Consistency of Polyphenol Oxidase (PPO) Thermostability in Ripening Apricots (*Prunus armeniaca* L.): Evidence for the Presence of Thermostable PPO Forming and Destabilizing Mechanisms in Apricots

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Destabilization of thermostable polyphenol oxidase (TS-PPO) during the ripening of peaches has been previously shown (Yemenicioğlu, A.; Cemeroğlu, B. *Tr. J. Agric. For.* **1998**, *22*, 261−265). This work studied the effect of ripening on thermal stability of apricot PPO for three different cultivars. Kabaaşı cultivar contained thermolabile PPO, whereas TS-PPO appeared in Hacıhaliloğlu and Çataloğlu cultivars. The TS-PPO showed biphasic inactivation curves, and its D and z values between 60 and 90 °C varied in the ranges of 357−1.12 min and 11.9−12.7 °C, respectively. In Hacıhaliloğlu cultivar the TS-PPO was very consistent and existed at all stages of ripening, whereas in Çataloğlu cultivar it appeared only at the half-ripe stage. The loss of consistent TS-PPO in Hacıhaliloğlu apricots after partial purification by acetone precipitation and DEAE-cellulose chromatography suggested the non-covalent nature of its stabilization. The main purified fractions (F1 and F2) showed monophasic inactivation curves with similar thermal inactivation parameters (z_{F1} = 10.4 °C, z_{F2} = 10.1 °C). However, their kinetic properties against catechol (K_{mF1} = 61 mM, K_{mF2} = 122.7 mM) and substrate specificities were considerably different. The results of this study showed the presence of TS-PPO forming and destabilizing mechanisms in apricots. Further studies are needed for the solution of these mechanisms and to develop some new strategies that may be utilized by molecular techniques for a planned production of apricot cultivars provided with heat labile but normal PPO activity.

KEYWORDS: *Prunus armeniaca*; apricot; ripening; polyphenol oxidase; heat inactivation; purification

INTRODUCTION

One of the biggest problems faced during the processing of fruits and vegetables is enzymatic browning, which is catalyzed by polyphenol oxidases (PPO). The use of sulfites is one of the most effective methods to prevent enzymatic browning reactions. However, due to the health concerns, there have been great efforts to minimize or eliminate the use of sulfites in food technology; this encouraged the use of sulfite alternatives, such as ascorbic acid and its derivatives, β-cyclodextrin, L-cysteine, and 4-hexylresorcinol (*1−3*). These chemicals are less effective compared with sulfites and need some complementary treatments such as packaging under nitrogen atmosphere and/or use in combination with heat treatments, acidic solutions, or polyphosphates (*3−7*).

A novel and promising approach for the control of PPO activity in fruits and vegetables without the use of chemicals is the use of molecular biology. By employing an antisense RNA

technique Bachem et al. (*8*) blocked PPO gene expression in potatoes and obtained very low PPO activity-containing cultivars. Coetzer et al. (*9*) also reduced the PPO activity in potatoes by using sense or antisense RNA techniques and successfully controlled their enzymatic browning. One of the concerns related to these methods is that PPO in plants is involved in disease and pest resistance (*10*). The inactivation of PPO enzymes in plants may cause a susceptibility increase to diseases and may encourage wider pesticide use. In fact, hypersusceptibility to pathogens was reported in tomatoes with reduced PPO activity induced by the use of an antisense technique (*11*). Thus, alternative strategies should be developed to increase the application potential of molecular techniques.

In addition to chemical and genetic methods, heat treatment is also used effectively to prevent undesirable enzymatic browning reactions during the processing of fruits and vegetables. Especially in the production of frozen or thermally processed fruit pulps, heating is a widely used method employed for the prevention of enzymatic browning. Also, because most of the emerging technologies are not very effective or cause some undesirable effects on food quality, many different studies

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have been conducted to combine moderate or mild heat treatments with high-pressure sterilization, irradiation, microwave heating, ultrasound, osmotic rehydration, or hurdle technologies (*11*−*17*).

In fact, PPO enzymes do not belong to an "extremely heat-stable enzyme" group, and short exposures of product to temperatures between 70 and 90 °C are sufficient to inactivate the enzymes. However, in some *Prunus* fruits such as cherries, plums, and apricots PPO may have a considerable thermostability (*18*). In particular, the thermostability of apricot PPO has been known for a very long time (*18*−*21*). The thermostability of PPO enzymes may be affected by the cultivar, the physiological state, or the environmental conditions (*18*). Thus, it is very difficult to determine the factors causing the formation of thermostable PPO (TS-PPO). However, it is well-known that the PPO in unripe fruits is generally more heat stable than the PPO in ripe fruits (*18*, *22*). This indicates the presence of some in situ mechanisms that reduce the consistency of TS-PPO enzyme during ripening. The primary objective of this study was to obtain some data about the consistency of PPO thermostability in different apricot cultivars during ripening and to characterize the TS-PPO enzyme form. Information about the possible mechanisms that cause the decrease or elimination of TS-PPO may help to develop a new strategy for researchers working in the field of molecular biology. The development of crops containing thermolabile, but normal, PPO activity may allow disease and pest resistance to be maintained, required exposure times to heat during processing to be shortened, and use of chemical additives to be minimized.

MATERIALS AND METHODS

Materials. The apricot cultivars used in the research were obtained form Malatya Fruit Research Institute, Turkey. The fruits were washed, drained, and classified according to their color. The green mature fruits with very little or no straw-yellow areas were designated unripe; straw-yellow fruits with very little green areas and light orange fruits with straw-yellow and green areas were designated half-ripe; and orange fruits were designated ripe. The average weight of samples was determined by weighing 30 randomly selected apricots. The pH, °Brix, and titrable acidity were determined according to the standard methods given in Cemeroğlu (*23*) by using the homogenates obtained from 10 fruits. The average value of three measurements was used for the determination of titrable acidity, whereas other measurements were done twice. The reagents catechol, (+)-catechin, 4-methylcatechol, and DEAE-cellulose (fast flow) were obtained from Sigma Chemical Co. (St. Louis, MO). Polyamide was obtained from Roth Chemical Co. (Karlsruhe, Germany), and pyrogallol and *p*-cresol were obtained from Merck (Darmstadt, Germany).

Extraction of Enzymes. Acetone powder, obtained according to the method described by Yemenicioğlu et al. (*24*), was used as enzyme source in this study. The crude enzyme extracts were obtained by mixing 2−5 g of acetone powder, 300−350 mL of cold 0.01 M sodium phosphate buffer at pH 6.5, and 0.5 g of polyamide for 2 h at 4 °C. This suspension was filtered through four layers of cheesecloth, centrifuged at 4 °C and 7000*g* for 30 min, and then used immediately in the heat inactivation experiments. In partial purification studies the extraction was carried out in the same above-mentioned phosphate buffer, but at pH 6.8, with the addition of 10 mL of 0.05 M $CaCl_2$ to induce the precipitation of pectic compounds.

Enzyme Precipitation. The acetone precipitation method, previously used in the purification of apricot PPO by Dijkstra and Walker (*25*), was performed in this study. For this purpose, the crude extract was mixed with 2 volumes of cold acetone at −35 °C and stirred slowly for 10 min at 4 °C. The colorless precipitate was collected by filtration through a tightly woven nylon cloth, dissolved in a minimum amount of extraction buffer, and dialyzed against the same buffer (3 × 1500 mL) for 48 h at 4 °C.

Ion Exchange Chromatography. For ion exchange chromatography we used two different DEAE-cellulose columns (5 × 2.5 cm) containing 10 or 20 g of DEAE-cellulose matrix. Before use, these loosely and tightly filled columns were washed and equilibrated with 0.01 M phosphate buffer. After the enzyme extracts had been loaded, the columns were eluted at 4 °C with a linear discontinuous gradient of pH 6.8 sodium phosphate buffers (0.01−0.34 M). The fractions (5 mL) collected during elution were assayed for their PPO activity, and their absorbance at 280 nm was determined to monitor the protein peaks.

Heat Inactivation Experiments. Heat inactivation studies were carried out according to the method of Yemenicioğlu et al. (*24*) within the temperature range of 60−90 °C by using thermal inactivation time (TIT) tubes (i.d., 9 mm; wall thickness, 1 mm). The pH values of the buffers used in these studies were 6.5 and 6.8 for the crude and partially purified enzyme extracts, respectively. To minimize the lag phase, TIT tubes containing 4 mL of sodium phosphate buffer were heated to inactivation temperature. Once the temperature of the buffer solution stabilized, 0.4 mL of enzyme extract was pipetted into TIT tubes, mixed with a vortex and immersed again into water bath. After heating for a given period, the tubes were cooled in an ice water bath and immediately assayed for PPO activity. Because of the limited amount of enzyme extract, the heat inactivation experiments of partially purified enzymes were conducted by pipetting 0.2 mL of enzyme extracts to preheated TIT tubes containing 2 mL of phosphate buffer.

Kinetic Properties. The effect of temperature on PPO activity was determined by measuring the enzyme activity at 20−60 and 20−45 °C for the crude and partially purified enzyme extracts, respectively. During measurements, to reduce the heating lag the enzyme extract was added to a preheated buffer and substrate mixture. Optimum pH was determined by using 0.1 M sodium phosphate buffer at different pH values (5.5−8) in reaction mixtures. Michaelis constants and maximum velocities of the enzyme fractions were determined by using the double-reciprocal plot. The final concentrations of catechol (3.2−143.2 mM) in cuvettes were kept around the K_m values of PPO reported in the detailed review of Vamos-Vigyazo (*18*). In the substrate specificity tests, the concentration of pyrogallol, catechol, 4-methylcatechol, hydroquinone, and *p*-cresol was 50 mM and the concentration of (+)-catechin was 2.5 mM.

PPO Activity Determination. The activity measurements were conducted at 420 nm by using a Pye Unicam SPG-550 UV−vis spectrophotometer equipped with a constant-temperature cell holder. After heat inactivation experiments, for the determination of their residual catecholase or cresolase activities the TIT tubes were incubated at 30 or 35 °C for 5 min, respectively. The catecholase activity was determined at 30 °C by mixing 2 mL of TIT tube content with 0.5 mL of 0.5 M catechol (at 30 °C) in a quartz cuvette (optical path length, 1 cm), whereas cresolase activity was determined at 35 °C by mixing 4 mL of tube content with 1 mL of 5 mM *p*-cresol in a glass cuvette (optical path length, 5 cm). For activity measurements during partial purification and kinetic studies, 0.2−0.3 mL of enzyme extract was mixed with 2 mL of 0.01 M, pH 6.8, sodium phosphate buffer in a test tube. After 5 min of incubation at 30 °C, 2 mL of tube content was transferred to a quartz cuvette and 0.2−0.3 mL of 0.5 M catechol at 30 °C was added to the cuvette to start the reaction. The absorbance values observed during the determination of catecholase and cresolase activity were recorded at every 10 (or 15) and 120 s, respectively. The catecholase activity was determined from the slope of the initial linear portion of absorbance versus time curves, whereas cresolase activity was determined from the slope of the linear portion of absorbance versus time curves coming after the initial lag period. The enzyme activities were given as unit (the amount of enzyme that caused 0.001 change in absorbance in 1 min) or $\Delta abs\ min^{-1}\ mL^{-1}$. In this study the catecholase activity was designated PPO activity unless otherwise indicated.

Protein Content. Protein was determined according to the Lowry method by using bovine serum albumin as standard (*26*).

RESULTS AND DISCUSSION

Consistency of PPO Thermostability during Fruit Ripening. The effect of ripening on the thermal stability of apricot PPO was investigated between 60 and 90 °C by using 0.01 M,

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Table 1. Some Characteristics of the Apricot Cultivars Used in This Study

cultivar	av wt (g)	pH	titrable acidity[a] (g kg⁻¹)	°Brix	ratio[b]
Hacıhaliloğlu	30.3				
unripe		4.92	17.0	15.8	0.93
half-ripe		4.98	17.4	19.6	1.13
ripe		4.96	15.4	20.6	1.34
Çataloğlu	21.4				
unripe		4.86	14.1	14.8	1.05
half-ripe		4.95	13.4	15.8	1.18
ripe		5.02	12.4	16.2	1.31
Kabaaşı	31.5				
unripe		4.75	15.4	11.6	0.75
half-ripe		4.97	13.0	14.4	1.11
ripe		4.94	13.0	17.2	1.32

[a] Titrable acidity is given as malic acid equivalent. [b] °Brix/titrable acidity.
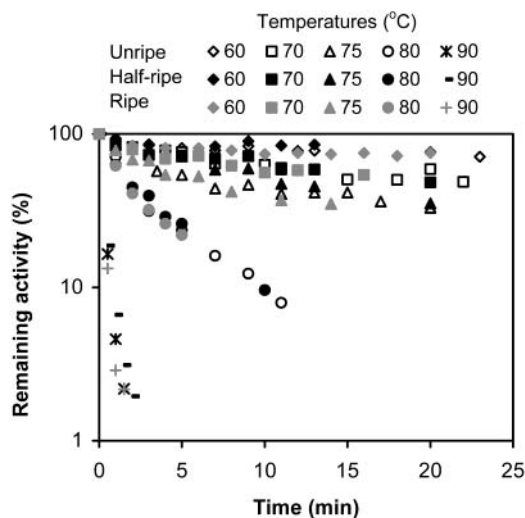
Figure 1. Heat inactivation of PPO from Hacıhalioğlu apricots at different ripening stages.

pH 6.5, sodium phosphate buffer as inactivation medium for the three different cultivars. Some characteristics of the apricot cultivars used in this study are given in **Table 1**. In all of the apricot cultivars the inactivation of PPO followed a first-order reaction kinetic. However, the courses of inactivation curves varied considerably. For example, in Hacıhaliloğlu cultivar at all stages of ripening the inactivation curves were biphasic (**Figure 1**) and the PPO enzyme was considerably thermostable (TS) (**Table 2**). In contrast, in Kabaaşı apricots at all stages of ripening the enzyme was very thermolabile (TL) and its heat inactivation curves were monophasic or showed tailing at high temperatures when PPO activity reduced to below 10% (**Figure 2**). PPO from Çataloğlu apricots, on the other hand, showed both monophasic (with or without tailing at high temperatures) and biphasic inactivation curves (**Figure 3**). In these apricots the enzyme was quite heat labile at unripe and ripe stages and showed inactivation curves similar to those of PPO in Kabaaşı cultivar. In contrast, in half-ripe Çataloğlu apricots the course of inactivation curves was biphasic and the enzyme was very thermostable. It is worth noting that the D and z values of PPO in half-ripe Çataloğlu apricots are almost in the range of D and z values of PPO in Hacıhaliloğlu apricots. Thus, these results clearly indicate the presence of consistent and inconsistent TS-PPO in Hacıhaliloğlu and Çataloğlu apricots, respectively.

The biphasic inactivation curves of PPO in Hacıhaliloğlu apricots and in half-ripe Çataloğlu apricots enabled us to calculate the percentages of their TS and TL enzyme fractions. This was done as described by Yamamoto et al. (*27*) by extending the heat stable portion of a heat inactivation curve and finding its intercept on the *y*-axis. As shown in **Table 3** between 60 and 80 °C the percentage of TS-PPO in unripe, half-ripe, and ripe Hacıhaliloğlu apricots varied between 54 and 87, between 67 and 91, and between 61 and 83, respectively. At 90 °C the percentages of TS-PPO enzyme fraction in unripe and half-ripe Hacıhaliloğlu apricots increased slightly from 42 to 44 and then dropped to 27 in ripe fruits. Because of the rapid loss of the TL enzyme's activity at 90 °C, the reduction in the percentage of the TS enzyme fraction in ripe fruits was more apparent. Also, in unripe and ripe Hacıhaliloğlu apricots the TS/TL ratio dropped below 3 above 70 °C. However, in half-ripe Hacıhaliloğlu apricots and half-ripe Çataloğlu apricots this occurred above 75 °C. These comparisons clearly showed the increase and then reduction of the TS/TL ratio in Hacıhaliloğlu apricots at half-ripe and ripe stages, respectively. However, unlike the TS-PPO in Çataloğlu apricots a considerable portion of TS enzyme form was consistent in Hacıhaliloğlu apricots and existed at all stages of ripening. Previously in our laboratory we showed the presence of inconsistent TS-PPO in Hale Haven peaches (*22*). In that study, by using the heat inactivation curves at 70 °C, we calculated the percentages of TS-PPO in unripe, half-ripe, and ripe peaches as 87, 48, and 55, respectively. Similar to the results obtained for Hacıhaliloğlu apricots, no considerable change was determined among the $D_{70°C}$ values of TS enzyme fraction in unripe, half-ripe, and ripe peaches. The disappearance of TS-PPO affected only the TS/TL ratio. Thus, these results confirm the presence of in situ TS-PPO destabilizing mechanisms in apricots and peaches.

In this study the effect of temperature on the activity of PPO from ripe apricots was also investigated. As seen in **Figure 4** PPO in Kabaaşı, Çataloğlu, and Hacıhaliloğlu apricots showed optimal activity at 35, 40, and 45 °C, respectively. The higher temperature optimum of TS-PPO in Hacıhaliloğlu apricots is not surprising. However, it is quite interesting to observe that TS-PPO in these apricots and TL-PPO in Çataloğlu and Kabaaşı apricots showed almost no activity at 65 °C. The heat inactivation studies showed that apricot PPO was quite heat stable at this temperature. Thus, the inability of enzymes to display their activity at 65 °C should be related to reversible modifications in PPOs' conformation that occurred before or after their interaction with the substrate so as to decrease or eliminate their activities. This result is in accordance with the general consideration that during processing, to control browning caused by PPO, the products should be heated very rapidly above 65 °C (*18*).

To obtain more data about the similarities and differences between the TS-PPO in Hacıhaliloğlu apricots and TL-PPO in Çataloğlu and Kabaaşı apricots, we also investigated the heat stability of their cresolase activities in ripe fruits. The inactivation of PPO cresolase activity in apricots between 65 and 80 °C also followed a first-order reaction kinetic. However, at high temperatures a lag phase had been observed in the inactivation curves before the monophasic inactivation started. In Çataloğlu apricots the lag phases were more apparent, and at 70 °C we also initially observed a considerable increase in enzyme activity. However, when we repeated the heat inactivation study at 70 °C, a lag phase was observed instead of an increase in enzyme activity (**Figure 5**). Thus, it is likely that the lag phase observed in heat inactivation curves was due to the activation of enzyme by heat. Comparison of the D values, calculated from the linear portions of inactivation curves coming after the lag

Table 2. Effect of Ripening on Thermostability of PPO from Different Apricot Cultivars

| cultivar | temp (°C) | D value (min) | | |
		unripe	half-ripe	ripe
Hacıhaliloğlu	60	278.0 (20.0)[a]	286.0 (33.3)	357.0 (25.0)
	70	92.0 (12.5)	82.0 (14.3)	81.0 (20.0)
	75	78.0 (10.0)	51.0 (16.7)	39.0 (9.1)
	80	13.5 (5.96)	11.8 (5.7)	11.1 (5.1)
	90	1.13 (0.64)	1.28(0.68)	1.27 (0.57)
		$z = 12.5$ °C (0.918)[b]	$z = 12.7$ °C (0.960)	$z = 12.2$ °C (0.985)
		$z^{c} = 20.8$ °C (0.845)	$z = 18.3$ °C (0.870)	$z = 18.1$ °C (0.881)
Kabaaşı	60	313.0	385.0	345.0
	70	37.6	47.8	35.8
	75	11.6	8.3	11.6
	80	3.93	2.1	2.86
	90	0.37	0.45	0.39
		$z = 10.2$ °C (0.999)	$z = 9.9$ °C (0.982)	$z = 10.1$ °C (0.998)
Çataloğlu	60	233.0	345.0 (33.3)	124.0
	70	21.9	112.0 (20.0)	17.2
	75	9.2	54.0 (14.3)	7.5
	80	2.7	13.3 (9.1)	2.0
	90	0.43	1.12 (0.99)	0.37
		$z = 11.0$ °C (0.997)	$z = 11.9$ °C (0.958)	$z = 11.8$ °C (0.997)
			$z = 20.4$ °C (0.865)	

[a] D values of thermolabile PPO. [b] Correlation coefficients. [c] z values of thermolabile PPO.

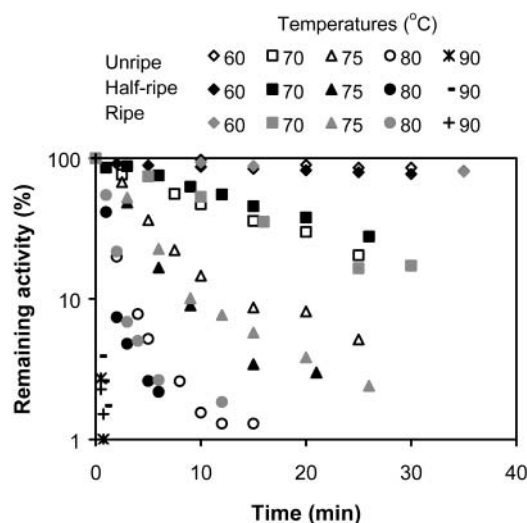**Figure 2.** Heat inactivation of PPO from Kabaaşı apricots at different ripening stages.

Figure 3. Heat inactivation of PPO from Çataloğlu apricots at different ripening stages.

phases, indicated that between 65 and 80 °C Kabaaşı apricots had the most thermostable PPO cresolase activity (**Table 4**). At low temperatures, the cresolase activity in apricots had considerably lower thermostability compared to their catecholase activity. In Kabaaşı and Çataloğlu apricots the difference between the thermostabilities of cresolase and catecholase activities reduced at higher inactivation temperatures. However, in Hacıhaliloğlu apricots the considerable difference between the thermostabilities of two activities existed even at high temperatures. Thus, it is clear that the presence of TS catecholase activity (TS-PPO) in Hacıhaliloğlu apricots did not yield also a thermostable cresolase activity.

Characterization of TS-PPO. To further understand the characteristics of consistent TS-PPO, we partially purified and characterized the enzyme from ripe Hacıhaliloğlu apricots (**Table 5**). In DEAE-cellulose column chromatography we applied two different procedures. In the first procedure we used the loosely filled column (5 × 2.5 cm, 10 g of DEAE-cellulose matrix) and collected most of the active fractions (33−41
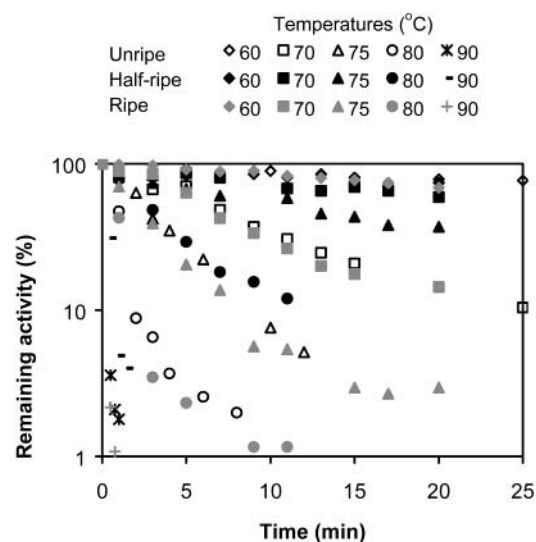
fractions for F1 and 44−52 fractions for F2) of two main peaks eluted without considering the minor fractions (**Figure 6A**). The heat inactivation of these main fractions followed a first-order reaction kinetic. However, enzymes showed monophasic inactivation curves, and they were very heat labile (**Figure 7**). In fact, their z values were in the range of those z values obtained for TL-PPO in Kabaaşı cultivar. Although F2 was slightly more heat stable than F1, there were no considerable differences between the thermostabilities of two fractions. However, their kinetic parameters, temperature optima, and substrate specificities were quite different (**Table 6**). It is worth noting that the F1 oxidized both p-cresol and the para-diphenolic compound hydroquinone. The presence of laccase enzymes having cresolase activity was reported in the detailed review of Vamos-Vigyazo (*18*). However, it is also likely that the broad substrate specificity of F1 was due to the presence of catechol oxidase isoenzymes in this fraction. The lack of monophenolase activity in F2 indicated the homogeneous character of isoenzyme forms
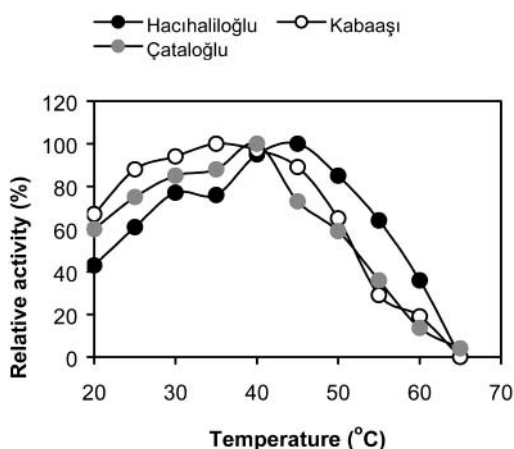
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Table 3. Effect of Ripening Stage and Cultivar on the Percentage of Thermostable and Thermolabile PPO Fractions in Apricots

cultivar	temp (°C)	% of fraction		TS/TL ratio
		thermo-stable (TS)	thermo-labile (TL)	
Hacıhaliloğlu (unripe)	60	87	13	6.7
	70	83	17	4.9
	75	60	40	1.5
	80	54	46	1.2
	90	42	58	0.72
Hacıhaliloğlu (half-ripe)	60	91	9	10.1
	70	84	16	5.3
	75	84	16	5.3
	80	67	33	2
	90	44	56	0.79
Hacıhaliloğlu (ripe)	60	83	17	4.9
	70	81	19	4.3
	75	74	26	2.8
	80	61	39	1.6
	90	27	73	0.37
Çataloğlu (half-ripe)	60	87	13	6.7
	70	90	10	9.0
	75	84	16	5.3
	80	73	27	2.7
	90	66	34	1.94

Figure 4. Effect of temperature on the activity of PPO from ripe apricots of different cultivars.
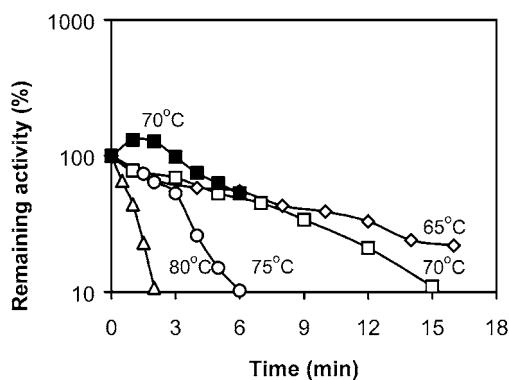
Figure 5. Heat inactivation of PPO cresolase activity from ripe Çataloğlu apricots.

found in this fraction. The labile character of PPO cresolase activity was reported by different workers (*28*, *29*). Thus, its loss during purification was not surprising.

In the second procedure of partial purification, to achieve further purity we used the densely filled column (5 × 2.5 cm,

Table 4. Thermostability of PPO–Cresolase Activity from Different Cultivars of Ripe Apricots

cultivar	temp (°C)	lag phase (min)	*D* value (min)
Hacıhaliloğlu	65		61.0
	70		22.0
	75	2	5.0
	80	0.5	1.8
			$z = 9.6$ °C (0.995)[a]
Kabaaşı	60		182.0
	65		70.0
	70		31.0
	75	3	7.1
	80	1	2.3
			$z = 10.4$ °C (0.990)
Çataloğlu	65		26.3
	70	9	12.5
	75	3	4.3
	80	1	1.6
			$z = 12.1$ °C (0.994)

[a] Correlation coefficients.

20 g of DEAE-cellulose matrix) and collected only the most active fractions of each peak. This time the first main fraction was sharply separated from the second fraction, which is the complex of four or five isoforms (**Figure 6B**). In the literature there are different reports about the number of PPO isoforms in apricots. For example, Vamos-Vigyazo (*18*) reported the existence of two main enzyme fractions in these fruits, whereas Chavalier et al. (*30*) and Fraignier et al. (*31*) reported the presence of a single main and several minor forms. Thus, it appears that the apricots may contain different numbers of PPO molecular forms. The temperature profiles of purified fractions determined by a 2 min incubation of enzyme extracts at different temperatures are given in **Figure 8**. Although some fractions (F3^I and F4^I) showed apparent activation between 50 and 60 °C, again all of the purified enzymes showed no thermostability and were inactivated almost one decimal above 75 °C.

The results of heating studies showed that the TS-PPO enzyme lost its thermostability during the purification. In our previous studies we found that the acetone precipitation does not affect the characteristic *z* value and biphasic inactivation curves of PPO in Hacıhaliloğlu apricots (*32*). Thus, although DEAE-cellulose is a weak anion exchanger and is recommended for the purification of labile proteins (*33*), the ion exchange processes occurring during column chromatography may cause the loss of enzyme thermostability. The results of Benjamin and Montgomery (*34*), who investigated the thermostability of cherry PPO, supported this hypothesis. These workers determined the half-lives of an acetone-precipitated fraction and DEAE-cellulose-purified DE 1 and DE 2 fractions at 75 °C as 8, 1.9, and 2.7 min, respectively. The reduction of the thermostability of DEAE-cellulose-immobilized enzymes is attributed to the disruption of critical internal salt bridges of enzyme (*35*). Thus, it seems that the thermostability of apricot PPO is due to ionic interactions.

Possible Mechanisms of TS-PPO Formation and Destabilization. Although this study shows that Hacıhaliloğlu apricots contain consistent TS-PPO enzyme, there is no proof that this enzyme form is constantly present. For example, in 1995 we investigated the thermal properties of PPO from ripe Hacıhaliloğlu apricots brought from the same region, but we did not observe the TS-PPO form in these apricots. The enzyme showed its characteristic *z* value ($z = 12.1$ °C) and biphasic inactivation curves even in apricot serum, but the *D* values of

Table 5. Partial Purification of PPO from Ripe Hacıhaliloğlu Apricots

purification step	volume (mL)	total activity (units)	total protein (mg)	specific activity (units mg^{-1})	yield (%)	purity (fold)
crude extract I[a]	309	2753715	57.8	47642	100	1
acetone precipitation	41	943582	7.4	127511	34	2.7
DEAE-cellulose column chromatography[b]						
F1	45	390398	2.24	174285	14	3.7
F2	45	432878	1.55	279276	16	5.9
crude extract II[a]	310	206770	35.6	5808	100	1
acetone precipitation	30	74520	4.2	17743	36	3.1
DEAE-cellulose column chromatography[c]						
F1'	15	5700	0.063	90476	2.8	15.6
F2'	5	1725	≪		0.8	
F3'	10	2370	≪		1.1	
F4'	5	1785	≪		0.86	
F5'	5	805	≪		0.39	

[a] Crude extracts I and II were prepared by using 5 and 2 g of acetone powder, respectively. [b] Loosely filled column. [c] Tightly filled column.
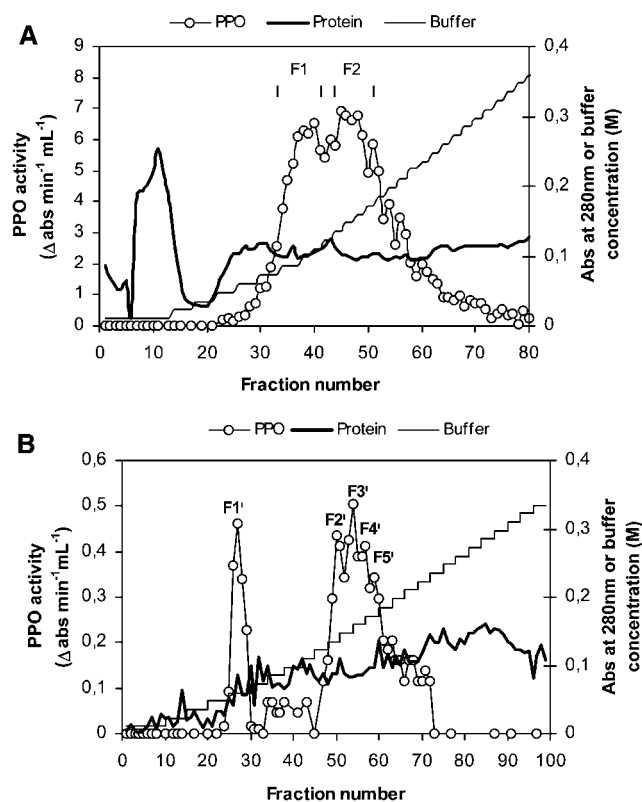

Figure 6. DEAE-cellulose chromatography of PPO from ripe Hacıhaliloğlu apricots: (A) loosely filled column (5 × 2.5 cm, 10 g of DEAE-cellulose matrix); (B) tightly filled column (5 × 2.5 cm, 20 g of DEAE-cellulose matrix).

Figure 7. Heat inactivation of partially purified main PPO fractions from ripe Hacıhaliloğlu apricots.

the heat stable portion in pH 6.8 phosphate buffer ($D_{55°C}$ = 134.2 min and $D_{70°C}$ = 21.4 min) indicated the thermolabile nature of the enzyme (*32*). These findings suggest the importance of environmental factors in the formation of TS-PPO enzyme and the risk of selecting heat labile PPO-containing cultivars. Thus, it is important to find the in situ mechanisms related to the formation and destabilization of TS-PPO enzyme.

In the early literature it has been reported that the active forms of PPO in plants having molecular masses in the range of 40−50 kDa derive from the in situ proteolysis of 60−70 kDa precursors (*36−38*). Fraignier et al. (*31*) reported that in apricots and in other *Prunus* fruits the main active PPO form is a 43 kDa enzyme. The possible high molecular weight precursor of this enzyme form detected with apple anti-PPO antibodies was
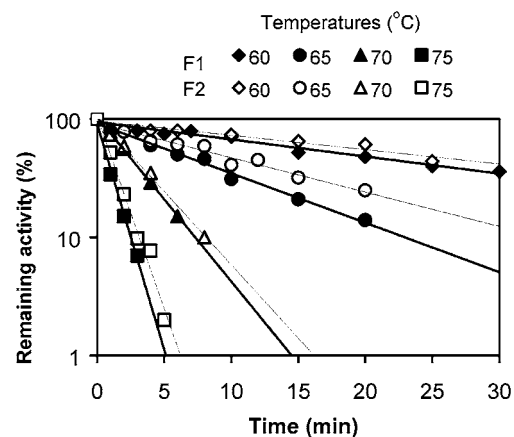
an inactive protein of 63 kDa molecular weight. In peaches and apricots in addition to the protease-resistant 43 kDa major form, these researchers found also different active PPO forms having molecular weights between 27 and 40 kDa. Thus, they considered these forms to occur as a result of in vivo proteolysis of the main form by native proteases having activities that vary according to the cultivar and physiological state of fruits. Chavalier et al. (*30*) calculated the molecular weight of the main active PPO form in apricots, under denaturing conditions in electrophoresis, as 60 kDa and claimed that the 43 kDa enzyme reported by Fragnier et al. (*31*) should have been formed by the proteolytic cleavage of the C-terminal end of this enzyme form. The same researchers also calculated the predicted molecular weight of PPO preprotein in apricots as 67.1 kDa. The results of our partial purification studies indicate that apricots contain multiple PPO forms with different kinetic properties. This suggests the action of proteases on PPO enzyme. Thus, the possible mechanism of TS-PPO formation and destabilization may simply be explained by the action of specific proteases that cause the modification of enzyme during ripening. In the literature the presence of at least five catalytic types of proteases was reported in plant tissues (*39*). It was found that some proteases cause only the modification of PPO, whereas some others cause a partial digestion of the enzyme and reduce its molecular weight (*40*). Thus, we hypothesized that the protease profile in apricot cultivars was different. The consistent TS-PPO existed only in Hacıhaliloğlu apricots, and this may be due to the presence of specific proteases (TS-PPO-forming

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Table 6. Characteristics of Partially Purified Main PPO Fractions from Ripe Hacıhaliloğlu Apricots

characteristic	F1 fraction	F2 fraction
heat inactivation parameters	$D_{60°C} = 69$ min, $D_{65°C} = 24$ min, $D_{70°C} = 7.2$ min, $D_{75°C} = 2.6$ min $z = 10.4$ °C (0.990)[a]	$D_{60°C} = 82$ min, $D_{65°C} = 34$ min, $D_{70°C} = 8$ min, $D_{75°C} = 3$ min $z = 10.1$ °C (0.996)
optimum pH range[b]	6.8–8	6.8–7.7
optimum temperature	35 °C	30 °C
kinetic parameters	$K_m = 61$ mM $V_{max} = 22.6$ Δabs min^{-1} mL^{-1} $V_{max}/K_m = 0.37$	$K_m = 122.7$ mM $V_{max} = 36.1$ Δabs min^{-1} mL^{-1} $V_{max}/K_m = 0.29$
substrate specificity	4-methylcatechol (100) (+)-catechin (88) catechol (64) pyrogallol (50) hydroquinone (0.6) *p*-cresol (0.3)	4-methylcatechol (100) (+)-catechin (35) catechol (45) pyrogallol (17) hydroquinone (0) *p*-cresol (0)

[a] Correlation coefficients. [b] In the given pH range fractions maintain ≥90% of their activity.
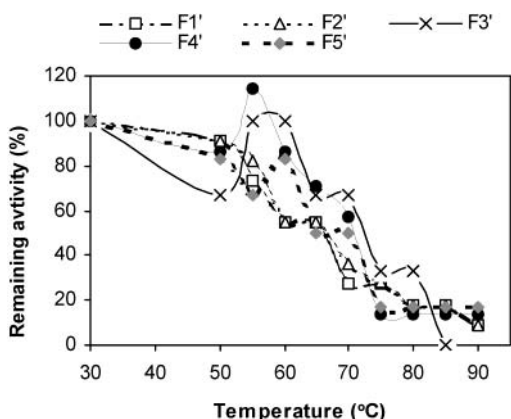
Figure 8. Temperature profiles of partially purified PPO fractions from ripe Hacıhaliloğlu apricots.

proteases) that solely determined the conformational changes necessary for TS-PPO formation. The consistency of the enzyme may be explained by the very low activity of digestive proteases (TS-PPO-destabilizing proteases) that destabilize the thermostable enzyme's conformation. The protease profile hypothesis can also be used to explain the inconsistent TS-PPO formation. This PPO form may appear when fruits contain less TS-PPO-forming but more abundant destabilizing enzymes. In this case, as occurred in Çataloğlu apricots, the TS-PPO may appear very late (at the half-ripe stage) and, because of the activity of destabilizing enzymes, it cannot maintain its unique conformation for a very long time. The lack of TS-PPO, on the other hand, may occur when the TS-PPO-destabilizing enzymes prevail over the TS-PPO-forming enzymes. If these assumptions are proved, they may provide some new strategies for the solution of the TS-PPO problem by use of molecular techniques. For example, overexpression of genes related to enzyme systems responsible from the destabilization of TS-PPO may be a strategy. Also, blocking the expression of genes related to enzyme systems that catalyze TL-PPO transformation to TS-PPO may be another strategy. Thus, further studies should be conducted to find the enzymes driving these mechanisms.

In addition to the specific proteases, nonproteinous substances may also contribute to the formation of TS-PPO enzyme. For example, the glycosylation may considerably increase the thermostability of a protein by affecting its hydrophilic/hydrophobic balance and its surface charges (*41*, *42*). Thus, artificial glycosylation has been used to increase the thermostability of some enzymes and proteins such as lysozyme, lipase, phosvitin, and β-lactoglobulin (*41*, *43*–*45*).

Many enzymes, including PPO and peroxidase (POD), may naturally contain a carbohydrate conjugate (*46*–*50*). In fact, all laccases described to date are glycoproteins and they contain more carbohydrate compared to catechol oxidases (*47*, *51*). Dijkstra and Walker (*25*) reported the presence of laccase enzyme activity in apricots. Also, in this study we determined the presence of laccase enzyme activity in one of the major fractions (F1) of PPO enzyme. Chavalier et al. (*30*) studied the molecular cloning and characterization of apricot PPO and reported the presence of a single putative glycosylation site in this enzyme. Thus, glycosylation may also be an important factor for the formation of TS-PPO enzyme.

Studies related to the glycosylation mechanisms of plant PPO and the roles of carbohydrate residues in the functions of these enzymes are scarce. In contrast, in their exhaustive review, Branza-Nichita et al. (*52*) reported that the N-glycosylation of mammalian PPO occurs in the very early stages of PPO synthesis and that the introduced carbohydrate residues contribute to the formation of the enzyme's specific conformation and to copper loading. Also, it has been reported that the nature of glycosylated carbohydrate and the degree of glycosylation may affect the affinity of proteases to their substrates (*53*, *54*). Thus, in addition to different protease profiles, PPO availability may also be effective on the formation and destabilization of TS-PPO enzyme in apricots. The solution of these mechanisms may be very complicated if modifications in carbohydrates by glycosidases and glycosyltransferases are effective on the enzymes' conformation and affinity to proteases. However, the studies should first be concentrated more on the effect of specific proteases and glycosylation on PPO thermostability.

In conclusion, the results of this study showed the presence of in situ TS-PPO forming as well as destabilizing mechanisms in apricots. The loss of PPO thermostability by partial purification indicated the non-covalent nature of TS-PPO stabilization. Further studies about the molecular transformations of PPO during ripening and the roles of specific proteases and enzyme glycosylation in these transformations are needed to develop some new strategies that may be utilized by molecular techniques for a planned production of apricot cultivars provided with heat labile but normal PPO activity.

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