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Purification and Characterization of Pear (*Pyrus communis*) Polyphenol Oxidase

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Three isoenzymes of polyphenol oxidase (PPO) were isolated from a local pear variety (Ankara Armutu) through ammonium sulfate precipitation, dialysis and gel filtration. The sample obtained from dialysis after ammonium sulfate precipitation was used for characterization of the partially purified enzyme. Optimum pH values of PPO were 8.2 for pyrogallol, 7.2 for 4-methylcatechol, 7.0 for catechol, 5.6 for D-tyrosine, 5.0 for p-cresol and 4.8 for L-dopa. The optimum temperature of PPO was 35 °C with 4-methylcatechol. Catechol was oxidized more rapidly than the other substrates; however, 4-methylcatechol, chlorogenic acid and caffeic acid were also good substrates. The effect of 6 different inhibitors on the PPO activity was investigated in this work. L-ascorbic acid, L-cystein and sodium diethyldithiocarbamate were the most effective inhibitors. K_m and V_{max} values of the enzyme were estimated as 5.55 mM and 344.5 IU/mL min respectively for catechol substrate. Thermal inactivation data indicated an apparent activation energy of 0.19 cal/mol. Three isoenzymes of Ankara pear PPO were detected by activity analysis in nondenaturing-polyacrylamide gel electrophoresis. Their molecular weights were determined as 60, 40 and 28 kDa by sodium dodecyl sulfate-PAGE.

Introduction

Polyphenol oxidase (monophenol dihydroxyphenylalanine:oxidoreductase: E.C. 1.14.18.1; PPO) is widely distributed in plants and it can also be found in microorganisms. The Ankara pear (*Pyrus communis*) has a very short shelf-life under normal ambient conditions due to skin color loss (browning) and deterioration during storage and transportation. For these reasons there are considerable losses in the market value of this fruit. The major reason for skin browning is the oxidation of phenolic compounds by molecular oxygen as a result of enzymatic catalysis of PPO.[1−7] The phenolic compounds and PPO are components of the skin tissue of fruit.

PPO has been widely studied in various fruits and vegetables such as potato tuber[8,9], peach [10,11], apple[12,13], apricot[14], banana[15,16,17], grape[18,19], pear[20,21] , green olive [22], strawberry[23], plum[24], kiwi[25] and mango[26].

The objective of our study was to isolate, purify and characterize the PPO enzyme from the Ankara pear. Thus, more effective preservation conditions and methods can be developed in order to prevent the enzymatic browning of this fruit.

Experimental

Materials Ankara pears were used as the research material. The fruit is smaller and fleshier than other pear species. Therefore they are preferred in the manufacture of jam and marmalade and also for fresh consumption. Pears at the firm green-yellow ripe stage were harvested from the Kızılcahamam region in Ankara province and stored at 4 °C until used. Most of the chemicals were obtained from Sigma Co. (Deisenhofen, Germany). 4-Methyl catechol was purchased from Fluka (Steinheim, Germany).

Enzyme Extraction and Purification. Enzyme extraction was performed at 4 °C, unless otherwise stated. For preparing the partially purified enzyme, 170 g of pear tissue was halved, cored and cut quickly into thin slices. Then the sliced tissue was homogenized with 300 mL of chilled 0.2 M phosphate buffer (pH 6.8) containing 0.05 M ascorbic acid and 0.5% polyvinylpolypyrolidone (PVP) in a Waring blender for 3 min. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at 30.000 x *g* for 30 min at 4 °C. The proteins in the supernatant were collected. The enzyme solution was fractionated with solid ammonium sulfate and the precipitate of 50–80% saturation was collected by centrifugation at 15.000 x *g* for 20 min. The pellet was redissolved in 10 mL of homogenization buffer (0.2 M phosphate buffer, pH 6.8) and dialyzed at 4 °C against the same buffer in cellulose dialysis tubing (mol. wt. cut off 12,000-14,000 Da). The dialysis buffer was changed 3 times with 8 h intervals. The dialyzed samples were kept in stoppered test tubes at –20 °C. The dialyzed solution was lyophilized, dissolved again in a small volume of 0.2 M phosphate buffer and applied to a Sephadex G-100 column.

The protein concentration of the samples at different stages of purification was determined by the method of Lowry et al.[27].

Gel Filtration. A column (1.2 x 70 cm) was prepared with Sephadex G-100 for gel filtration chromatography and equilibrated with 0.1 M phosphate buffer (pH: 6.8) containing 10% glycerin. The dialyzed enzyme solution was loaded into the column and the elution rate was adjusted to 0.5 mL/min. The eluates were collected as 3 mL volumes in tubes by a fraction collector. The elution process continued until no absorbance was observed at 280 nm. The protein and PPO activities of each fraction were estimated. The fractions having PPO activity were collected and purification degrees were determined by measuring specific activity.

PPO Activity Measurement. PPO activity was assayed according to the pyrocatechin method of Traverso-Rueda and Singleton[28]. PPO activity was determined spectrophotometrically by measuring the absorbance increase at 420 nm within 2 min by means of a spectrophotometer (Jenway 6105 UV/vis). Dialyzed enzyme solution was diluted 1/10 (v/v) with phosphate buffer. The sample cuvette contained 2.8 mL of substrate in phosphate buffer and 0.2 mL of enzyme solution. The blank cuvette contained only 3 mL of substrate solution in phosphate buffer. The reaction was carried out at various temperatures and pH values with the substrates. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as the amount of enzyme that caused an increase of 0.001 unit of absorbance per minute at room temperature. The experiments were duplicated.

Characterization of PPO

Substrate Specificity. Nine different commercial grade substrates (catechol, 4-methyl catechol, D-tyrosine, L-dopa, pyrogallol, chlorogenic acid, p-cresol, gallic acid and caffeic acid) were used for investigation of the substrate specificity of enzyme. All substrates were used in 0.02 M concentrations except for D-tyrosine and

caffeic acid, which were 0.002 M due to their limited solubilities.

Effect of pH and temperature. The effect of pH on PPO activity was determined with 7 different substrates (catechol, 4-methyl catechol, L-dopa, pyrogallol, D-tyrosine, caffeic acid and p-cresol). Catechol, 4-methyl catechol and pyrogallol were used in 10 mM; D-tyrosine was used in 2 mM; and caffeic acid, p-cresol and L-dopa were used in 5 mM concentrations. Appropriate buffers (0.1 M citrate pH 4.2-5.2; 0.2 M phosphate pH 5.2-7.0 and 0.05 M Tris-HCl pH 7.0-9.5) were used determine the optimum pH of PPO. The optimum pH values obtained from this assay were used in all the other experiments.

The effect of temperature on PPO activity was measured within the 10-75 °C interval using the 7 substrates indicated above.

Thermal Inactivation. Heat treatments of PPO were carried out at 60, 70, 75, 80 and 85 °C for varying periods of time in a temperature-controlled waterbath. Ten milliliters of enzyme solution was placed in a prewarmed tube at the specified temperature and 0.5 mL of the sample portions was withdrawn at various time intervals, cooled and assayed for residual activity. The stabillity of the enzyme was expressed as remaining activity. The activation energy for denaturation of the enzyme was determined by an Arrhenius plot of log reaction rate constants (ln k) vs. the reciprocal of the absolute temperature.

Enzyme Kinetics. Initial reaction rates of PPO at optimal pH and temperature values were measured using 7 different substrates at different concentrations (1, 1.25, 2.5, 4, 7.5 and 10 mM). $K_m$and V_{max} values for each substrate were calculated from the Lineweaver-Burk plot[29]. The initial reaction rate was estimated by the assay described in the "PPO Activity Measurement " section.

Effect of Inhibitors. The inhibitory effect of L-cysteine, L-ascorbic acid, sodium azide, sodium diethyl dithiocarbamate, β-mercaptoethanol and thiourea on the PPO activity at fixed concentrations was estimated at 20 °C and pH 7.0 by using catechol as substrate at different concentrations (1, 1.25, 2.5, 4, 7.5, 10 mM). Inhibition constants (K_i) of each inhibitor were estimated from Lineweaver-Burk graphs.

Electrophoresis. Polyacrylamide gel electrophoresis was performed according to the method of Laemmli[30] for separating PPO isoenzymes of the Ankara pear in nondenaturing conditions. Electrophoresis was performed with a discontinuous buffer system using 4% stacking gel and 7.5% acrylamide separating gel. The enzyme samples obtained from dialysis (20 μL) were applied to the space of wells in the stacking gel. The gel was run at the constant current of 30 mA at 4 °C until the bromophenol blue marker had reached the bottom. After completion of the run, the gel was cut into 3 pieces. One gel piece was then immersed in 0.2 M phosphate buffer (pH 7.2) containing 20 mM catechol and 0.05% o-phenylendiamine. The other pieces were immersed into the solution containing 20 mM 4-methyl catechol and 10 mM pyrogallol. The isoenzyme bands were developed in 100 min and each gel piece was then rinsed in 1 mM ascorbic acid solution for 4 min. Then gel pieces treated with catechol, 4-methyl catechol and pyrogallol substrates were stored in 30% ethanol and photographed. For SDS-PAGE, enzyme solutions were denaturated by adding denaturation solution containing 2.5% glycerol, 0.05% SDS, 1.25% β-mercaptoethanol and 0.001% bromophenol blue in 0.06 M Tris buffer. After the samples were denaturated, electrophoresis was carried out as described above. After the electrophoretic run gels were stained with Coomassie brilliant blue. Then the gels were destained in 7% acetic acid solution and photographed. The molecular weight of the PPO was calculated by comparing the relative migration distances of the enzyme and marker proteins at different molecular weights.

Results and Discussion

Purification of PPO. Polyvinylpolypyrrolidone (PVP) was used during the enzyme extraction due to its binding ability to the phenolics in order to prevent phenol-protein interactions. PVP is an inhibitor of PPO [31], but its removal by centrifugation avoided this effect. Several precipitations with solid ammonium sulfate between 40 and 80% were tested to find the proper saturation point. As a result, the PPO activity of the precipitate of 80% $(NH_4)_2SO_4$ saturation was found the highest and this saturation point was used in all the extraction processes. The oxidation of phenolics by PPO produces quinones during extraction [32]. Therefore ascorbic acid was used to reduce the formation of quinones. The results of the purification of the PPO from the Ankara pear are shown in Table 1. The elution profile of PPO from Sephadex G-100 revealed 3 fractions (S_1, S_2, S_3) (Figure 1). The data in Table 1 show that the specific activities of fractions S_1 and S_2 increased slightly with gel filtration. The purification degrees of PPO were 13.3-, 11.3- and 8.7-fold after gel filtration, showing that Ankara pear PPO has 3 isoenzymes.

Table 1. Purification of PPO from Ankara pear.

Purification steps	Volume (mL)	Activity (EU/mL)	Total activity	Protein (mg/mL)	Total protein (mg)	Specific activity (EU/mg of protein)	Yield (%)	Purification n-fold
Crude Extract	350	255.4	89401.4	0,882	308.7	289.57	100	0
Partially purified extract (40-80% $(NH_4)_2SO_4$ fractionation) (Redissolved and dialyzed)	190	462.5	87871.2	0.461	87.6	1003.25	98	3.5
Sephadex G-100 Fraction S_1	94	388.6	36530.2	0.101	9.5	3847.52	41	13.3
Fraction S_2	56	298.7	16727.2	0.091	5.09	3282.42	19	11.3
Fraction S_3	74	314.0	23236.0	0.124	9.18	2532.26	26	8.7

Characterization of Ankara pear PPO.

Enzyme characterization was performed with the dialyzed enzyme solution obtained from ammonium sulfate fractionation. The specific activity and protein concentration of the enzyme were 1003.25 U/mg and 0.461 mg/mL respectively.

Substrate Specificity. The results of the PPO substrate specificity study are shown in Table 2. The PPO from the Ankara pear showed higher affinity against diphenolic compounds than those of tri-hydroxy phenols like pyrogallol and gallic acid, and monophenols like p-cresol and D-tyrosine. Catechol was the best substrate of this enzyme. Similar results were also obtained by Oktay et al.[12] for the PPO of Amasya pear. Tate et al.[33] observed very high activity against o-diphenolic substrates of Bartlett pear PPO. Siddiq et al.[24], also demonstrated that 4-methyl catechol was oxidized much more rapidly by PPO of Stanley plums

than that of other substrates. Cash et al.[18] found only catecholase activity with concord grape PPO and no activity when monohydroxy phenols were used as substrates. Several other researchers have reported only activity against ortho-diphenolic compounds in other fruits[20,34,35,36]. The literature findings indicate that the PPO enzyme systems of most plants are specific for ortho-diphenolic substrates. However, Yue-Ming [37] has also found specificity for both diphenolic and triphenolic compounds.

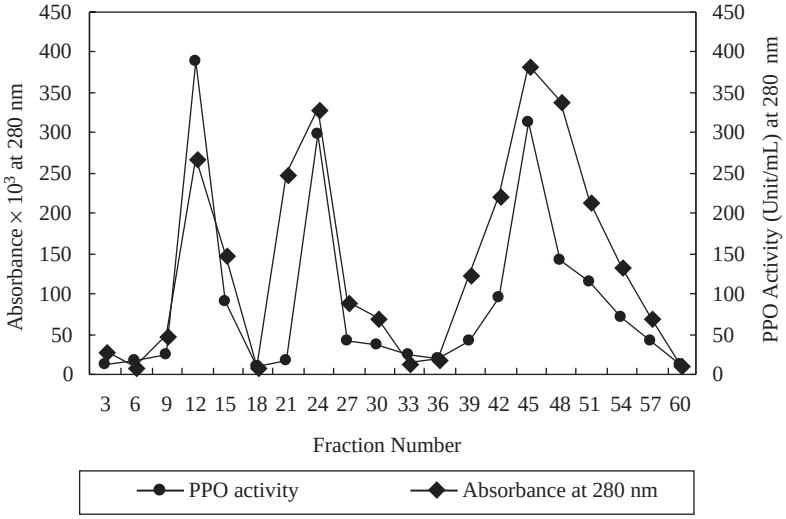

Figure 1. The gel filtration chromatography of the partially purified enzyme.

Table 2. Effect of phenolic substrates on PPO.

Substrate	Type of phenolic compound	Relative activity
Catechol	Dihydroxy	100
4-Methylcatechol	Dihydroxy	68.4
Chlorogenic acid	Dihydroxy	53.6
Caffeic acid*	Dihydroxy	50.0
L-Dopa	Dihydoxy	44.3
Gallic acid	Trihydroxy	20.1
Pyrogallol	Trihydroxy	12.5
p-Cresol	Monohydroxy	11.0
D-Tyrosine*	Monohydroxy	10.4

*Except for D-tyrosine and Caffeic acid (0.002 M) all substrates are 0.02 M.

Effect of pH and temperature. The optimal pH and temperature values of the PPO of the Ankara pear were estimated by using 7 different substrates and the results are shown in Table 3. In general, the PPO of most fruits shows maximum activity at neutral or near to neutral pH values[18,24,35,38,39]. The pH optima of PPO from the cherry [34], Clingstone and Halford peaches[35,40], banana[41] and longan fruit[42] were found to be between 6.2 and 7.2 but for the PPO of Lula avocado[43] the pH optimum was 4.7-4.8. Şakiroğlu et al.[44] reported that the optimum pH of pear PPO may vary between 5.8 and 6.4. The optimum pH of the PPO of different fruits may vary depending on the origin of material, extraction method, the maturity of the fruit and the substrate.[40] Ripened Ankara pears were used in this study. Consequently the optimal pH of enzyme

can change when it is extracted from fruits having different maturation stages. The optimal temperatures of Ankara pear PPO changed depending on the substrate used. Above 45 °C activity declined as temperature increased, but the enzyme was not completely inactivated even at 75 °C. Cash et al.[18] and Nakamura et al.[45] reported 25 °C and 30 °C optimal temperatures respectively for grape PPO. Furthermore, 37 °C was reported as the optimal temperature for peach PPO[10]. Guadarrama and Rivas[46] reported a temperature optimum of 35 °C for the PPO of cocoyam tubers.

Table 3. Optimum pH and temperature of the PPO from Ankara pear.

Substrate	Optimum pH	Optimum temp (°C)
Pyragallol	8.2	30
4-Methylcatechol	7.2	35
Catechol	7.0	20
D-tyrosine	5.6	55
Caffeic acid	5.6	25
p-Cresol	5.0	40
L-Dopa	4.8	45

Thermal Inactivation Kinetics. The results of the thermal inactivation of Ankara pear PPO at 60, 70, 75, 80 and 85 °C are presented in Figure 1. The enzyme showed a relatively high heat stability. The rate of heat inactivation was greater with increasing temperature and followed first order kinetics. The same kinetic order has also been shown for PPO from several plant sources. [39,47] The PPO of Clingstone peaches[35], Royal Ann cherries[34] and Bartlett pears[20] are characterized by a relatively high stability to heat.

The corresponding reaction rate constants at various temperatures were obtained by regression analysis of activity data (Figure 1a). An activation energy (Ea) of 15.38 kcal/mol for the heat inactivation of PPO was then estimated from the Arrhenius plot of Figure 1b. Park and Luh[25] determined that the Ea for the thermal inactivation of 2 PPO isoenzymes purified from kiwifruit was 4.0 and 7.0 kcal/mol, respectively.

The Arrhenius Ea was calculated to be 63.76 cal/mol from the slope of the ln k - 1/T plot (Figure 2b). Inactivation rate constants (k_i) and half-lives ($t_{1/2}$) of the PPO were determined (Table 4).

Table 4. Inactivation rate constants (k_i) and half-lives ($t_{1/2}$) of the PPO.

Temperature (°C)	k_i	$t_{1/2}$
60	1.11	0.63
70	1.83	0.38
75	2.8	0.25
80	5.15	0.14
85	9.95	0.07

Enzyme Kinetics. K_m and V_{max} values of Ankara pear PPO were calculated from the Linewear-Burk graphs and the results are shown in Table 4. As reported previously, Ankara pear PPO is primarily an o-diphenol oxidase, showing the highest activity towards 4-methyl catechol and catechol. Substrate concentration exhibited apparent Km values of 5.5 and 7.82 mM for catechol and 4-methyl catechol, respectively. The substrate affinity of PPO generally changes depending on the source of the enzyme. For example, Amasya apple PPO[12] has more affinity for 4-methylcatechol than for the other substrates. In an earlier work, Walker[32] reported a 1.6 mM $K_m$value for apple PPO with chlorogenic acid substrate. Guadarrama and Rivas[46] found 40 mM for the K_m value of the PPO of cocoyam tubers with catechol as substrate.

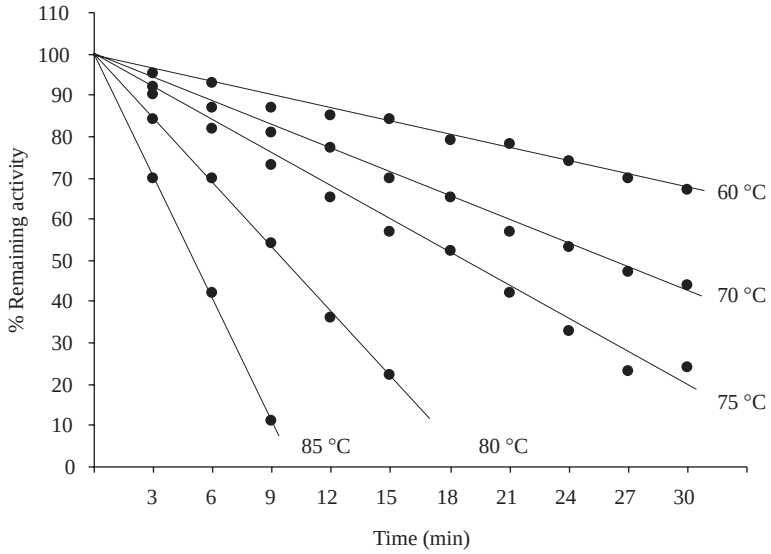

Figure 2a. Thermal inactivation of partially purified PPO at pH 7.2 in 10 mL 0.2 M phosphate buffer containing PPO enzyme.

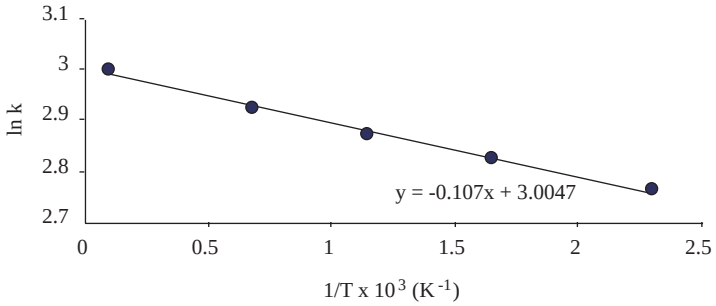

Figure 2b. Arrhenius plot for partially purified PPO at pH 7.2 in 10 mL 0.2 M phosphate buffer containing PPO enzyme.

Effect of Inhibitors. The type of inhibition and Ki values obtained for 7 different inhibitors of PPO are shown in Table 5. Sodium diethyldithiocarbamate was the strongest inhibitor of the Ankara pear PPO. L-cystein was reported as a strong inhibitor of apple PPO[13]. Şakiroğlu et al.[44] also showed that L-cystein and ascorbic acid are effective inhibitors of dog rose PPO. Cystein has also been suggested to prevent browning in processed fruit products[47]. Since L-cystein and ascorbic acid are naturally occurring substances and nontoxic, they may be useful in preventing the enzymatic browning of pear products.

Table 5. K_m and V_{max} values of the PPO.

Substrate	$K_{m,}$(mM)	$V_{max,}$(IU/mL.min)
Pyragallol	29.00	112.78
4-Methylcatechol	7.82	41.67
Catechol	5.55	344.50
D-tyrosine	32.67	97.15
Caffeic acid	16.00	124.25
p-Cresol	45.50	142.68
L-Dopa	24.50	298.72

Inhibitors like sodium diethyldithiocarbamate (DETC) and thiourea, which combine with copper in the enzyme, are generally potent inhibitors of PPO[48,49]. Knapp[43] reported that DETC and 1-phenyl 2-thiourea were inhibitors of Lula avocado PPO. These inhibitors are copper chelating agents and they suppress browning activities, thus supporting theories that copper is directly involved in the oxidation of phenolic compounds[50].

Table 6. K_i values and inhibition modes inhibitors.

Inhibitors	Concentration (M)	Average values of K_i (M)	Type of inhibition
L-Ascorbic acid	2.0×10^{-4}	7.5×10^{-11}	competitive
	2.5×10^{-4}		
Sodium azide	5.0×10^{-2}	3.47×10^{-9}	competitive
	1.5×10^{-1}		
L-Cysteine	6.5×10^{-5}	3.77×10^{-11}	competitive
	2.0×10^{-4}		
Sodium diethyl dithiocarbamate	2.5×10^{-5}	1.11×10^{-11}	competitive
Mercaptoethanol β	6.5×10^{-4}	1.18×10^{-3}	noncompetitive
Thiourea	1.5×10^{-1}	2.42×10^{-2}	noncompetitive

Storage Stability of Ankara Pear PPO. Partially purified PPO extracts were kept in a small Erlenmayer flask at 4 °C and 20 °C for 9 days. Samples were taken every day in order to determine the activity loss. The results are shown in Figure 2. PPO activity decreased sharply during the first 2 days at 20 °C. The decrease was slow in the subsequent days. Activity losses were lower at 4 °C than those at 20 °C. This indicates that the enzyme is more stable at low temperatures.

Electrophoresis. Three isoenzymes of Ankara pear PPO were obtained by polyacrylamide gel electrophoresis using catechol and pyrogallol substrate. Three isoenzymes were also obtained from gel filtration chromatography. Previous studies indicate that 3 PPO isoenzymes have been shown in apples, [12,51] pears [20] and cocoyam tubers[46]. Nondenaturing-PAGE band patterns of Ankara pear PPO isoenzymes are shown in Figure 4. Figure 4 (A and B) shows the nondenaturing electrophoretic pattern of Ankara pear PPO with catechol and pyrogallol as substrates.

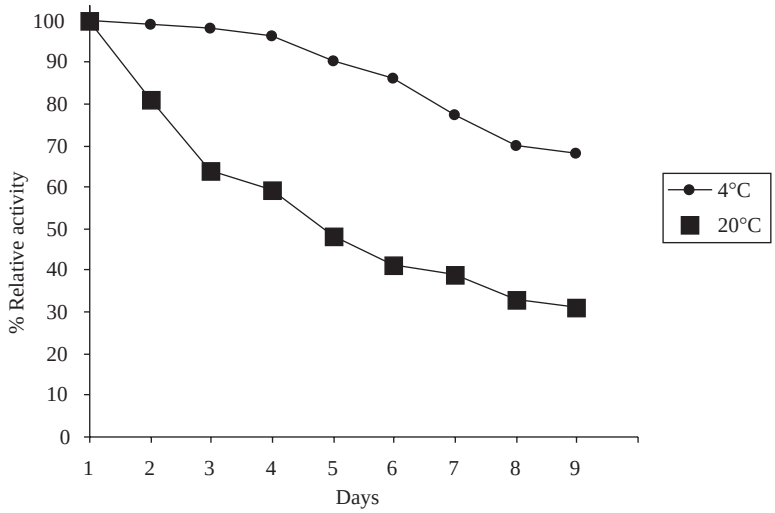

Figure 3. Stability of crude PPO enzyme during storage at pH 7.2 in 10 mL 0.2 M phosphate buffer containing PPO enzyme.

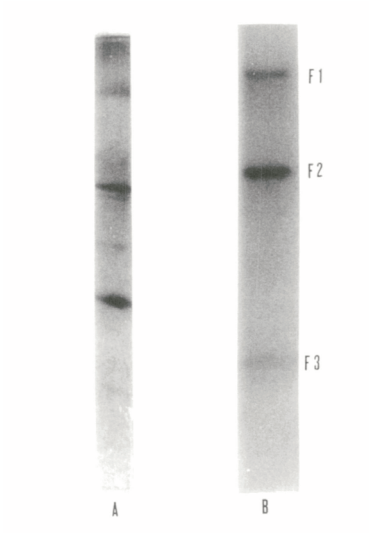

Figure 4. Nondenaturing electrophoretic pattern of Ankara pear PPO A-Catechol substrate. B-Pyrogallol substrate.

Three isoenzymes were determined by staining the PAGE gels with Coomassie brilliant blue R-250. The stained bands of isoenzymes was shown as F1, F2 and F3 in Figure 4. F1 and F2 bands appeared to be most active, followed by the moderately active band F3 for both substrates. Halim and Montgomery[47] reported 8 active bands for the isoenzymes of the PPO of d'Anjou pears. Constantinides and Bedford [53] showed that mushroom PPO was composed of 9 distinct isozymes.

The electrophoretic pattern of dialyzed Ankara pear PPO enzyme extract in denaturing conditions is presented in Figure 5. SDS-PAGE revealed 3 dominant bands with the molecular weights of 60, 40 and 28 kD (marked with arrows).

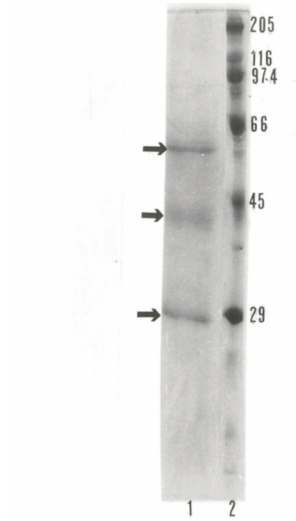

Figure 5. SDS-PAGE pattern of Ankara pear PPO. **Line 1:** Dialyzed Ankara pear PPO, **Line 2:** Line Molecular weight marker (Myosin 205 kDa, β-Galactosidase 116 kDa, Phosphorylase 97.4 kDa, Bovin Serum Albumin 66 kDa, Albumin Egg 45 kDa, Carbonic Anhydrase 29 kDa).

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