Purification and Characterization of Dog-rose (*Rosa dumalis* Rechst.) Polyphenol Oxidase

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Polyphenol oxidase (PPO) of dog-rose fruit was extracted and purified through $(NH_4)_2SO_4$ precipitation, dialysis, gel filtration, and DEAE-Sephacel ion-exchange chromatography. The sample obtained from ammonium sulfate precipitation and dialysis was used for characterization of the PPO. For this aim, optimum conditions, i.e., pH, temperature, and ionic strength, were determined with eight substrates. The best substrate of the PPO was found to be 4-methylcatechol. Optimum pH and temperature were found at pH 8.5 and 20 °C, and K_M and V_{max} values were 8.64 mM and 431.96 with 4-methylcatechol, respectively. Eleven inhibitors were tested in the study and the most effective was found to be sodium metabisulfide as competitive inhibitor. The PPO has showed renaturation property after it denatured, as well. Therefore, heat inactivation process for preventing enzymatic browning of dog-rose fruit products is not recommendable. Two isoenzymes of the PPO were detected by polyacrylamide slab gel electrophoresis.

Keywords: *Dog-rose fruit; Rosa dumalis Rechst.; polyphenol oxidase; kinetics; electrophoretic properties*

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INTRODUCTION

Dog-rose fruit in Rosaceae family has valuable importance because of existing vitamin C (Halasova and Jicinska, 1988; Halasova, 1988; Sojak and Hricovsky, 1986; Iskenderov and Ragimov, 1973). In addition, it contains carotene, B_1, B_2, E, and K vitamins, too. This fruit is also rich in terms of minerals.

Dog-rose fruit has been exploited in the cure of some diseases for centuries. For instance, cures of some inflammation diseases in the time of Hippocrates, of lung diseases in the Middle Ages, of kidney stones, and of chest pains are some uses of this fruit in medical treatments (User, 1967). Therefore, Russia and some European countries have been employing it in the food and medicine industries as a raw material because of its valuable properties. In these countries and in Turkey, this fruit is used in the production of marmalade, fruit juice, rose hip cream, and tea of dog-rose fruit. In addition, it is also employed as an additive product for fruit and vegetable juice which have a low level of ascorbic acid (Halasova and Jicinska, 1988; Halasova, 1988; Sojak and Hricovsky, 1986; User, 1967). This fruit has received more attention from growers because it has high yield, resistance against diseases and pest, hardiness, and easy propagation. All these make dog-rose very important, a fruit of future.

One other important point is that this fruit contains an enzyme called polyphenol oxidase (PPO). This is a major enzyme responsible for enzymatic browning by oxidizing some phenolic substrates to quinones (Mathew and Parpia, 1970; Mayer and Harel, 1979; Vamos-Vigyazo, 1981; Matheis, 1983; Mayer, 1987). PPO has been investigated in several fruits such as peach (Jen and Kahler, 1974; Flurkey and Jen, 1980), banana (Khan, 1985), grape (Cash et al., 1976; Valero et al., 1988; Lamikanra et al., 1992), pear (Halim and Montgomery, 1978; Wisseman and Montgomery, 1985), green olive (Ben-Shalom et al., 1977), kiwi (Park and Luh, 1985), strawberry (Ebelling and Montgomery, 1990), plum (Siddiq et al.,1992), and apple (Janovitz-Klapp et al., 1990; Keles, 1986; Oktay et al., 1995). However, we have not encountered any article about PPO of dog-rose fruit, even though it is necessary to characterize dog-rose PPO for controlling enzymatic browning of fruit products. Therefore, in this work, isolation, and purification of the PPO and its characterization have been studied.

EXPERIMENTAL PROCEDURES

Plant Material. Fruits of dog-rose (*Rosa dumalis* Rechst.) have been used as research material. The fruits of the species are bigger and fleshier than that of other species. Therefore, these are preferred in the manufacture fruit juice and marmalade and, also, for fresh fruit consumption. Fully mature fruits of the dog-rose plants grown in the campus of Atatürk University were harvested in September and stored at 4 °C until used in the study.

Enzyme Extraction and Purification. For preparing the crude extract, 20 g of the fruit was homogenized in 100 mL of 0.5 M phosphate buffer (pH 7.3) containing 0.5% polyethylene glycol and 10 mM ascorbic acid by using Waring blender for 2 min. The crude extract was filtered and the filtrate was centrifuged at 48 000*g* for 1 h at 5 °C. The supernatant was brought to 80% $(NH_4)_2SO_4$ saturation with solid $(NH_4)_2SO_4$. The precipitated PPO was separated by centrifugation at 48 000*g* for 1 h. The precipitate was dissolved in a small amount of 5 mM phosphate buffer (pH 6.3) and dialyzed at 4 °C in the same buffer for 24 h with three changes of the buffer during dialysis.

For further purification of PPO, the dialyzed enzyme aliquot was fractionated by both gel filtration and DEAE-Sephacel ion-

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Table 1. Purification of Polyphenol Oxidase from Dog-rose Fruit

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	460			0.59	271.4			
$(NH_4)_2SO_4$ precipitation and dialysis	130	755	98150	0.98	127.4	770.4	100	0
gel filtration chromatography (first fraction)	637	76.5	48730	0.07	44.6	1092.8	49.6	1.4
gel filtration chromatography (second fraction)	1106	42.5	47005	0.04	44.2	1062.5	48.6	1.3
ion-exchange chromatography (first fraction)	9424	5.0	47120	0.004	37.6	1250.0	48.0	1.6
ion-exchange chromatography (second fraction)	9730	4.4	42812	0.003	29.2	1466.6	43.6	1.9

exchange chromatography methods. In gel filtration chromatography, a column with 100 mL bed volume was prepared using Sephadex G-100 and equilibrated with 0.1 M phosphate buffer (pH 6.3) containing 10% glycerin. The dialyzed enzyme solution was then passed through the column. The elution rate was adjusted to 15−20 mL/h.

The eluates were collected as 4 mL volumes in tubes by a fraction collector. Elution process was continued until ∼0 absorbance at 280 nm was obtained. Qualitative protein determination was done at 280 nm on the obtained eluates, and PPO activity was measured in eluates showing absorbance at 280 nm. Thus, the obtained values were graphed against the tube number. The fractions having PPO activity were collected and purification degrees were determined by measuring specific activity before and after purification. For determining specific activity, PPO activity and quantitative protein measurements were carried out. Protein contents were determined by the protein dye binding method (Bradford, 1976).

In ion-exchange chromatography, two fractions obtained from gel filtration were combined and applied to a DEAE-Sephacel column (1.2 cm^2 × 70 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 10). The elution rate was adjusted to 10−15 mL/h by the aid of a peristaltic pump. The elution process was performed in the presence of a decreasing pH gradient in range of pH 10 to 4. The other steps of the ion-exchange chromatography were continued by performing the same steps as indicated in the previous paragraph.

Determination of PPO Activity. PPO activity was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (LKB Biochrom Ultraspec II). The sample cuvette contained 0.1 mL of the enzyme and 2.9 mL of substrate solution in various concentrations. The blank sample contained only 3 mL of substrate solution. The reaction was carried out at various temperatures and pH values with the substrates mentioned as follows. PPO activity was calculated from the linear portion of the curve (Wong et al., 1971). One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001/min.

Characterization of PPO. *(a) Effect of pH.* PPO activity was determined with eight different substrates (catechol, 4-methylcatechol, L-dopa, dopamine, gallic acid, pyrogallol, L-tyrosine, and *p*-cresol) at a concentration of 10 mM, only L-tyrosine, 5 mM. Appropriate buffers (0.1 M citrate/0.2 M phosphate for pH 4.0−5.5, 0.2 M phosphate for pH 5.5−7.0, and 0.2 M Tris-HCl for pH 7.0−10.0) were used for determining optimum pH of PPO. The optimum pH values obtained from this assay were used in all the other experiments.

PPO activities were measured with various buffers [0.1 M citrate/0.2 M phosphate (pH 5.0), 0.1 M phosphate (pH 6.2 and 7.0), and 0.2 M Tris-HCl (pH 8.3)] to determine the PPO's stability at different pH values. The activity measurements were performed with varying day intervals of days using 4-methylcatechol substrate in optimum conditions.

(b) Effect of Temperature. For determining optimum temperature values of the enzyme, PPO activity was measured at different temperatures in the range from 5 to 85 °C using the eight substrates indicated above. The desired temperatures were provided by using an ice bath for temperatures under 20 °C and a constant temperature circulator for the temperatures above 20 °C.

(c) Heat Stability. The denaturation property of the enzyme was determined by measuring PPO activity at different temperatures in range from 50 to 90 °C for 1 h duration at certain intervals using 4-methylcatechol. Renaturation property of the enzyme was investigated by measuring the activity with the certain intervals at lowered temperature to 22 °C from the studied temperatures indicated above.

(d) Effect of Ionic Strength. Ionic strength effect on the enzyme was studied with 0.01 M substrate concentration using different concentrations of the buffers.

(e) Enzyme Kinetics. For determination of Michaelis constant (K_M) and maximum velocity (V_{max}) values of the enzyme, PPO activities were measured with the eight substrates at varying concentrations (0.1, 0.2, 0.5, 1.0, and 2.5 mM for *p*-cresol; 0.15, 0.25, 0.50, 1.0, 2.5, and 5.0 mM for L-tyrosine; and 1.0, 2.5, 5.0, 7.5, 10.0 and 15.0 mM for the other six substrates) in optimum conditions of pH, ionic strength, and temperature. K_M and V_{max} values of PPO for each substrate were calculated from a plot of $1/V$ vs $1/[S]$ by the method of Lineweaver and Burk (Lineweaver and Burk, 1934).

(f) Effect of Inhibitors. Inhibitor effects on PPO activity were determined by using the following inhibitors: sodium metabisulfite, L-cysteine, L-ascorbic acid, glutathione, thiourea, sodium azide, sodium diethyl dithiocarbamate (DIECA), dithioerithritol, tannic acid, benzoic acid, and β-mercaptoethanol at five different concentrations of inhibitors with 10 mM 4-methylcatechol substrate at 20 °C and pH 8.5. Percent activity graphs were drawn from these results to find both I_{50} values and three constant inhibitor concentrations which show about 50% inhibition effect. Later, using five different concentrations of the substrates, PPO activities were measured at these three constant inhibitor concentrations with the inhibitors indicated above. $1/V$ and $1/[S]$ values obtained from these activity measurements were used for drawing Lineweaver−Burk graphs. Finally K_i constant values were found from the graphs.

(g) Electrophoresis. Polyacrylamide slab gel electrophoresis was performed according to the method of Laemmli (Laemmli, 1970) for separating PPO isoenzymes of dog-rose fruit in natural conditions. The experiment was conducted with the electrode buffer tris/glycine (pH 8.3) using 3% stacking gel and

Table 2. Substrate Specificity of Dog-rose PPO

substrate	concentration (M)	activity (units/mL)
catechol	0.01	49.00
4-methylcatechol	0.01	207.50
L-dopa	0.01	45.40
dopamine	0.01	70.00
gallic acid	0.01	86.50
pyrogallol	0.01	90.09
L-tyrosine	0.0025	100.00
p-cresol	0.0025	41.32

Table 3. Optimum pH and Temperature, and K_M and V_{max} Values of the PPO

substrate	optimum pH	optimum temp (°C)	K_M (M)	V_{max} (EU/mL min)
catechol	8.5	25	7.41×10^{-3}	41.66
4-methylcatechol	8.5	20	8.64×10^{-3}	431.96
L-dopa	8.0	45	6.06×10^{-4}	45.45
dopamine	8.5	45	2.82×10^{-3}	42.48
gallic acid	8.8	35	2.64×10^{-3}	111.11
pyrogallol	7.0	15	2.79×10^{-3}	111.73
L-tyrosine	7.0	65	8.27×10^{-4}	142.85
p-cresol	5.0	60	8.63×10^{-5}	40.00

Table 4. K_i Values and Inhibition Modes for 11 Inhibitors

inhibitors	[I](M)	K_i (M)	average values of K_i (M)	type of inhibition
sodium metabisulfide	1.5×10^{-5}	6.01×10^{-9}		
	3.0×10^{-5}	6.12×10^{-9}	5.19×10^{-9}	competitive
	4.5×10^{-5}	3.44×10^{-9}		
L-cysteine	7.0×10^{-5}	5.28×10^{-8}		
	1.0×10^{-4}	4.76×10^{-8}	5.79×10^{-8}	competitive
	2.5×10^{-4}	7.35×10^{-8}		
ascorbic acid	2.0×10^{-4}	9.89×10^{-8}		
	2.5×10^{-4}	9.67×10^{-8}	9.83×10^{-8}	competitive
	3.0×10^{-4}	9.95×10^{-8}		
glutathion	8.0×10^{-5}	3.64×10^{-8}		
	1.5×10^{-4}	3.59×10^{-8}	3.67×10^{-8}	competitive
	3.0×10^{-4}	3.80×10^{-8}		
thiourea	5.0×10^{-2}	2.80×10^{-5}		
	1.0×10^{-1}	3.84×10^{-5}	3.52×10^{-5}	competitive
	1.5×10^{-1}	3.92×10^{-5}		
sodium azide	6.0×10^{-2}	3.98×10^{-5}		
	1.0×10^{-1}	5.01×10^{-5}	5.77×10^{-5}	competitive
	2.0×10^{-1}	8.33×10^{-5}		
sodium diethyl dithiocarbamate	2.0×10^{-5}	4.35×10^{-5}		
	4.0×10^{-5}	4.15×10^{-5}	4.35×10^{-5}	uncompetitive
	6.0×10^{-5}	4.56×10^{-5}		
dithioerythritol	2.0×10^{-5}	1.29×10^{-8}		
	4.0×10^{-5}	1.88×10^{-8}	1.53×10^{-8}	competitive
	8.0×10^{-5}	1.44×10^{-8}		
tannic acid	7.5×10^{-6}	4.89×10^{-9}		
	1.5×10^{-5}	6.62×10^{-9}	7.28×10^{-9}	competitive
	3.0×10^{-5}	10.33×10^{-9}		
benzoic acid	1.0×10^{-1}	9.26×10^{-2}		
	2.0×10^{-1}	1.17×10^{-1}	1.21×10^{-1}	noncompetitive
	3.0×10^{-1}	1.56×10^{-1}		
β-mercaptoethanol	2.5×10^{-5}	2.67×10^{-4}		
	5.0×10^{-5}	2.47×10^{-4}	2.38×10^{-4}	noncompetitive
	7.5×10^{-5}	2.01×10^{-4}		

10% separating gel. The enzyme samples obtained after from ammonium sulfate precipitation and dialysis were loaded on to the each space of the stacking gel at 0.1 mL. Initially, an electric current of 80 v was applied until the bromphenol dye had reached into the separating gel. It was then increased to 150 V for 5−6 h until the tracking dye migrated to 1−2 cm from the bottom. Electrophoresis was carried out in a cold room at 4 °C. After completion of the run, the slab gel was cut into the proper parts according to the applied sample. Gel parts were, then, immersed separately in 15 mM catechol solutions containing 0.05% *o*-phenylenediamine prepared in 0.2 M tris buffer (pH 8.5). After 1−1.5 h of incubation of the gels, isoenzyme bands were developed. The gels were shaken in 1 mM ascorbic acid solution for 5 min and stored in 30% ethanol and then their photographs were taken.

RESULTS AND DISCUSSION

Extraction and Purification of PPO. Extraction of PPO was carried out in 0.5 M phosphate buffer pH 7.3, containing 0.5% polyethylene glycol and 10 mM ascorbic acid and then precipitated by $(NH_4)_2SO_4$ method. Several precipitations with solid $(NH_4)_2SO_4$ between 0−20%, 20−40%, 40−60%, 60−80% and 80−90% were tested to find the proper saturation point. As a result, PPO activity of the precipitate of 80 % $(NH_4)_2SO_4$ saturation was found the highest, and this saturation point was used all the extraction processes. Polyethylene glycol was used during extraction to bind the phenols which may inactivate the PPO. It is well documented that oxidation of phenolics by PPO produces quinones which would inhibit PPO (Walker, 1964). Therefore, ascorbic acid was, also, used to reduce quinones to phenolic substrates during extraction.

After ammonium sulfate precipitation, the dialyzed enzyme extract was used for purification of the PPO.

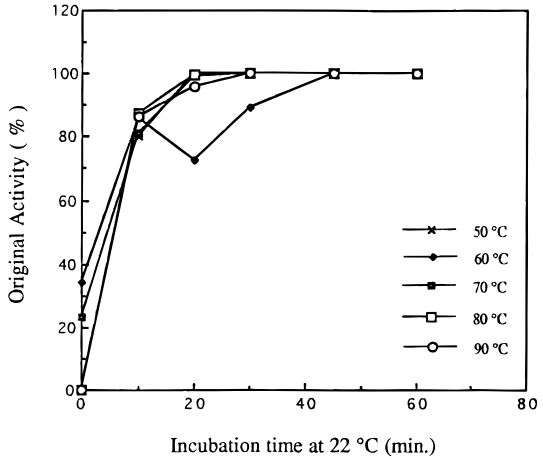


Figure 1. Renaturation property of the PPO denatured at different temperatures.

Results on purification of the PPO are shown in Table 1. PPO activity of crude extract is not shown. Since we could not measure the PPO activity in crude extract, it is not presented in Table 1. The reason for this is the existence of abundant ascorbic acid in the extract which may inhibits PPO. Therefore, it was possible to compare the yield and purification degrees of PPO with crude extract. The purification degrees of PPO were found 1.3−1.4-fold after gel filtration and 1.6−1.9-fold after ion-exchange chromatography, showing that dog-rose PPO has two isoenzymes.

Characterization of Dog-rose PPO. The enzyme samples obtained from ammonium sulfate precipitation and dialysis were used in characterization of the PPO.

Because the activity in eluates of the both chromatography was quite low that the characterization was not possible.

(a) Substrate Specificity. Dog-rose PPO has showed activity with all of the phenolic substrates used in the study. The substrate with the highest activity was found 4-methylcatechol but the poorest was *p*-cresol (Table 2).

(b) Effect of pH. PP0 activities were measured with the eight substrates to determine pH optimum for each substrate (Table 3). Maximum PPO activities were recorded at pH 8.8 for gallic acid; pH 8.5 for catechol, 4-methylcatechol, and dopamine; pH 8.0 for L-dopa; pH 7.0 for pyrogallol and L-tyrosine; and pH 5.0 for *p*-cresol. Overripe fruits of dog-rose were used in this study. If activity is measured in earlier stages of maturation, it is likely possible that the pH optima will be change. This case has been reported for peaches (Luh and Phithakpol, 1972).

Stability of the enzyme at different pH (5.2, 6.2, 7.0, and 8.3) was, also, investigated using 4-methylcatechol. PPO was more stable at pH 6.2 than other at pH values.

(c) Effect of Temperature. The effects of temperatures between 15 and 80 °C on PPO activity were assayed and optimum temperatures with the each substrate are showed in Table 3. As seen in the table, optimum temperatures are quite substrate dependent and an interesting result is that with monohydroxyphenolic substrates (L-tyrosine and *p*-cresol) maximum PPO activity at higher temperatures 65 and 60 °C, respectively, have been shown whereas with the other substrates, the temperatures were 45 °C or lower.

Heat stability of the PPO was also investigated between 50 and 90 °C with a 60 min duration using 4-methylcatechol as substrate. A decrease in PPO activity of 20%, 67%, 78%, and 100% was found at 50, 60, 70, and 80 °C, respectively. The enzyme was completely inactivated at 90 °C after 30 min.

On the other hand, renaturation property of the PPO was also studied by lowering the temperatures to 22 °C. When the denatured enzyme had incubated at 22 °C for a 10−60 min duration, it recovered activity again (Figure 1). Thus, dog-rose PPO shows renaturation property. This is an undesirable case from point of food processing. Therefore, we do not recommend the heat inactivation method for preventing enzymatic browning of dog-rose fruit products.

(d) Enzyme Kinetics. K_M and V_{max} values calculated from the Lineweaver−Burk graphs were shown in Table 3. As seen in the table, the PPO shows the greatest affinity for L-dopa among the dihydroxyphenols and *p*-cresol among the monohydroxyphenols. Substrate affinity of PPO, generally, changes depending on the obtained source. For example, PPO has more affinity for 4-methylcatechol than for the other substrates in Amasya apple (Oktay et al., 1995). V_{max} values of dog-rose PPO indicate that the PPO is most effective on 4-methylcatechol (Table 3). Thus, the PPO activity with 4-methylcatechol is 10-fold higher than with catechol found in this study. The result is similar to the work on strawberry PPO (Wesche-Ebelling and Montgomery, 1990).

(e) Effect of Inhibitors. K_i values and inhibition modes for 11 inhibitors used in the study are shown in Table 4. From the K_i constants, it has been concluded that inhibition modes of the inhibitors are as follows: sodium diethyl dithiocarbamate, uncompetitive; benzoic acid and β-mercaptoethanol, noncompetitive, and the others, competitive. The most strong inhibitor was found to be sodium metabisulfite. On the other hand, inhibition effects of sodium chloride, NaEDTA, ethylene glycol, and thiodiglycol were, also, studied but their effects were found to be weaker than the inhibitors shown in Table 4, even sodium chloride showed activator effect (Şakiroğlu, 1994).

(f) Electrophoresis. Two isoenzymes of dog-rose PPO were obtained by polyacrylamide slab gel electrophoresis using 4-methylcatechol substrate. Two isoenzymes were obtained from both ammonium sulfate precipitation and gel filtration chromatography samples. The existence of two peaks in gel filtration chromatography proves this result, as well. Generally, 2−3 PPO isoenzymes have been detected in fruits, such as apple (Constantinides et al., 1967), pear (Rivas and Whitaker, 1973), cherry (Pifferi and Cultrera, 1974), and banana (Palmer, 1963). Since, there is no research on dog-rose PPO isoenzymes, it is not possible to compare our results, but there are similarities with previous studies on other fruits indicated above.

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