning. Oxidative browning has an adverse effect on color, flavor, and aroma of white wines, which is considered undesirable in young table and sparkling wines. It can lead to loss of color, flavor and aroma, and increase of astringency, as well as loss of nutritional value of wine. The initiating mechanism of browning of wine can be enzymatic or nonenzymatic. Enzymatic browning is catalyzed by PPO upon crushing or processing of grapes during vinification of white wine. Nonenzymatic browning can occur both in grape must and wine [3], [4]. However, enzymatic browning almost entirely occurs in grape must [5]. PPOs are cupric oxidoreductases, catalyzing the hydroxlylation of monophenols to *o*-diphenols and the dehydrogenation of *o*-diphenols to *o*-quinones. Subsequent polymerization of o-quinones leads to formation of brown-black pigments. Browning intensities of individual phenolics show variation.

The major phenolic compounds in freely expressed grape juice are caffeoyl tartaric (caftaric) acid and coumaroyl tartaric (coutaric) acid, which are also good substrates for PPOs. Enzymatic oxidation of caftaric and coutaric acid results in the formation of caftaric acid o-quinone. Reaction of o-quinones with glutathione

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results in the formation of 2-S-glutathionyl caffeoyl tartaric acid (also called grape reaction product, GRP). GRP is a colorless compound and not oxidizable by PPO [4]. Therefore, the formation of GRP is believed to limit the must browning. Following glutathione depletion, the exceeded caftaric acid quinones are involved in coupled oxidation mechanisms in which the glutathionyl adduct or catechin can serve as reductant [5]. Glutathione is a naturally occurring tripeptide in grapes with an –SH group. It has been postulated that cysteine, which has long been known to be a PPO inhibitor, can have similar effects. Susceptibility of grape varieties to browning varies, which might be attributed to the differences in the content of reductive species that can react with quinines, such as glutathione and ascorbic acid, as well as cysteine [4].

Conversely, non-enzymatic oxidation occurs in wine in the presence of transition metal ions and involves polyphenols with ortho-dihydroxybenzene or trihydroxybenzene moieties. The quinones formed from polyphenols oxidation are unstable and may undergo further reactions combining nucleophilic compounds (i.e. phenols, thiols, amines) to produce colored dimers. Subsequently, these products may rearrange to form new dihydroxybenzene moieties, which are more easily oxidized because they have lower redox potentials than their initial phenols [5].

The Emir grapes are one of the important grape varieties grown in Turkey. Emir, which is cultivated in the Nevşehir-Urgup (Cappadocia) region. This variety is an important variety for the wine industry in Turkey. It comprises around 25% of the total vineyards of the region [4], [6]. Therefore, it is important to control the PPO activity, as well as to determine its characteristics associated with the variety. This work was undertaken to study the effect of harvest year on the characteristics of PPO from Emir grapes in terms of pH and temperature optima, thermal inactivation, substrate specificity and potency of some PPO inhibitors.

2. Material and Methods

2.1. Material

Emir grapes cultivated in Cappadocia region of Turkey was used in this study. Catechol was obtained from Sigma-Aldrich (St. Louis, USA). Acetone was purchased from Merck, (Darmstadt, Germany). All other chemicals were of analytical grade.

2.2. Preparation of Crude Enzyme Extract

200 g of cold berries, which were chosen to be free of any visible mould or rot, were homogenized in 200 ml of cold acetone (-25^{o}C) using a pre-chilled Waring blender for 2 min at maximum speed. The slurry was vacuum-filtered through filter paper. The residue was re-extracted with 120 ml of cold acetone and filtered again. This procedure was repeated until a white powder was obtained. The residue from the repeated extractions was called acetone powder, which was dried overnight at room temperature and stored at -25^{o}C [1]. In order to obtain enzyme extract, 0.5 g of acetone powder was suspended in 37.5 ml of prechilled 0.1 M phosphate buffer (pH 6.8) and then stirred for 1 h at 4^{o}C. The suspension was centrifuged at 7500 g for 30 min at 4^{o}C. The supernatant was used as crude PPO. From here on polyphenol oxidase from the year 2006 will be denoted as PPO_{2006} and that from the year 2007 as PPO_{2007}.

2.3. 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^{o}C. The initial rate was calculated from the slope 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 0.2 M citric acid buffer (pH 5.49) for PPO_{2006} and PPO_{2007}. 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 [1].

2.4. pH and Temperature Optima

PPO activity was determined in a pH range of 3.04-5.80 in0.2 M citric acid buffer and 6.30-6.70 in0.2 M phosphate buffer. PPO activity was assayed, using the standard reaction mixture but changing the buffer. The activity of PPO was determined at temperatures ranging from 20^{o}C to 80^{o}C. PPO activity was calculated in the form of percent residual PPO activity at the optimum temperature and pH.

2.5. Enzyme Kinetics

In order to determine Michaelis constant (K_m) and maximum velocity (V_{max}), PPO activities were measured using catechol, 4-methylcatechol, pyrogallol and caffeic acid as substrate at various concentrations (0.1875–400 mM). K_m and V_{max} values of the enzyme were calculated from a plot of 1/V versus 1/S by the method of Lineweaver and Burk.

2.6. Heat Inactivation

Thermal inactivation of crude PPO was studied at the selected temperatures (65, 70, 75 and 80^{o}C) for various times (2, 4, 6, 8, 10, 20 and 30 min) using screw-cap tubes. The inactivation constant (k), half-life ($t_{1/2}$), decimal reduction (D), the energy of activation of denaturation (E_a) and Z values were calculated according to Ünal [7].

2.7. Effects of Inhibitors

Inhibitors examined were L-cysteine, ascorbic acid, citric acid and sodium metabisulfite. 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.

3. Results and Discussion

3.1. pH Optima

The pH activity profiles for PPO_{2006} and PPO_{2007} are depicted in Fig. 1. Increasing pH from 3.04 to 4.98 resulted in the activity of PPOs. The maximal activity occurred at pH 4.98 for PPO_{2006} and PPO_{2007}. After the optimum pH the activity of PPOs started to decline until pH 6.70. Some of the reported optimum pH values for PPO from various sources include between 6.0 for Thomson seedless grapes [8], 6.5 for Red Globe grape [9], 5.49 for Narince grape [10], 6.3 for Muscat Bailey A [11], 3.4 for Sultaniye [12]. The pH optimum obtained in this study is within the reported values.

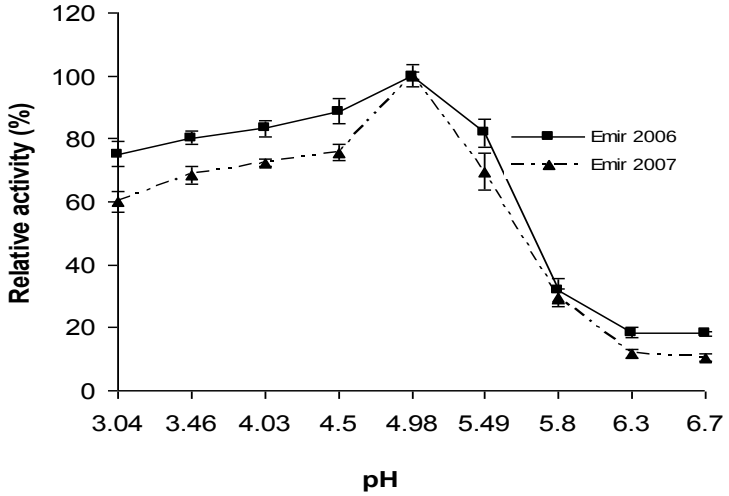

Fig. 1: Activity of Emir grape PPO as a function of pH. Each data point is the mean of three determinations.

3.2. Temperature Optima

Effect of temperature on PPO activity was investigated in the range 20-80 ℃ and the results are depicted in Fig. 2. The optimum temperature for PPO_{2006} was 30 ℃, whereas that for PPO_{2007} was 35 ℃. After the optimum temperatures the enzyme activities started decline. PPO_{2006} retained more than 40% of the maximum activity at 80 ℃ whereas that for PPO_{2007} was more than 80%. The optimum temperature of PPO shows great variability depending on the enzyme source and the substrate used in the assay [1]. The reported optimum temperature values were 30 ℃ for banana PPO [7], 40 ℃ for honeydew peach [13], 45 ℃ for grape PPO [9], 55 ℃ for persimmon PPO [14]. Our results are within the range of these values.

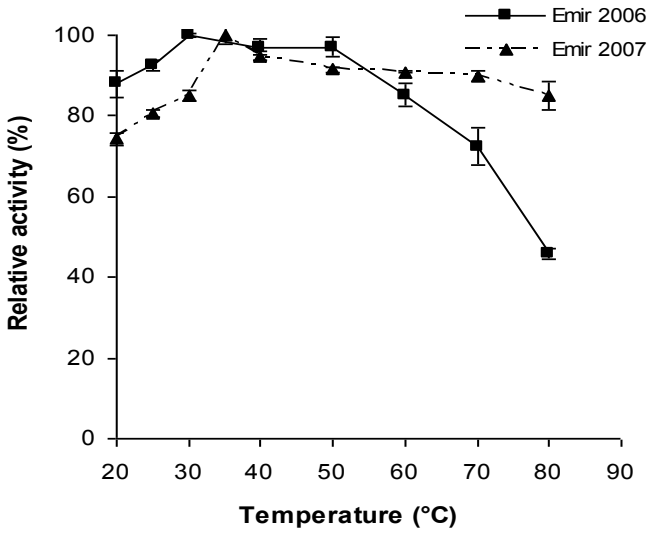

Fig. 2: Activity of Emir grape PPO as a function of temperature. Each data point is the mean of three determinations.

3.3. Kinetic Parameters

K_m and V_{max} values for PPO_{2006} and PPO_{2007} for different substrates are presented in Table 1. The enzymes exhibited different kinetic properties. PPO_{2007} had a higher affinity for catechol and 4-methylcatechol, whereas PPO_{2006} had a higher affinity towards pyrogallol and caffeic acid as evidenced by lower K_m values. The criterion for the best substrate is the V_{max}/K_m ratio. From the V_{max}/K_m ratios in Table 1, the best substrate for PPO_{2006} and PPO_{2007} are caffeic acid. It was reported that PPO from Sultaniye grape had a K_m value of 44.5 mM with catechol as substrate [12]. In a study carried out by Goyeneche et al [15], the radish PPO had a great affinity for gallic acid (K_m 4.2mM), followed by pyrogallic, chlorogenic and L-tyrosine (K_m 6.3, 7.2 and 9.3 mM, respectively). Km values for PPO from *Solanum lycocarpum* fruits were found to be 6.47 mM for catechol and 0.15 mM for 4-methylcatechol [16]. As can be seen, affinity of PPOs from various sources for various substrates varies widely.

Table 1: Kinetic parameters of Emir grape PPO

Substrate	2006			2007		
	K_m (mM)	V_m (units/mg protein)	V_m/K_m	K_m (mM)	V_m (units/mg protein)	V_m/K_m
Catechol	41.70	0.76	0.018	38.60	3.01	0.078
4-Methylcatechol	5.77	1.21	0.209	4.57	2.84	0.621
Pyrogallol	55.70	0.1	0.001	91.36	0.68	0.007
Caffeic acid	0.55	0.62	1.1272	0.85	2.18	2.565

3.4. Effect of Inhibitors

Inhibitory effects of cysteine, ascorbic acid, citric acid and sodium metabisulfite on grape PPO activity were studied at various concentrations using catechol as the substrate and the results were reported as percentage inhibition in Table 2. There was year-to-year variation in the inhibitory effects of the inhibitors. The effects of inhibitors varied in a dose dependent manner. Citric acid was the least effective of the inhibitors tested. Cysteine, citric acid and sodium metabisulfite effectively inhibited the PPOs in both years. From these results it can be concluded that enzymatic browning in juice and wine can be effectively controlled by the use of sulfite, ascorbic acid and cysteine.

In a study carried out by Zaini et al [17] on the purification and characterization of PPO from Snake fruit [*Salacca zalacca* (Gaertn.) Voss], it was found that L-cysteine showed the best inhibitory effect, with an IC50 of 1.3±0.002 mM followed by ascorbic acid (1.5 ± 0.06 mM), glutathione (1.5 ±0.07 mM), EDTA (100 ±0.02 mM) and citric acid (186±0.16 mM). It was reported that Ginseng PPO activity was inhibited by ascorbic acid, sodium metabisulfite, and kojic acid, using catechol as the substrate, whereas 1 mM citric acid showed slight activation rather than inhibition for the ginseng PPO [18].

Table 2: Effect of inhibitors on Emir grape PPO activity

Inhibitor	Concentration (mM)	Inhibition* (%)	
		2006	2007
Cysteine	0.01	14.2 ±2.8	32.8 ±2.4
	0.10	33.4 ±1.4	13.4 ±2.9
	1.00	100.0 ±0.0	100.0 ±0.0
Ascorbic acid	0.01	8.6 ±4.4	2.4 ±1.2
	0.10	14.6 ±0.8	4.7 ±2.7
	1.00	100.0 ±0.0	100.0 ±0.0
Citric acid	0.01	4.8 ±3.3	1.4 ±0.7
	0.10	5.9 ±3.1	2.6 ±2.0
	1.00	7.8±0.8	5.7 ±5.0
Sodium metabisulfite	0.01	10.3 ±3.0	1.9 ±1.0
	0.10	31.6 ±2.0	23.5 ±0.8
	1.00	100.0 ±0.0	100.0 ±0.0

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

3.5. Thermal Inactivation

PPO is generally considered as an enzyme of low thermostability. Thermal inactivation parameters obtained in study are summarized in Table 3. The results indicate that PPO_{2006} and PPO_{2007} grape PPO exhibited different heat sensitivities, PPO_{2007} being more heat sensitive. 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 $t_{1/2}$ and D values. E_a and Z values in 2006 were calculated to be 250.4 kj/mol K and 9.13 ℃ (0.9904), whereas those in 2007 were 260.1 kj/mol K (0.9764) and 8.92 ℃ (0.9738). As seen, there was a year to year variation in thermal inactivation parameters. Ünal and Şener [10] reported a Z value of 15.4 ℃ and an E_a value of 150.8 kJ/mol for Narince grape PPO, which are different than those found in this study. Tao et al [19] reported E_a value of 103.3 kJ/mol for PPO from Jackfruit (*Artocarpus heterophyllus*) bulbs.

Table 3: Thermal inactivation parameters of Emir grape PPO

Temperature (℃)	2006				2007			
	k_D (min^{-1})	R^2	$t_{1/2}$ (min)	D (min)	k_D (min^{-1})	r^2	$t_{1/2}$ (min)	D (min)
65	0.0101	0.983	68.6	228.0	-	-	-	-
70	0.0445	0.973	15.6	51.7	0.0214	0.971	32.4	107.6
75	0.1688	0.961	4.1	13.6	0.1122	0.972	6.2	20.5
80	0.4329	0.967	1.6	5.3	0.2826	0.859	2.5	8.1

4. Conclusion

PPOs from Emir grape which is suitable for white wine production in 2006 and 2007 were characterized in terms of temperature and pH optima, heat inactivation, potency of some PPO inhibitors, substrate specificity and effect of harvest year on biochemical characteristics of PPOs was investigated. pH activity profile was similar in both years, the maximum activity occurring at pH 4.98. Temperature profile, on the other hand, showed year-to-year variation. The optimum temperature for PPO_{2006} was 30 ℃, whereas that for PPO_{2007} was 35 ℃. Inhibitory effects of the inhibitors tested showed variation in both harvest years. Cysteine, citric acid and sodium metabisulfite effectively inhibited the PPOs, indication that enzymatic browning in juice and wine can be effectively controlled by the use of sulfite, ascorbic acid and cysteine. The best substrate for PPO_{2006} and PPO_{2007} was caffeic acid. There were marked differences between the two enzymes in terms of thermal stability.

5. Acknowledgement

This study was supported by the Scientific and Technical Research Council of Turkey (TÜBİTAK 106O340).

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