Polyphenoloxidase from Amasya Apple

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ABSTRACT

Polyphenoloxidase (PPO) of Amasya apple was partially purified by $(NH_4)_2SO_4$ precipitation and dialysis. The sample was used for characterization of the PPO. Optimum pH were 7.0, 9.0, 8.6 and 6.6 on substrates catechol, 4-methyl catechol, pyrogallol and L-dopa respectively. Catechol was the most suitable for Amasya apple PPO. The optimum temperature for maximum PPO activity was 18°C with catechol. Of seven inhibitors tested, the strongest was L-cysteine. Effectiveness of inhibitors increased in the order: thiourea, glutathione, β-mercaptoethanol, sodium cyanide, ascorbic acid, sodium metabisulfide, and L-cysteine. The K_M was 34 mM of catechol. The activation energy with catechol was 107 cal/mol. In electrophoretic separation, three isoenzymes were detected with both catechol and L-dopa substrates.

Key Words: Amasya apple, polyphenoloxidase, isozymes, physical stability

INTRODUCTION

ENZYMATIC BROWNING catalyzed by PPO occurs when plant tissues are damaged, and is an economic problem for processors and consumers. The main step 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 (Matheis, 1983).

PPO is a copper-containing enzyme widely distributed in plants (Mayer and Harel, 1979; Mayer, 1987). It has been related to enzymatic browning in several plant tissues, including potato tubers (Patil and Zucker, 1965; Matheis and Belitz, 1978; Walter and Purcell, 1980; Kocaçalişkan and Özbay, 1987) peaches (Jen and Kahler; 1974; Flurkey and Jen, 1980), bananas (Kahn, 1985), grapes (Cash et al., 1976; Valero et al., 1988; Lamikanra et al., 1992), pears (Halim and Montgomery, 1978; Wissemann and Montgomery, 1985), green olives (Ben-Shalom et al., 1977), kiwis (Park and Luh, 1985), strawberries (Ebeling and Montgomery, 1990), plums (Siddiq et al., 1992) and apples (Janovitz-Klapp et al., 1977; Keles, 1986; Coseteng and Lee, 1987). Little has been reported about the Amasya apple PPO. We observed that Amasya apple has one of the highest rates of enzymatic browning among several apple cultivars. PPO from different plant tissues shows different substrate specificities and degrees of inhibition (Vamos-Vigyazo, 1981). Therefore, characterization of the enzyme could help to develop more effective methods in controlling browning of Amasya apples and products. Our objective was to isolate PPO from Amasya apple and study characteristics of the enzyme at different pH and temperatures. Substrate and inhibitor effects were also studied.

MATERIALS & METHODS

Isolation of PPO

Amasya apples (*Malus sylvestris* Miller cv. Amasya) were harvested fresh (in Tortum, Turkey) and stored at 4°C. For preparing the crude extract, 100g of apple tissue was cut quickly into thin slices and homogenized with 200 mL of chilled 0.1M phosphate buffer (pH 7) containing 10 mM ascorbic acid and 0.5% PVP in a Waring Blender for 2 min. The homogenate was filtered and the filtrate was centrifuged at 20,000 × *g* for 30 min 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 20,000 × g for 30 min. The precipitate was dissolved in a small amount of homogenization buffer and dialyzed at 4°C in the same buffer for 3 hr with three changes of buffer during dialysis. The dialyzed sample was used as the PPO enzyme source in the following experiments.

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Assay of PPO activity

PPO activity was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (LKB-Biochrom). The sample cuvette contained 2.9 mL of substrate in various concentrations prepared in the homogenization buffer, and 0.1 mL of the enzyme. The blank sample contained only 3 mL of substrate solution. The reaction was carried out at four temperatures (18, 56, 58 and 70°C), and pH values of 7.0, 9.0, 8.6 and 6.6 using catechol, 4-methyl catechol, pyrogallol and L-dopa substrates, respectively. Enzyme 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. PPO activity was assayed in triplicate measurements. For determining substrate specificity, commercial grades of the substrates were used. L-dopa,catechol,4-methyl catechol and pyrogallol each were added to the standard reaction mixture to give a final concentration of 0.05M.

Effect of pH and temperature

PPO activity was determined using four different substrates (L-dopa, catechol, 4-methyl catechol and pyrogallol) at 0.01M using appropriate buffers (0.1M citrate/0.2M phosphate, 0.1M phosphate and 0.1M Tris-HCl) between pH 3.5 and 10. The determined optimum pH was used in all other experiments. PPO activity was measured at temperatures ranging from 15°C to 80°C by using different substrates. Substrate solution was heated to the tested temperature and the enzyme was added.

Enzyme kinetics

Michaelis constant (K_M) and maximum velocity (V_{max}) were determined using four substrates (L-dopa, catechol, 4-methylcatechol and pyrogallol) in varying concentrations (0.002, 0.004, 0.006, 0.008, and 0.01M) and in optimum conditions (pH, temperature and ionic strength). The reaction was followed in a spectrophotometer and data were plotted according to Lineweaver and Burk (1934).

Effect of inhibitors

To determine effects of inhibitors, PPO activity was measured using seven different inhibitors at three concentrations with catechol substrate (Table 3). Results were expressed as Ki constants and percent catechol inhibition.

Activation energy

Enzyme activity log values from temperature assays for each substrate were plotted as related to the reciprocal of absolute temperature. The activation energies of reactions were calculated from the slopes. The Arrhenius equation was used for determining activation energies.

Electrophoresis

Polyacrylamide gel slab electrophoresis was performed according to the method of Laemmli (1970) to separate PPO isoenzymes of Amasya apple. The electrode buffer was tris/glycine (pH 8.3) using 3% stacking gel and 10% seperating gel. The enzyme sample was loaded (0.08 mL, 0.044 mg protein) into each space of the stacking gel. Initially, an elec-

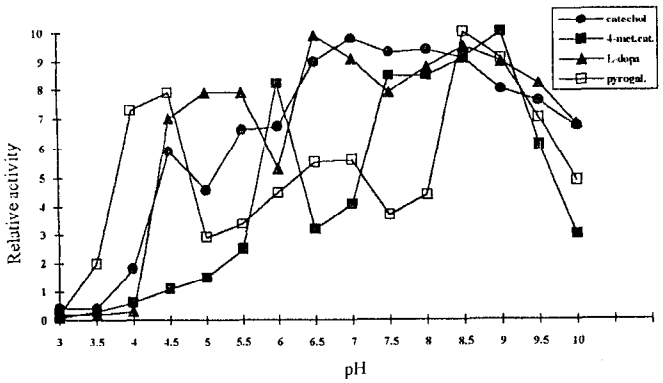


Fig. 1—Effect of pH on PPO activity.

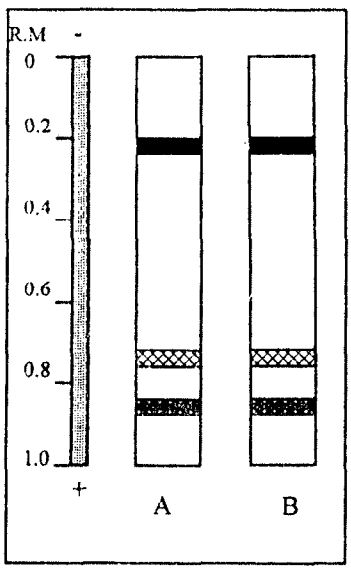


Fig. 2—Relative mobilities of Amasya apple PPO on polyacrylamide gel electrophoresis. Relative mobility was based on migration of bromophenol marker dye. Isoenzyme bands were developed using catechol (A), and L-dopa (B) substrates. Color intensities of the bands: ■ strong, ▒ moderate, ▪ weak (R.M: Relative mobility).

Table 1—Substrate specificity of Amasya apple PPO

Substrate (0.05 mM)	Activity (units/mL)
Catechol	1187.0
4-Methyl catechol	520.2
L-dopa	24.2
Pyrogallol	781.0

tric voltage (80 V) was applied until the bromphenol dye reached the seperating gel. Then it was increased to 200 V for 3 hr. Electrophoresis was carried out at 4°C. After completion of the run, the gel was cut into two symmetrical parts. One part was immersed in 0.1M phosphate buffer (pH 6) containing 1.5 mM L-dopa substrate, and the other part immersed in phosphate buffer (pH 7.2) containing 15 mM catechol and 0.05% o-phenylenediamine. The isoenzyme bands were developed for 1 hr and each gel then rinsed in 1 mM ascorbic acid solution for 5 min. The gels treated with L-dopa and catechol substrates were stored in distilled water or 30% ethanol respectively, and photographed.

RESULTS & DISCUSSION

Effect of pH

Different pH optima were determined for each substrate (Fig. 1). Maximum PPO activities were at pH 7.0 for catechol, 9.0 for 4-methyl catechol, 8.6 for pyrogallol, and 6.6 for L-dopa. In general, most fruit showed maximum activity at or near neutral pH values (Betrosian et al., 1960; Chan and Yong, 1971; Wong et al., 1971; Cash et al., 1976; Siddiq et al., 1992). In our study, we found similar results for catechol and L-dopa substrates but pH optima of the other two substrates were alkaline. We found three peaks in the pH-activity graphs. This may indicate different isoenzymes, since we found three isoenzymes of Amasya apple PPO (Fig. 2).

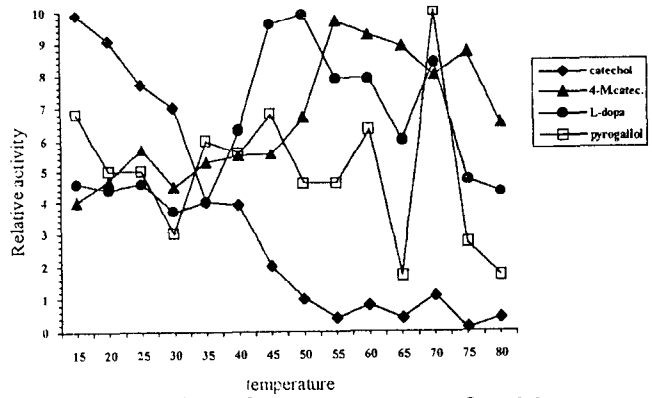


Fig. 3—Effect of temperatures on PPO activity.

Table 2—K_M and V_{max} values for each substrate

Substrate	K_M (mM)	V_{max} (units/mL)
Catechol	34.0	2000
4-Methyl catechol	3.1	552
L-dopa	6.5	27
Pyrogallol	27.0	1215

Substrate specificity

Amasya apple PPO showed activity with all the o-diphenolic substrates (Table 1). The substrate with highest activity was catechol, followed by 4-methyl catechol, pyrogallol and L-dopa. In earlier studies, however, the most suitable substrate for PPO was reported to be 4-methyl catechol and the least effective was pyrogallol in apple chloroplasts (Harel et al., 1965) and peel (Stelzig et al., 1972).

Effect of temperature

Optimum temperatures for maximum PPO activity (Fig. 3) were 15°C for catechol, 56°C for 4-methyl catechol, 50°C for L-dopa, and 70°C for pyrogallol. When catechol was substrate, PPO showed maximum activity at 15°C and decreased gradually with increasing temperatures. Above 60°C PPO showed almost no activity. With other substrates, however, PPO showed fluctuations in activity with increasing temperatures even as high as 80°C.

Enzyme kinetics

Values calculated from Lineweaver-Burk graphs (Table 2) showed K_M values were 34 mM for catechol, 3.1 mM for 4-methyl catechol, 6.5 mM for L-dopa, and 27 mM for pyrogallol. From these results, we suggest Amasya apple PPO has more affinity for 4-methyl catechol than for the other substrates. In an earlier work (Walker, 1964), K_M of apple chloroplast PPO was reported to be 1.6 mM with chlorogenic acid substrate. However, K_M of apple flesh PPO has been indicated as 4.6 mM with catechol substrate (Vamos-Vigyazo, 1981).

Effect of inhibitors

Percent inhibition and Ki values for the inhibitors we used (Table 3) indicated that thiourea and glutathione show uncompetetive inhibition whereas the other inhibitors were compete-

Table 3—K_i constants and percent inhibition values of the PPO with different inhibitors

Inhibitor	Concentrations (M)	K_i (M)	% Inhibition
β-mercaptoethanol	1.38×10^{-3}	1.8×10^{-2}	4.0
	5.50×10^{-3}	1.5×10^{-2}	22.3
	8.30×10^{-3}	1.1×10^{-2}	36.8
Thiourea	3.3×10^{-5}	7.8×10^{-4}	2.5
	9.9×10^{-5}	6.5×10^{-4}	4.4
	1.32×10^{-4}	4.6×10^{-4}	6.3
Sodium cyanide	8.9×10^{-5}	1.4×10^{-4}	33.1
	1.8×10^{-4}	1.2×10^{-4}	53.1
	2.6×10^{-4}	1.1×10^{-4}	65.6
Sodium metabisulphide	1.7×10^{-5}	9.6×10^{-6}	15.8
	3.3×10^{-5}	8.1×10^{-6}	76.7
	4.9×10^{-5}	6.7×10^{-6}	85.4
Glutathion	2.04×10^{-5}	4.0×10^{-4}	1.3
	4.08×10^{-5}	2.1×10^{-4}	3.5
	8.17×10^{-5}	1.1×10^{-4}	12.2
L-cysteine	9.7×10^{-6}	9.8×10^{-6}	42.5
	2.9×10^{-5}	6.5×10^{-6}	77.6
	4.8×10^{-5}	4.2×10^{-6}	90.0
Ascorbic acid	1.7×10^{-5}	9.2×10^{-5}	12.5
	3.5×10^{-5}	7.3×10^{-5}	26.9
	7.1×10^{-5}	5.1×10^{-5}	74.1

Table 4—Activation energies (E_A) of PPO for different substrates

Substrate	Slope ($-E_A/2.303R$)	Activation energy (cal/mol)
Catechol	0.235	−107.3
4-Methyl catechol	−0.062	28.3
Pyrogallol	0.044	−20.0
L-dopa	−0.046	20.9

tive. The most effective inhibitor was L-cysteine. In a reported study (Janovitz-Klap et al., 1990), cysteine was a strong inhibitor of apple PPO. Since L-cysteine is a naturally occurring amino acid and nontoxic, it may be useful in preventing enzymatic browning of apple products.

Activation energy

For each substrate, a plot was constructed by using temperature-activity values from the temperature optimum study. Activation energy (E_A) was calculated from the slopes (Table 4). Activation energies of catechol and pyrogallol were negative whereas all others were positive. This may be explained by inactivation of the PPO by high temperature. Activation energy values are, generally, not published, therefore comparisons are not possible. Only one study of PPO in kiwifruit states its activation energy was 7 kcal/mol with catechin substrate (Park and Luh, 1985).

Electrophoresis

Three isoenzymes were separated by polyacrylamide slab gel electrophoresis and detected using catechol and L-dopa substrates (Fig. 3). In both cases, the slow moving first bands were more active than the fast moving second and third bands (indicated by color intensity). Previous studies indicate three PPO isoenzymes were found in apples (Harel et al., 1965; Constantinides and Bedford, 1967).

CONCLUSIONS

PPO OF AMASYA APPLE showed different pH optima with different substrates. Its thermostability was quite high, even as high as 80°C. The most effective inhibitor of PPO activity was L-cysteine. Since L-cysteine is a naturally occurring amino acid and nontoxic, it may be of use in preventing enzymatic browning of apple products. Three PPO isoenzymes were detected by electrophoresis.

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