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Purification and kinetic characterization of polyphenol oxidase from Barbados cherry (*Malpighia glabra* L.)

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Abstract

Polyphenol oxidase (PPO) of Barbados cherry was extracted and purified through ammonium sulfate precipitation, gel filtration, and affinity chromatography. The purification factor for PPO was 60% with 8.3% yield. The enzyme was characterized for thermal stability, pH and kinetic parameters. The molecular mass of PPO was approximately the sum of 52 and 38 kDa estimated by SDS–PAGE. The purity was checked by native PAGE, showing a single prominent band. The optimum pH was 7.2. The enzyme had a temperature optimum at 40 °C and was relatively stable at 60 °C, with 55% loss of activity. Sodium diethyl dithiocarbamate (SDDC), L-cysteine and ascorbate significantly inhibited PPO activity. 4-Methyl catechol and catechol were found to be efficient diphenolic substrates for cherry PPO, considering the V_{max}/K_m ratio. The data obtained in this study may help to understand cherry fruit browning.

Keywords: Polyphenol oxidase; Barbados cherry; Affinity chromatography; Inhibition; Browning; Kinetics

1. Introduction

Polyphenol oxidase (E.C. 1.14.18.1) is a copper-containing enzyme, widely distributed in plants and also in microorganisms. In fruits, the key enzyme responsible for browning reactions is polyphenol oxidase (PPO), which uses molecular oxygen to catalyze the *o*-hydroxylation of monophenols to *o*-diphenols and their further oxidation to coloured and highly reactive *o*-quinones. These *o*-quinones readily polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments. This leads to organoleptic and nutritional modifications, thus depreciating the food value. This has been a serious problem in the food industry (Yang, Fujita, Ashrafuzzaman, Nakamura, & Hayashi, 2000). PPO has been isolated from various sources, such as banana, pear and apple (Jharna, Santhoor, & Lalitha, 1997; Kahn, 1977; Ziyan & Perkyardimci, 2004). The Barbados cherry (*Malpighia glabra* L.) has a very short shelf life under ambient conditions due to skin colour loss and quality deterioration during storage. However, no work has been carried out on PPO of cherry fruit. Therefore, the objective of our study was to isolate, purify and characterize the PPO from the Barbados cherry.

2. Materials and methods

2.1. Materials

Barbados cherry fruits were harvested fresh from the garden and stored at −20 °C.

2.2. Isolation and purification of PPO

All the purification steps were carried out at 4 °C, as described by Jiang (1999). The cherry peel was homogenised with 0.1 M sodium phosphate buffer (Na–P buffer) (pH 6.8) with Poly-Clar AT (insoluble high molecular weight grade of polyvinylpyrrolidone; 1% of fruit weight). After filtration, the filtrate was centrifuged at 15 000*g* for 20 min and the supernatant was collected. The enzyme protein was

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fractionated with solid ammonium sulfate (50–80% saturation) and the precipitate was recovered by centrifugation at 15000*g* for 20 min, redissolved in 0.01 M Na–P buffer (pH 6.8) and dialyzed against the same buffer for 24 h. Following dialysis, the extract was loaded onto a Sephadex S-200 column pre-equilibrated with 0.01 M sodium phosphate buffer (pH 6.8). The enzyme solution was eluted with the same buffer and the fractions with highest enzymatic activity were pooled, lyophilized and redissolved in small volume of 0.01 M sodium phosphate buffer (pH 6.8). After dialysis, the concentrate was loaded onto a Phenyl Sepharose column equilibrated with 0.01 M sodium phosphate buffer containing 1 M ammonium sulfate, 1 M KCl, pH 6.8. The PPO was eluted with a gradient of 100%, 80%, 60%, 40%, 20%, 10% to 0% of the same equilibrium buffer (pH 6.8). The active fractions from the Phenyl Sepharose column were pooled, lyophilized, and dissolved in a small volume of 0.01 M sodium phosphate buffer (pH 6.8). After overnight dialysis against the same buffer, the dialyzed solution was collected as enzyme source.

2.3. Assay of PPO activity

PPO activity was determined by measuring the increase in absorbance at 420 nm. The reaction mixture contained 0.2 ml of enzyme solution and 2.8 ml of 100 mM catechol solution in 0.1 M Na–P buffer, pH 7.0, at 40 °C. The blank contained only 3 ml of substrate solution. One unit of enzyme activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 min^{-1}. The protein concentration was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951).

2.4. Effect of pH and temperature

The effect of pH on PPO activity was determined with seven different substrates (catechol, 4-methyl catechol, L-dopa, pyrogallol, D-tyrosine, caffeic acid, and ferulic acid). Catechol, 4-methyl catechol, and pyrogallol were used at 10 mM; D-tyrosine was used at 2 mM and caffeic acid, ferulic acid, and L-dopa were used at 5 mM concentrations. Appropriate buffers (0.1 M citrate, pH 4–5; 0.2 M phosphate, pH 5–7 and 0.05 M Tris–HCl, pH 7–10) were used to determine the optimum pH. 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 °C to 75 °C interval using the seven different substrates indicated above.

2.5. Thermal inactivation

Heat treatments of PPO were carried out at 45 °C, 55 °C, 65 °C, 75 °C, and 85 °C for varying periods of time in a temperature controlled water bath; 10 ml of enzyme solution were placed in a pre-warmed tube at the specified temperature and 0.05 ml aliquots were withdrawn at various time intervals, cooled and assayed for residual activity. The stability of the enzyme was expressed as % residual activity and was calculated by comparison with untreated enzyme.

2.6. SDS–PAGE

The purified PPO, after affinity chromatography, was run on SDS–PAGE, using 10% polyacrylamide gel (Laemmli, 1970). The purified enzyme was also subjected to native PAGE and the activity was localized by incubating in 15 mM catechol in 0.1 mM phosphate buffer (pH 7.0) at 35 °C for 1 h, followed by 1 mM ascorbate solution until appearance of bands occurred.

2.7. Effect of inhibitors

L-Cysteine, L-ascorbate, sodium diethyldithiocarbamate, β-mercaptoethanol, thiourea, EDTA, $CaCl_2$ and sodium metabisulphite were evaluated for their effectiveness as inhibitors of PPO activity, using catechol as the substrate. The results were reported as % catechol inhibition. The compounds showing highest inhibitions were further assessed by studying the kinetics of interaction of PPO with inhibitors. In separate experiments, inhibitors at various concentrations (1, 2, 3, 5, and 10 mM) were added and were monitored at time intervals of 2 min (up to 10 min). First and second order rate constants were calculated.

2.8. Enzyme kinetics and substrate specificity

PPO activity was assayed using catechol, L-dopa, 4-methyl catechol and pyrogallol in buffers at optimum pH value for each substrate. The K_m value and maximum velocity V_{max} were determined by the Lineweaver–Burk plot. Substrate specificity V_{max}/K_m was calculated by using the data obtained from the above plot (Lineweaver & Burk, 1934).

3. Results and discussion

3.1. Purification

PPO was purified from Barbados cherry using Phenyl Sepharose affinity chromatography (Jiang, 1999). Table 1 summarizes the purification profile of PPO. The crude extract of PPO showed a specific activity of 669 U/mg protein and the profile of purification was further increased to near homogeneity by Phenyl Sepharose. Thus, the protocol yielded a purified PPO with specific activity 40,192 U/mg, with a low protein content of 0.12 mg. Overall, the specific activity increased about 60 fold with 8.3% yield of activity. This is significantly higher than that obtained for guava (Augustin, Ghazali, & Hashim, 1985) and pear (Zhou & Feng, 1991). The activity of PPO was checked by gel assay which showed a thick band, indicating the localization site of the PPO activity, as well as homogeneity of enzyme protein (Fig. 1a). This was similar to those of mulberry (Ar-

Table 1
Purification profile of Barbados cherry PPO[a]

Purification steps	Enzyme activity (U/g) tissue	Yield (%)	Total protein (%) mg/g tissue	Specific activity (U/mg) protein	Fold purification
Crude extract	58240	100	87.1	669	0
Ammonium sulfate 80%	28583	49.1	10.4	2748	4.1
Sephadex G-100	9829	15.9	1.6	7741	11.6
Phenyl Sepharose	4823	8.3	0.12	40192	60.0

[a] Enzyme activity was assayed using 100 mM catechol and 0.1 Na–P buffer, pH 7.0, and enzyme solution purified by various steps.

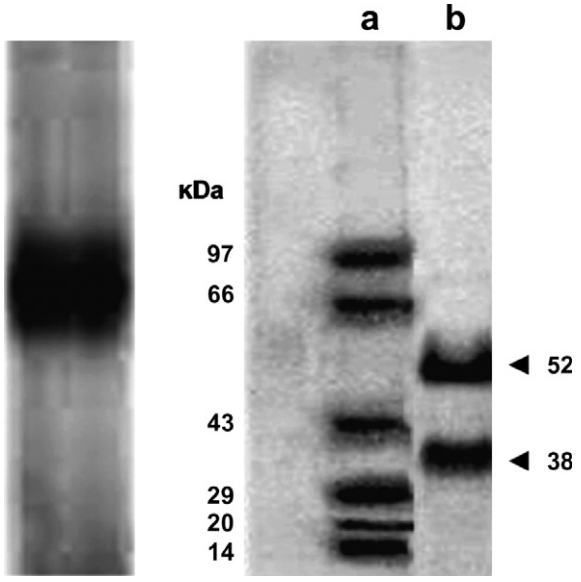

Fig. 1. (a) Native PAGE of PPO localized using 15 mM catechol (b) SDS–PAGE of the purified PPO protein stained with Coomassie Brilliant Blue. Lane a, marker; lane b, purified protein.

slan, Erzengin, Sinan, & Ozensoy, 2004) but different from those of pear (Ziyan & Perkyardimci, 2004). The electrophoretic pattern of cherry PPO in SDS–PAGE revealed two dominant bands with molecular masses of 52 and 38 kDa (Fig. 1b). Thus, this enzyme differs from PPO isolated from pear (Ziyan & Perkyardimci, 2004) and Chinese cabbage (Nagai & Suzuki, 2001), which were reported to be monomers.

3.2. Effect of pH and temperature

Optimal pH and temperature of the PPO were estimated by using seven different substrates (Table 2). The pH optimum for enzyme-catalyzed oxidation of catechol in phosphate buffer was found to occur at pH 7.0. At pH above 8.0, the activity decreased very rapidly. This value was different from those of Brassica (Nagai & Suzuki, 2001), avocado, (Jiang, 1999) and field bean (Paul & Gowda, 2000). In order to determine pH stability, the enzyme solution was incubated in appropriate buffer solutions, ranging from 4.0 to 10.0 for 24 h at 4 °C. Residual PPO activity was determined as percent residual PPO activity at the optimum pH by mixing 100 mM catechol as substrate

Table 2
Optimum pH and temperature of cherry PPO

	pH	Temperature (°C)
Pyrogallol	8	30
4-Methyl catechol	7	36
Catechol	7	40
D-Tyrosine	5.2	47
Caffeic acid	5.4	24
L-Dopa	6.6	41
Ferulic acid	5.6	25

40 °C with the incubated enzyme solution. Fig. 2 shows the effect of pH on the stability of the enzyme. The pH stability profile for the enzyme shows that 72% of the PPO activity was retained between pH 5.5 and 8.0 (Dincer, Colak, Aydin, Kadioglu, & Guner, 2002). The enzyme is not stable below pH 5.0 or above 8.5. The PPO system in fruits has been shown to be most active at a near neutral pH value (Vamos-Vigyazo, 1981). According to Lee, Kagan, Jaworski, and Brown (1990), the rapid inactivation of the enzyme above pH 8.0 may be due to conformational changes in the enzyme under the alkaline conditions and/or the enzyme may react rapidly with quinones through Maillard reactions and/or Strecker degradation.

The activity of PPO was measured at different temperatures at optimum pH for 5 min (Table 2). The enzyme showed the highest activity at 40 °C with the substrate catechol. This value was different from those of plum PPO (Siddiq, Sinha, & Cash, 1992) and medlar (Dincer et al., 2002).

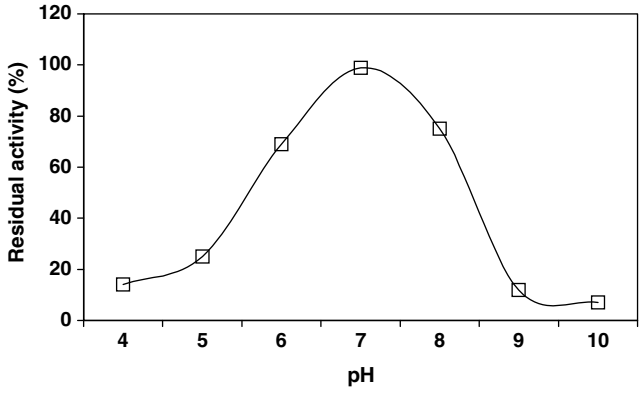

Fig. 2. pH stability of cherry PPO after a pre-incubation period of 24 h at the indicated pH.

3.3. Thermal inactivation kinetics

The enzyme was incubated at different temperatures (45–85 °C) for 30 min and, after cooling, the residual enzyme activity was measured (Fig. 3). Consequently, it was found that the enzyme was stable at 45 °C but was unstable at temperatures above 75 °C. It has been reported that pear PPO was stable at 60 °C and plum PPO at 70 °C (Ziyan & Perkyardimci, 2004; Siddiq et al., 1992). The decrease in percentage residual activity at higher temperatures is due to the unfolding of the tertiary structure.

3.4. Effects of Inhibitors

The effects of various inhibitors on the purified PPO are shown in Table 3. It was markedly inhibited by sodium diethyl dithiocarbamate (SDDC), L-cysteine and ascorbate. EDTA and $CaCl_2$ showed minimum inhibition. L-Cysteine was reported to be a strong inhibitor of apple PPO (Oktay, Küfrevioğlu, Kocacaliskan, & Sakiroğlu, 1995) and ascorbates are effective inhibitors of dog rose PPO (Jiang, 1999). Since cysteine and ascorbate are naturally occurring substances and non-toxic, they may be useful for preventing the enzymic browning of Barbados cherry. Inhibitors such as SDDC and thiourea, which combine with the copper moiety in the enzyme, are generally potent inhibitors of PPO. The inhibitors are copper-chelating agents and they suppress browning activities in which copper is directly involved in the oxidation of phenolic compounds.

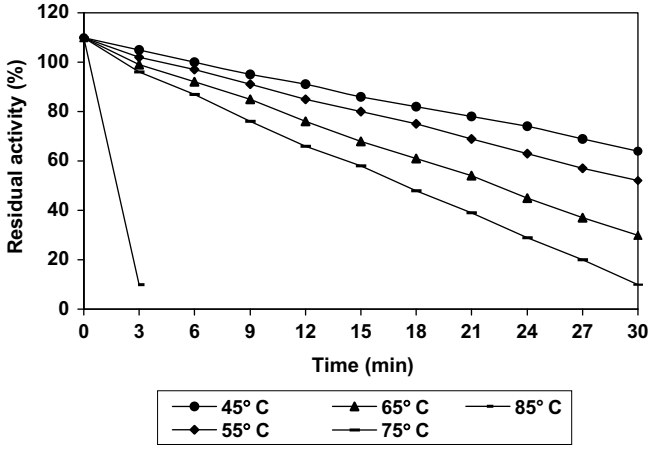

Fig. 3. Thermal stability of cheery PPO at varying temperatures (45–85 °C) versus time.

Table 3
Effect of inhibitors on cherry PPO activity

Inhibitors	% of inhibition	
	1 mM	10 mM
L-Ascorbate	12	93
Sodium azide	6	64
L-Cysteine	10	98
Mercapto ethanol	5	72
Thiourea	60	81
EDTA	2	29
$CaCl_2$	–	1
Sodium metabisulphite	2	26
Sodium diethyl dithio carbamate	18	100

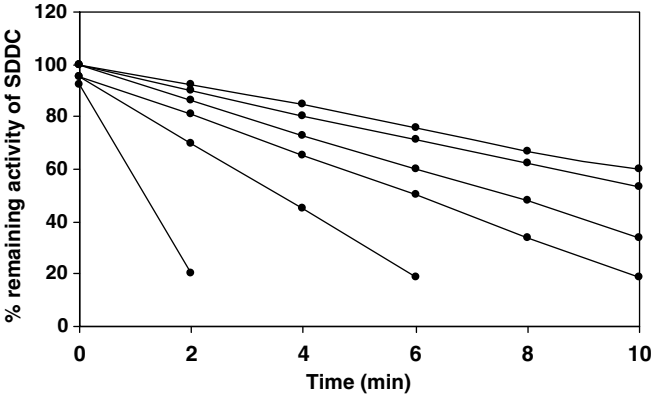

Fig. 4a. % Remaining activity of varying concentration of SDDC against time.

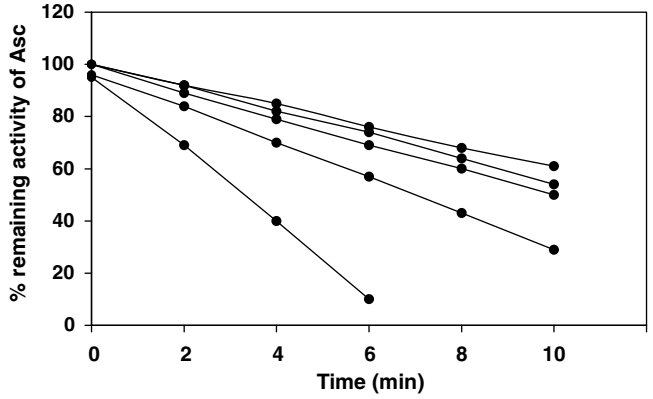

Fig. 4b. % Remaining activity of varying concentration of ascorbate (Asc) against time.

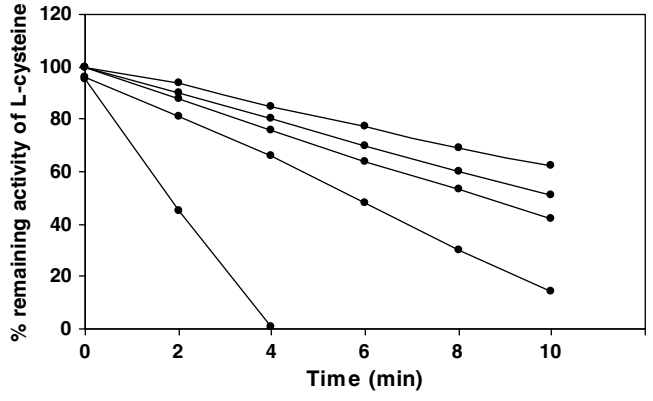

Fig. 4c. % Remaining activity of varying concentration of L-cysteine against time.

Table 4
First order rate constant for inhibition of activity of PPO by sodium diethyl dithio carbamate (SDDC), L-cysteine and L-ascorbate (ASC)

Inhibitor (concentration mM)	1	2	3	5	10
SDDC ($K\ s^{-1}$)	1.39×10^{-2}	1.66×10^{-2}	2.14×10^{-2}	2.97×10^{-2}	3.8×10^{-2}
L-Cysteine ($K\ s^{-1}$)	1.49×10^{-2}	2×10^{-2}	3.6×10^{-2}	5.9×10^{-2}	8.3×10^{-2}
Asc ($K\ s^{-1}$)	1.02×10^{-2}	1.2×10^{-2}	1.6×10^{-2}	2.3×10^{-2}	2.6×10^{-2}

3.5. Kinetics of interaction of SDDC, L-cysteine and ascorbate with PPO

It is clear from Table 3 that SDDC, L-cysteine and ascorbate inhibit PPO activity significantly. It is of interest to examine the kinetics of interaction of inhibitors with enzyme by monitoring the activity change. The enzyme was incubated for 10 min with different concentrations of SDDC, L-cysteine and ascorbate. At intervals of 2 min, the residual enzyme activity was measured. The enzyme was incubated for 0 min in the absence of inhibitor and there was no loss of activity. In the second control, the enzyme was assayed when both the inhibitor and the substrate were added together and monitored for 5 min for any loss in activity during the course of the assay. The value was normalized to 100% in each case and used to calculate the amount of remaining activity with the enzyme alone. At different concentrations of the inhibitor, the % remaining activity was plotted against the period of incubation (see Figs. 4a–4c). The slopes of the curves gave the first order rate constants summarized in Table 4. Increase in the extent of inhibition of enzyme activity was reflected in increased values of rate constants. The log of the first order rate constant was plotted against log SDDC, log L-cysteine, and log Asc concentrations and a second order rate constant was calculated as $64.4\ M^{-1}\ s^{-1}$, $29.3\ M^{-1}\ s^{-1}$, and $1.54\ M^{-1}\ s^{-1}$, respectively. The results demonstrate that SDDC was a potent inhibitor of cherry PPO.

3.6. Substrate specificity

K_m and V_{max} values of cherry PPO were calculated from the Lineweaver–Burk graphs and the results are shown in Table 5. All the substrates, namely catechol, 4-methyl catechol, L-dopa, pyrogallol, and caffeic acid, were oxidized significantly by the enzyme, displaying simple Michaelis–Menton kinetics. Linear regression analysis of v versus [S] determined V_{max} and K_m values for each substrate (Table 5). The highest K_m values were shown by 4-methyl catechol and catechol and the lowest by pyrogallol. In order to evaluate the substrate specificity, V_{max}/K_m ratio was taken as a criterion (Dincer et al., 2002). This result was consistent with the previous report on plant PPOs (Walker, 1995). It appears that the substrate-binding site of cherry PPO has a high affinity for small *o*-diphenols, such as catechol, 4-methyl catechol or L-dopa, and less affinity for the larger *o*-diphenols, caffeic acid, and triphenol-pyrogallol.

Table 5
Kinetic parameters for the oxidation of diphenolic substrates by the cherry PPO

Substrate	V_{max} (μM/min)	K_m (mM)	V_{max}/K_m (min^{-1})
Catechol	79.5	5.2	0.0153
4-Methyl catechol	125	6.9	0.0181
L-Dopa	56.5	4.1	0.0138
Pyrogallol	8.8	1.24	0.0071
Caffeic acid	48.2	3.6	0.0134

In this study we have purified and characterized polyphenol oxidase from Barbados cherry. The fruit is an excellent source of ascorbate and other nutraceuticals. Browning in fruits and vegetables is recognized as a serious problem in the food industry. Further studies are warranted to understand the PPO inhibitor in relation to the browning reaction of fruit during storage and processing.

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