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EPR Spectroscopy of Dog-Rose Polyphenoloxidase

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Polyphenoloxidase(PPO) of dog-rose fruit was extracted and purified through $(NH_4)_2SO_4$ precipitation and dialysis. The optimum conditions for this purpose, i.e., pH and temperature, were determined with 4-methylcatechol optimum pH and temperature and found to be pH 8.5 and 20° C, respectively. The inhibition effect of ascorbic acid was investigated and the I_{50} value was found to be 1.57×10^{-4} M. The activity of dog-rose PPO was investigated by electron paramagnetic resonance (EPR) at different pH levels and temperatures. The optimum conditions obtained from both spectrophotometry and EPR were compared.

Introduction

Enzymatic browning catalysed by PPO occurs when plant tissues are damaged, and this causes an economic problem for both processors and consumers. The most important factor in enzymatic browning is the oxidation of phenolic compounds to corresponding quinones by PPO in the presence of oxygen. The quinones then condense to form darkened pigments[1].

PPO is a copper-containing enzyme widely distributed in plants[2,3]. It is involved in enzymatic browning in several plant tissues, including potato tubers[4,5,6,7], peaches[8,9], bananas[10], grapes[11,12,13], pears[14,15], kiwis[16], strawberries[17], plums[18], and apples[19,20,21,22]. PPO from different plant tissues shows different substrate specificities and degrees of inhibition[23,24]. Kinetic study and isoenzyme determination of dog-rose PPO were carried out for the first time by Şakiroğlu et al. The results of PPO of dog-rose were different from those of PPO from other sources. other sources[24].

Ascorbic acid is widely used to inhibit the enzymatic browning of fruits and vegetables because it is nontoxic at the levels employed. The inhibition of PPO by ascorbic acid is complex. Inhibition of brown colour formation in the reaction of mushroom PPO and o-dihydroxyphenols has been demonstrated previously[25,26]. The mechanism of ascorbic acid inhibition has generally been attributed to the reduction of o-quinone back to the phenolic substrate[27]. It has been suggested that ascorbic acid neither inhibits nor activates the enzyme. In constrast to this, other reports[28,29] suggest that there is a direct interaction between ascorbic acid and PPO. The mechanism of inactivation is not clear from these results. A K-type interaction of PPO with ascorbic acid was reported[30]. With this type of inhibition the product of the

reactions reacts with the enzyme to form a covalent enzyme derivative which is inactive.

Although ascorbic acid is the first choice for anti-browning in fresh fruits and vegetables, its instability leads us to investigate other acids that may have greater stability and increased effectiveness in preventing browning.

In the course of these investigations, electron paramagnetic resonance spectroscopy (EPR) was utilised to establish the mechanism for that aspect of the inactivation of ascorbic acid on PPO of dog-rose. The EPR spectra of PPO extracted from different fruits and vegetables have been investigated[23,26,31]. Hsu and et al. found that Cu^{2+} of PPO was reduced to Cu^{+1} by ascorbic acid. In this study, PPO of dog-rose was extracted and purified, and its EPR spectra and inhibition by ascorbic acid were investigated for the first time. In addition, the effects of various pH and temperature on PPO of dog-rose activity were examined, and the effectiveness of these on the inhibition of PPO of dog-rose was determined by EPR.

Experimental Procedure

Plant Material

Dog-rose fruits (Rosa dumalis rechst.) were used as research materials. The fruit of the species is bigger and fleshier than that of other species. Fully mature fruits of dog-rose plants were grown on campus at Atatürk University, harvested in September, and stored at 4° C until their use in the study.

Enzyme Extraction

For preparation of crude extract, 20 g of the fruit was homogenised in 100 ml of 0.5M phosphate buffer (pH 7.3) containing 0.5 % polpethylene glycole and 10 mM ascorbic acid with a Waring blender for two minutes. 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.

Assay of PPO Activity

PPO activity was determined with a spectrophotometer (Pharmacia LKB Ultraspec III) measuring the absorbance at 420 nm. The sample cuvette contained 0.1 ml of the enzyme and 2.9 ml 4-methylcatechol solution in 10 mM concentraiton. The blank sample contained only 3 ml of substrate solution. The reaction was carried out at various temperatures and pH values with the substrate. PPO activity was defined as the amount of enzyme that caused an increase of 0.001/min. in absorbance.

a) Effect of pH

PPO activity was determined with 4-methylcatechol substrate. 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 pH optima of PPO (Figure 1).

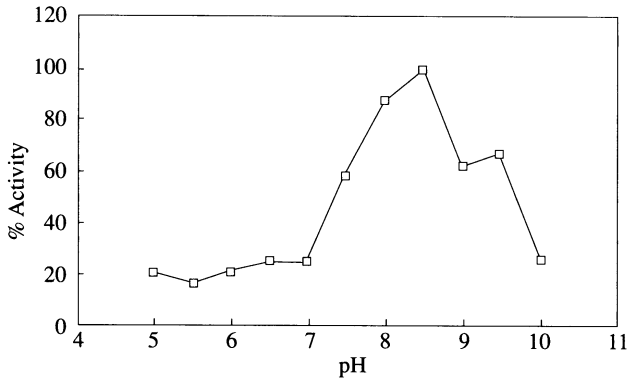

Figure 1. Activity % graph according to pH of dog-rose PPO

For determining the optimum pH values of the enzyme, PPO activity was studied at different pH levels in a range from pH 7.5 to pH 9.5 using by EPR (Figure 2a,2b,2c).

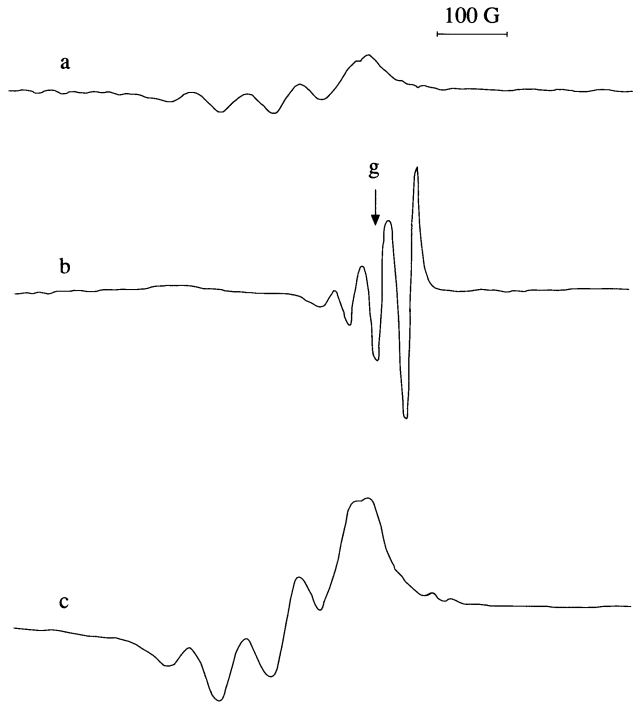

Figure 2. The EPR spectra of dog-rose polyphenoloxidase in the presence of 4-methyl catechol at 20° C and pH. a) 7.5, b) 8.5, c) 9.5

Effect of Temperature

In order to determine the optimum temperature values of the enzyme, PPO activity was measured at different temperatures in a range from 5° C to 85° C by spectrophotometry (Figure 3). EPR spectra were taken in a range from 20° C to 60° C (Figure 4a, 4b, 4c, 4d).

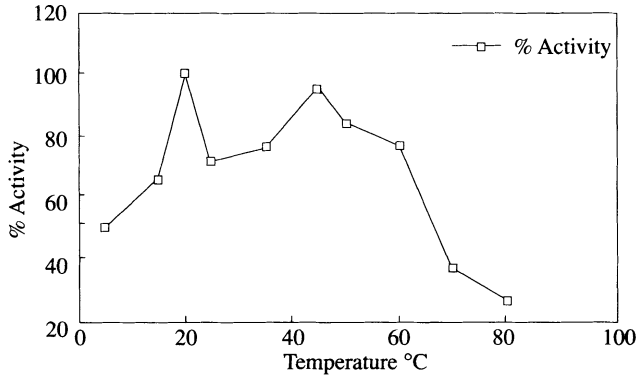

Figure 3. Activity % graph according to temperature of dog-rose PPO

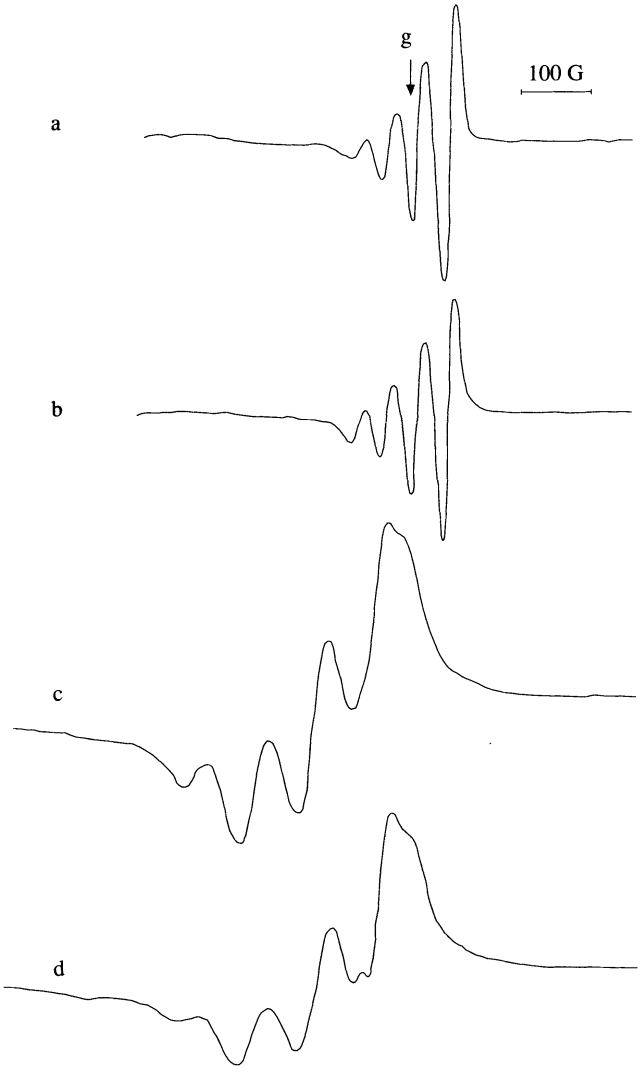

Figure 4. The EPR spectra of dog-rose PPO in the presence of4-methyl catechol at pH 8.5 and at. a) 20 °C, b) 45 °C, c) 55 °C, d) 60 °C.

c) Inhibition Effect of Ascorbic Acid

Inhibitor effects on PPO activity were determined through the use of ascorbic acid at five different concentrations of inhibitors with a 10 mM 4-methylcatechol substrate. Percent activity graphs were drawn from these results to find the I_{50} value (Figure 5).

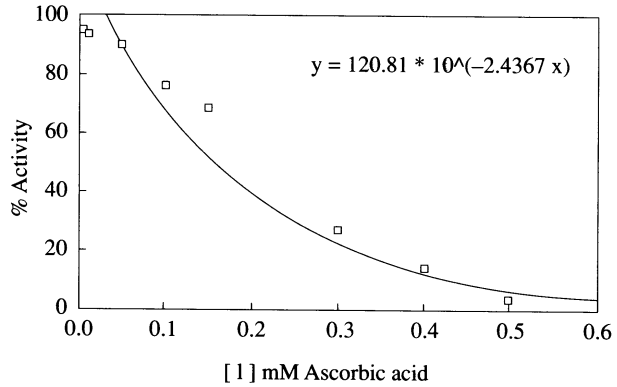

Figure 5. Activity % graph according to ascorbic acid of dog-rose PPO

EPR Measurements

A Varian E-104 spectrometer was used to record spectra at 9 GHz (X band). The spectrometer was equipped with an E-257 variable temperature accessory unit and the temperature was maintained within $\pm 1\,^{\circ}C$ by heating precooled nitrogen gas which was passed through the Dewar assembly placed in a E-231 cavity operating at 9.51 GHz. A copper-constantan thermocouple was used to monitor cavity temperature throughout the runs.

For spectra in which the line widths were quite narrow, the modulation amplitude was varied so that the transitions were not artifically broadened by over-modulation. The microwave power was controlled to avoid saturation of the individual spectral lines. The gain and filtering controls were varied to produce the best possible spectra.

The EPR spectrometer settings were as follows: Microwave power, 100 mw; frequency, 9.51 GHz; modulation amplitude, 40 G; time constant, 1.0 s; scan time, 2 min with 32 scans accumulated.

Interpretation of Cu^{2+} EPR Spectra

PPO enzyme spectra would be consistent with a structure in which Cu^{2+} forms three strong bonds to the protein[31]. One might expect that this spectrum (and such a structure) could be altered by the binding of inhibitors. The spectrum is composed of resonance signals arising from one copper ion bond to PPO.

Cu^{2+} has a $3d^9$ electronic configuration and the values S = 1/2 and I = 3/2. Because the allowed EPR transitions involve a change of $\Delta Ms = \pm 1$ $\Delta MS = 0$, a solution EPR spectrum of four hyperfine lines can be observed, where m_S and m_I are the quantum numbers for the components in the magnetic field direction of the electron and nuclear spin angular momentum, respectively.

Results and Discussion

The EPR spectra of PPO of dog-rose are markedly pH-dependent (Figure 2a, 2b, 2c) and characterized by EPR spectrum changes in line intensity, line width, line position (g) and line splitting (A). Figure 2b shows a mobile solution spectrum of Cu^{2+} while the others (Figure 2a, 2c) show its immobile solution spectra. This is in agreement with the optimum pH of 8.5 obtained by spectrophotometric measurements in a previous study of ours (Figure 1)[24]. Therefore, 4-methylcatechol was taken as a substrate. The optimum pH and temperature were also found to be 8.5 and $20\,^{\circ}C$ (Figure 3), respectively, for PPO of dog-rose in this study. Although the optimum pH depends on the substrate used for the experiments, it is interesting that the

optimum pH for PPO of dog-rose was found to be high compared to the PPO of other fruits. However, it was reported that plum PPO was still active at a pH as high as 8.0[32]. The temperature dependence of the EPR spectra of Cu^{2+} in PPO of dog rose (Figure 4a, 4b, 4c, 4d) gives an optimum temperature of 20°C, which is the same as the optimum temperature obtained by spectrophotometric measurements (Figure 3). As seen in these spectra, as the temperature increases, the spectra turn to immobile solution spectra of Cu^{2+} because the activity decreases. The obtained g and A value for mobile solution spectrum (at optimum temperature and pH) of Cu^{2+} in PPO of dog-rose approximately 2.150 and 40 G, respectively.

Figure 6 shows the EPR spectra of PPO of dog-rose in the presence of different ascorbic acid concentrations and in a sodium phosphate buffer. It can be seen from these spectra that the Cu^{2+} signal intensity decreases as ascorbic acid concentration increases because of the inhibition increase[31]. It disappears altogether in the sodium phosphate buffer alone (Figure 6c).

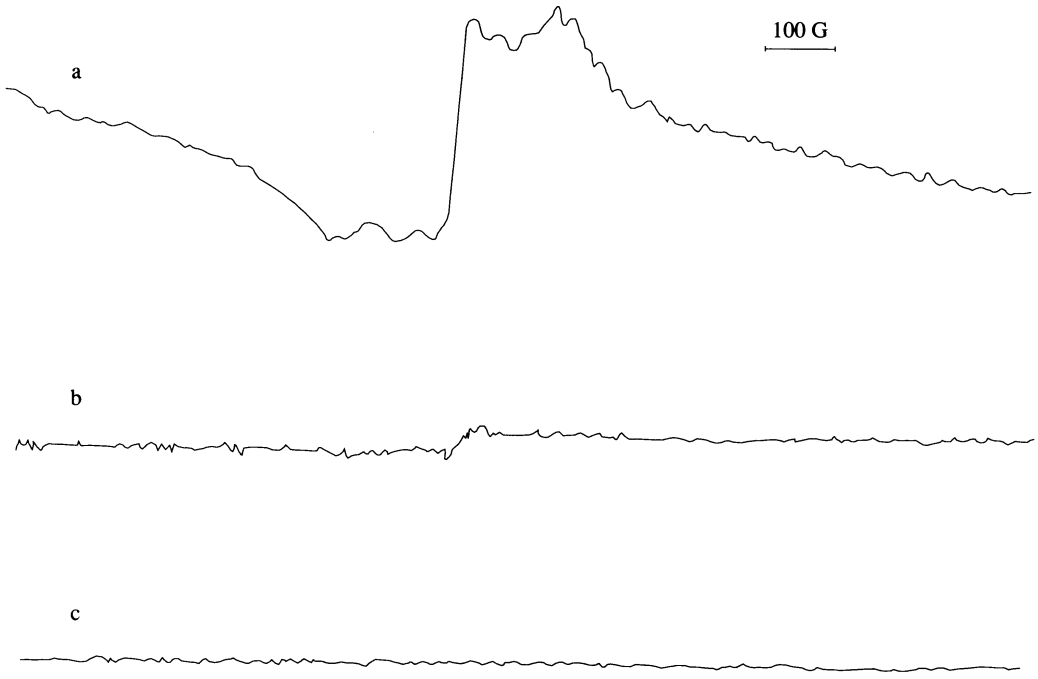

Figure 6. EPR spectra of dog-rose PPO in the presence of ascorbic acid at pH 8.5; a) 0.075 mM ascorbic acid (1.32×10^{-4} g/10 ml), b) 0.6 mM ascorbic acid (1.056×10^{-3} g/10ml), c) sodium phosphate buffer 55 mM, pH 6.3) alone.

The pH and temperature dependence of the EPR spectra of PPO of dog-rose show that the optimum temperature is 20°C and the optimum pH value is 8.5. The optimum conditions obtained from spectrophotometric measurements are in agreement with the EPR results.

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