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Purification of mulberry (*Morus alba* L.) polyphenol oxidase by affinity chromatography and investigation of its kinetic and electrophoretic properties

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Abstract

Polyphenol oxidase (PPO) was isolated from mulberry (*Morus alba* L.) fruit using a Sepharose 4B-L-tyrosine-*p*-amino benzoic acid affinity column. The purified enzyme was migrated as a single band on native and SDS–poliacrylamide gel electrophoresis. The molecular weight of the purified enzyme was estimated to be 65 kDa. Optimum PPO activity as a function of pH and temperature was determined using catechol, 4-methyl catechol and pyrogallol as substrates. The optimum pH and temperatures values of mulberry PPO for the used three substrates ranged between the pH 4.5–8.0 and 20–45 °C. At the optimum pH and temperature, the K_M and V_{Max} values of mulberry PPO towards catechol, 4-methyl catechol and pyrogallol were determined by Lineweaver–Burk method. The values V_{Max}/K_M showed that mulberry has the greatest reactivity towards pyrogallol among the substrates used. On the other hand mulberry PPO showed no activity toward the monophenols, *p*-cresol and L-tyrosine, suggesting the absence of monophenolase (cresolase) activity. Beside the classical PPO inhibitors, for the first time the inhibitory effect of some sulfonamide compounds on the mulberry PPO activities was also tested. Sulfonamide compounds exhibited considerable inhibition on mulberry PPO enzyme.

Keywords: Mulberry (*Morus alba* L.); Polyphenol oxidase; Affinity chromatography; Inhibition; Enzymatic browning

1. Introduction

Browning reactions in fruits and vegetables are recognized as a serious problem in the food industry. In fruits, the principal enzyme responsible for the 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 colored and highly reactive *o*-quinones. These *o*-quinones readily polymerize and/or react with endogenous amino acids and proteins to form complex brown pigments. Because such browning decreases their marketability, the enzyme has been extensively studied (Arslan, Temur, & Tozlu, 1998; Laurila, Kervinen, & Ahvenainen, 1998; Lee, Kagan, Jaworski, & Brown, 1990; Ragazzi & Veronese, 1967; Yang et al., 2001). Studies on PPO have included the isolation and characterization of PPO from avocado, banana, apples, eggplant, grape, apricot, raspberry and blackberry (Gonzales, Ancos, & Cano, 1999; Jharna, Santhoor, & Lalitha, 1997; Kahn, 1977; Sojo, Nunez-Delisado, Garcia-Carmona, & Sanchez-Ferrer, 1999). There have also been studies on the subject of PPO from plants. However, we have not encountered any article about PPO of mulberry fruit, even though it is necessary to characterize of mulberry PPO for controlling enzymatic browning of fruit products. Therefore, in this study, mulberry PPO was purified by

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using catechol, 4-methyl catechol and pyrogallol as substrates, and some properties of the purified enzyme were investigated.

2. Materials and methods

Mulberry (*Morus alba* L.) fruits used in this study were harvested in June from a field near Balikesir in Turkey. All chemicals used in this study were the best grade available and were used without further purification because of obtaining from Sigma Chem. Co. Enzyme assays were measured with the aid of a Cary |1E| g UV–Visible Spectrophotometer (Varian). Affinity gel which is used in this study was synthesized at Balikesir University, Research Center of Applied Sciences (BURCAS/Balikesir, Turkey) in Biology section laboratory (Arslan & Erzengin, 2002).

2.1. Purification of PPO

The extraction procedure was adopted from Wesche-Ebeling and Montgomery (1990). Firstly mulberry fruits were washed with distilled water three times. Secondly to prepare the crude extract, 10 g of sample tissue was cut quickly into thin slices and homogenized in a warring blender for 2 min using 100 ml of 0.1 M phosphate buffer (pH 6.5) containing 5% poly (ethylene glycol) and 10 mM ascorbic acid. After filtration of the homogenate through a muslin, the filtrate was centrifuged at 15,000*g* for 20 min, and the supernatant was collected. The enzyme solution was fractionated with solid ammonium sulfate and the precipitate was collected by centrifugation at 15,000*g* for 20 min, redissolved in 5 mM phosphate buffer (pH 6.30) and dialyzed against the same buffer (Wesche-Ebeling & Montgomery, 1990).

The enzyme solution was applied to the affinity column (1×10 cm), pre-equilibrated with 5 mM phosphate buffer (pH 5.0). The affinity gel was washed with the same buffer. PPO was eluted with the solution of 5 mM phosphate buffer/1 M NaCI (pH 8.5).

2.2. Assay of PPO activity

Enzyme activity was determined by measuring the increase in absorbance at 420 nm when using catechol, 4-methyl catechol and pirogallol as substrates with a spectrophotometer (Oktay, Küfrevioğlu, Kocaçalışkan, & Şakiroğlu, 1995). One unit of PPO activity was defined as the amount of enzyme that causes an increase in absorbance of 0.001 $ml^{-1}\,min^{-1}$.

2.3. Determination of protein

Protein was determined by the method of the Bradford (1976) using bovine serum albumin as a standard. In chromatography, protein was expressed with absorbance at 280 nm.

2.4. Polyacrylamide gel electrophoresis

Polyacrylamide gel slab electrophoresis (PAGE) of purified enzyme was carried out according to the method of Laemelli (1970).

2.5. Effect of pH

PPO activity as a function of pH was determined by in the pH range 4.0–9.0 by using phosphate buffer adjusted with 0.1 M Na OH or 0.1 M HCl. The optimum pH for the PPO was obtained using three different substrates (0.05 M of catechol, 0.05 M of 4-methyl catechol and 0.05 M of pyrogallol).

2.6. Heat inactivation of PPO

The effects of temperature and incubation time on PPO activity were determined. Enzyme extracts (0.2 ml) were subjected to 40–80 °C using a water bath, for times ranging from 10–60 min. They were then transferred into buffer solutions containing catechol (0.1 M) that were prewarmed to the corresponding temperatures. Reaction rates of these enzymes were assayed as previously described in 1 cm cuvettes around which water circulated at the respective temperatures of reaction.

2.7. Kinetic studies and determination of I_{50} and K_i values

In order to obtain K_M and V_{Max} values separately for catechol, 4-methylcatechol and progallol, at optimum pH and temperature the enzyme activity was measured at five different substrate cuvette concentrations. K_M and V_{Max} values were determined by means of Lineweaver–Burke graphs.

I_{50} and K_i values of different inhibitors (glutatyon, L-cystein, *p*-aminobenzene sulfonamide and sulfosalicilik acid) were determined on PPO. In order to determine I_{50} values 10 mM catechol was used as a substrate. At first the activity of enzyme assay without any inhibitor. This measure was accepting 100% activity for graph and than enzyme activity assay with different inhibitor concentration. In order to determine using % activity-inhibition graph drew I_{50} values. The inhibitor concentrations causing up to 50% inhibition were determined from the graphs. This way was followed to determine K_i values. In the media with or without inhibitor, the substrate concentrations were 0.6, 0.8, 1.0 and 1.2 mM. For this purpose, the substrate was used between 0.6 and 1.2 ml. Inhibitor solutions were added to the reaction medium as 0.1, 0.2 and 0.3 ml resulting in three different fixed concentrations of inhibitor. The Lineweaver–Burke graphs were obtained and K_i values were calculated.

3. Results and discussion

In this study, PPO was purified from mulberry fruits using a Sepharose 4B-L-tyrosine-*p*-aminobenzoic acid affinity column (Arslan & Erzengin, 2002). Fig. 1 shows the typical elution pattern of the enzyme activity on affinity column. The enzyme activity showed a single peak and the peak fractions were pooled as purified PPO.

The purified PPO migrated as a single band during native and SDS–polyacrylamide gel electrophoresis. On SDS–polyacrylamide gel electrophoresis, MPPO produced a single band of ~65 kDa (Fig. 2). The molecular weight of PPO from other species has been reported as follows: Chinese cabbage, 65 kDa (Nagai & Suzuki, 2001); banana, 62 kDa (Galeazzi, Sgarbieri, & Constantinides, 1981); cabbage 39 kDa, (Fujita et al., 1995); and field bean seed, 120 kDa (Beena & Gowda, 2000). Our results indicate that the molecular weights of MPPO were similar to those of chinese cabbage and banana but was different from those of cabbage and field bean seed.

As shown in Table 1, at the end of the chromatography on affinity column, 74-fold purification was achieved. This is higher than 17.2-, 10.8-, 9.0-, 4.9- and 6.5-fold obtained for guava (Augustin, Ghazali, & Hashim, 1985), yali pear (Zhou & Feng, 1991) and yam (Martin & Ruberte, 1976).

pH is a determinant factor for the expression of enzymatic activity. Therefore, the pH profile of PPO was determined between 4.5 and 9.0. As shown in Fig. 3, the optimum pH for maximum MPPO activity dependent on the substrates used for assay. The optimal pH values obtained from this study were 5.0, 7.0 and 7.5 for used 4-methyl catechol, catechol, and pyrogallol as substrates, respectively. This values were different from those of raspberry pH 8.0 (Gonzales et al., 1999), *Allium* sp. pH 7.5 (Arslan, Temur, & Tozlu, 1997), Stanley plum pH 6.0 (Siddiq, Sinha, & Cash, 1992), kiwi pH 6.8 and 7.3 (Park & Luh, 1985) and field bean seed pH 4.0 (Vamos-Vigyazo, 1981) using catechol as substrate. On the other hand, the optimum pH of mulberry PPO was similar to that of Amasya apple pH 7.0 (Oktay et al., 1995) using catechol as substrate. Differences in pH optima have been reported for partially purified Gum Arabic pH 5.3 (Mihalyi, Vamos-Vigyazo, Kiss-Kutz, & Babos-Szcbenyl, 1978), sogo log pH 4.5 (Onsa, saari, Selamt, & Bakar, 2000; Vamos-Vigyazo, 1981), seed of field bean pH 4.0 (Beena & Gowda, 2000) using 4-methyl catechol as substrate and for partially purified Chinese cabbage pH 5.0 (Halder, Tamuli, & Bhaduri, 1998) using pyrogallol as substrate. However, the optimum pH of MPPO was similar to that of sago log (*Metroxylon sagu*) pH 7.5 (Vamos-Vigyazo, 1981) using pyrogallol as substrate.

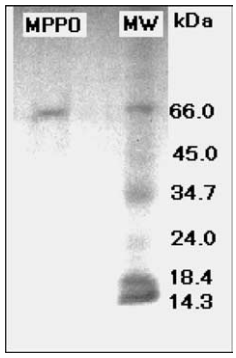

Fig. 2. SDS–PAGE gel electrophoresis of MPPO purified by affinity gel MPPO, mulberry polyphenol oxidase; MW, molecular weight marker.

Fig. 4 shows the effect of temperature on the activity and stability of the enzyme at optimum pH. The optimum temperature for mulberry PPO activity has been found to vary with substrate of the enzyme. Whereas the optimum temperature of enzyme for 4-methyl cathechol

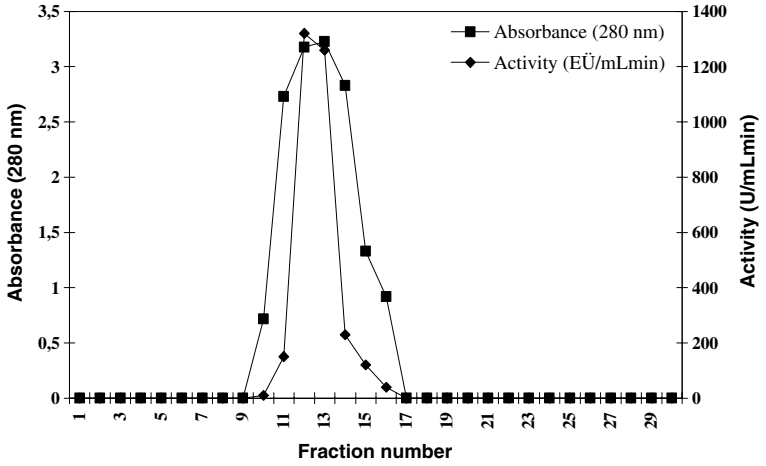

Fig. 1. Purification of MPPO by the balanced of Na_2HPO_4 buffer (pH 6.00) affinity gel.

Table 1
Purification of polyphenol oxidase from mulberry

Purification step	Volume (ml)	Total activity	Activity (U/ml min)	Total protein (mg)	Specific activity (U/mg protein)	Purification fold
Extract	5	15040	3123	10.335	1510.88	–
Affinity chromatography pH 6.0	3	3195	1065	0.0285	112105.26	74.20

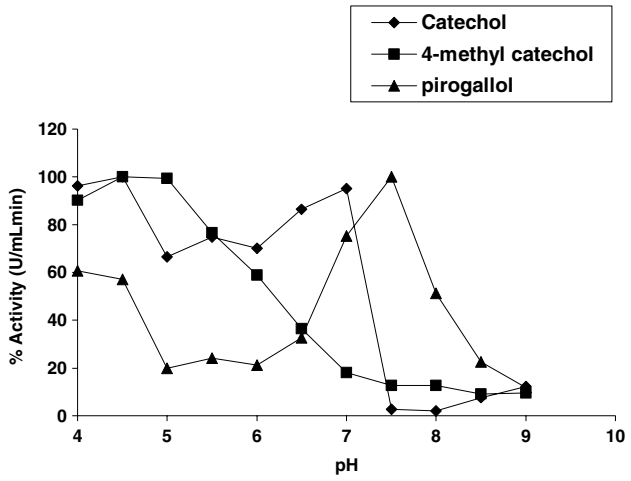

Fig. 3. Effect of pH on the activity of purified mulberry polyphenol oxidase.

and progallol oxidation was 20 °C, for cathecol it was 45 °C. The enzyme was relatively stable at 40 and 50 °C. The times required for 50% inactivation of activity at 50, 60 and 80 °C were found to be about 50, 30 and 20 min, respectively. Similar high thermal stability has been found for PPOs in peach (Mihalyi et al., 1978) and banana (Yang et al., 2001), quince (Vamos-Vigyazo, 1981).

The enzyme strongly oxidized pyrogallol. However, the oxidation rates of catechol and 4-methyl catechol by MPPO were below 1–4 that of pyrogallol. This substrate specificity is different from most reported PPO (Doğan, 2002; Gauillard & Richard, 1997). MPPO had no activity toward the monophenols, *p*-cresol and L-tyrosine, suggesting the absence of monophenolase (cresolase) activity (Beena & Gowda, 2000; Doğan, 2002). Therefore, mulberry PPO could be a diphenol oxidase. This is similar to the finding for aubergine (Beena & Gowda, 2000). Michaelis constant (K_M) for the purified enzyme were 19.81, 9.18 and 1.24 mM with catechol, 4-methyl catechol and progallol, respectively. The value for catechol is higher than 5 mM for olive enzyme (Ben-Shalom, Kahn, Harel, & Mayer, 1977). The K_M value for catechol is close to values reported for PPO from field bean seeds (Beena & Gowda, 2000) and yali pear (Zhou & Feng, 1991).

Inhibitor properties of purified PPO solution by glutation, L-cystein, *p*-aminobenzene sulfonamide and sulfosalicilic acid were investigated at the optimum pH of PPO activity with catechol as the substrate. Lineweaver–Burk plots of $1/V$ versus 1/[S] at three inhibitor concentrations determined the type of inhibition. The inhibition constant K_i for these inhibitors was deduced from the Dixon plots. Table 2 shows the inhibition results with catechol as the substrate. The most effective inhibitor was *p*-aminobenzenesulfonamide, followed by sulfosalicilik acid, L-cystein and glutation, in that order.

Table 2
Effects of some inhibitors on the activity of MPPO using as a catechol substrate

Inhibitors	K_i Values	Type of inhibition
Glutation	0.148	Competitive
L-Cystein	00.35	Competitive
p-Aminobenzene sulfonamide	7.50×10^{-5}	Uncompetitive
Sulfosalicilik acid	11.68×10^{-4}	Uncompetitive

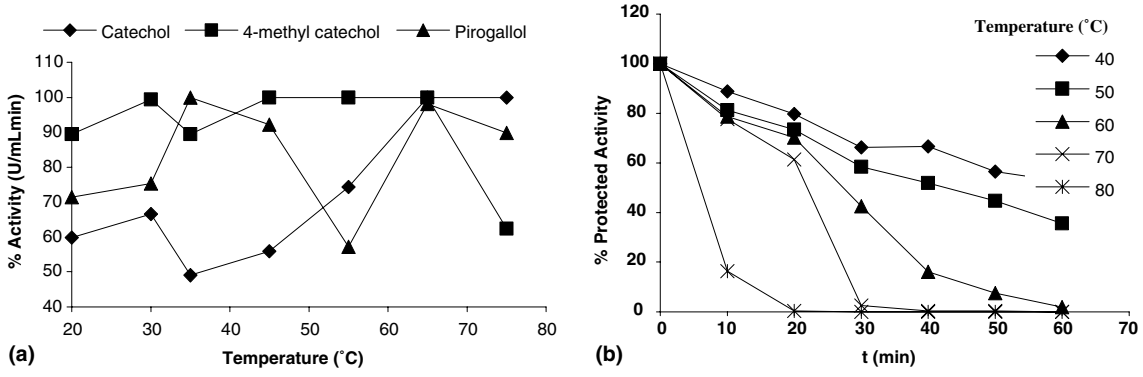

Fig. 4. The effect of temperature on the purified MPPO activity: (a) using different substrates; (b) heating inactivation.

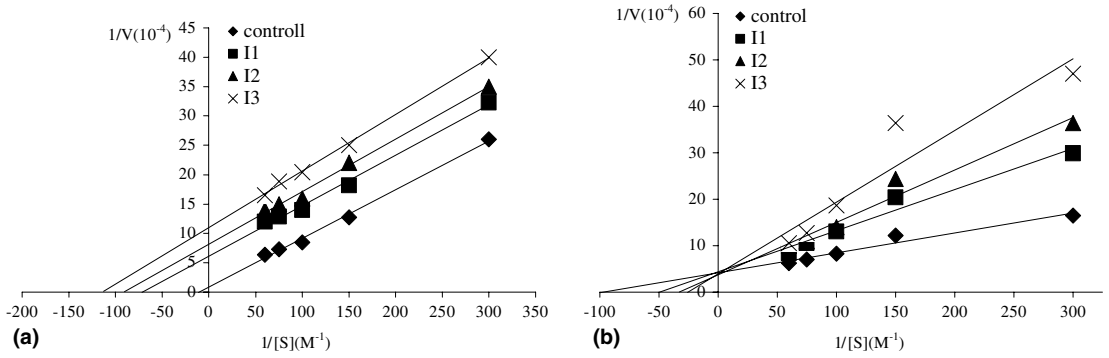

Fig. 5. Inhibition of MPPO from mulberry by *p*-amino benzene sulfonamide: $[I_1]$, 3.33×10^{-3} M; $[I_2]$, 4.17×10^{-3} M; $[I_3]$, 5.00×10^{-3} M K_i; 7.50×10^{-5} (a) by L-cystein $[I_1]$, 4.17×10^{-5} M; $[I_2]$, 6.25×10^{-5} M; $[I_3]$, 8.33×10^{-5} M K_i; 0.035 (b) using as a catechol substrate.

p-Aminobenzenesulfonamide and sulfosalicilic acid were found to be uncompetitive inhibitors of the catecholase activity of MPPO, as illustrated in the Fig. 5 for one of them. K_i value for *p*-aminobenzenesulfonamide is very similar to those potassium metabisulfide and ascorbic acid, which are specific inhibitors (Beena & Gowda, 2000; Billaud, Lecornu, & Nicolas, 1996). Therefore, these compounds can be used to prevent enzymatic browning in mulberry products.

MPPO was also inhibited by the presence of L-sistein in the reaction medium; although this inhibitor was less effective than *p*-aminobenzenesulfonamide as shown by the K_i value obtained from the Dixon plots (Fig. 5). This compound behaved as a classical competitive inhibitor. The similar inhibition type was found in purified PPO of bean seed (Beena & Gowda, 2000). The catecholase activity of MPPO is also inhibited competitively by glutation (data not shown). Among these inhibitors, glutation was the most ineffective inhibitor (K_i: 0.148). Inhibition of thiol compounds is attributed to either the stable colorless products formed by an addition reaction with *o*-quinones or binding to the active center of PPO.

In this study, we have investigated polyphenol oxidase enzyme from mulberry (*M. alba* L.) fruit. This fruit is most often used dried or fresh in some countries. Because it is a kind of nourishing tonic medicine that can broadly be used to cure some debility symptoms when used with other restoratives. And mulberry contains plentiful nutritious elements such as minerals and vitamins, it can cure chronic diseases of the digestive tract, promote gastric juice secretion, strengthen the ability for digesting and assimilating, improve the appetite and eliminate abdominal distension and constipation. For these reasons mulberry is still used in the form of mulberry syrup concentrates in many countries as in Turkey, Azarbejian. While this syrup makes a tasty sweet, it is also used as a medicine to protect against diseases of the liver, gall bladder and heart. Despite of all these applications, browning reactions in fruits and vegetables are recognized as a serious problem in the food industry. The purification of PPO from mulberry also provides a way to study the molecular mechanism underlying the defense role of PPO in mulberry. Further studies of MPPO is necessary to understand its behavior toward the browning reaction on food industry during storage and processing, for this the compounds which have been used in this study as PPO inhibitors can be used to prevent enzymatic browning in mulberry products.

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