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Partial purification and characterization of cresolase and catecholase activity of Black mulberry (Morus nigra)

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Abstract

Polyphenol oxidase from Black mulberries was extracted and partially purified through $(NH_4)_2SO_4$ precipitation, dialysis and ion exchanged chromatography. P-cresol was the best substrate for cresolase activity with a km Michaelis constant (11.5 mM) and catechol for catecholase activity with km (6.4 mM) at PH 5. km for cresolase and the catecholase activities of IsoPPOII at pH 6.5 were recorded as 12 and 8.5 and for IsoPPOIII at pH 8 were 9.5 and 7.5 mM, respectively. Maximum of catalytic efficiency was obtained from the ceaseless activity of IsoPPOIII (92.4 unit. mg^{-1}. mM^{-1}) and minimum of catalytic efficiency was obtained from the catecholase activity of IsoPPOII (42.9 unit. mg^{-1}. mM^{-1}). The enzyme showed high activity over a broad pH range of 3 – 9, so the optimum pH for PPO activity was found to be 5, 6.5 and 8. The optimal temperature for catecholase was gained to be 45 °C for IsoPPOIII but 40 °C for IsoPPOII and IsoPPOI. The affinity of PPOs for various substrates varied widely. The enzyme showed a broad activity over an extended pH and a temperature range. The thermal inactivation studies showed that the IsoPPOIII was more heat-resistant than IsoPPOII and IsoPPOI. The most potent inhibitors were Kojic acid. Kojic acid is a potent inhibitor from IsoPPOIII > IsoPPOII > IsoPPOI, respectively.

Keywords: Inhibition; purification; cresolase; catecholase; Black mulberry; thermal inactivation.

Introduction

Many vegetables and fruits become discolored during storage or processing, an action mediated by the enzyme polyphenol oxidase (PPO) [1,2]. PPO (tyrosinase, EC 1.14.18.1) is a copper-containing enzyme that is wide preaded in plants, and synthesized early in tissue development and stored in chloroplasts [3]. The enzyme is a copper protein distributed widely in a multitude of organisms, from bacteria to mammals [4] Enzymatic browning which is the main function of PPOs in fruit and vegetables, but it is often undesirable and responsible for unpleasant sensory qualities as well as losses in nutrient qualities [5,6]. When cell membrane integrity is disrupted, phenolic substrates encounter the enzyme and are converted to o-quinones in a two-step process of

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hydroxylation of monophenols to diphenols (monophenolase activity), followed by the oxidation of diphenols to o-quinones (diphenolase activity). These highly reactive quinones polymerize with other quinones, amino acids and proteins to produce coloured compounds, and nutrient quality and attractiveness are reduced. PPO from different plant tissues show different substrate specificities and degrees of inhibition. Therefore, characterization of the enzyme could enable the development of more effective methods for controlling browning in plants and plant products. Our objective was to characterize PPO from Black mulberry cultivated in Kurdistan, Iran under different conditions. Substrate and temperature effects were also studied. One unusual characteristic of this enzyme is its ability to exist in an inactive or latent state [7]. PPO can be released from latency or activated by various treatments or agents, including urea [8], polyamines [9], anionic detergents such as Sodium Dodecyl Sulfate (SDS) [10], proteases and fatty acids [11].

Experimental

General

The Black mulberries used in this study were obtained from Kurdistan of Iran and frozen at -25 °C until they were used. Catechol, polyvinyl pyrolidone (PVPP), p-cresol, tyrosine was purchased from Merck (Darmstadt, Germany). Acetone, ammonium sulphate, L-cysteine, kojic acid, L-glycine, polyethylene glycol (PEG), phenyl methyl sulfonyl fluoride (PMSF), cellulose membrane (76x49mm) and DEAE-cellulose were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were of analytical grade.

Enzyme extraction

500 Grams of Black mulberries were homogenized in 500 mL of 0.1M phosphate buffer (pH 6.7) containing 10 mM ascorbic acid and 0.5% polyvinyl pyrrolidone with the aid of a magnetic stirrer for 1 h. The crude extract samples were centrifuged at 30000 g for 20 min at 4 ºC. Solid ammonium sulphate $(NH_4)_2SO_4$ was added to the supernatant to obtain 30 and 80% $(NH_4)_2SO_4$ saturation, respectively. After 1 h, the precipitated proteins for each stage were separated by centrifugation at 30000 g for 30 min. The precipitate was redissolved in a small volume of distilled water and dialyzed at 4 ºC against distilled water for 24 h with 4 changes of the water during dialysis.

Ion exchange chromatography

The dialysate was applied to a column (2.5 cm x 30 cm) filled with DEAE-cellulose, balanced with 10 mM phosphate buffer, pH 6.7. In order to remove non-adsorbed fractions the column was washed with 200 mL of the same buffer at the flow rate of 0.5 mL/min. Then, a linear gradient of phosphate buffer concentration from 20 to 180 mM was applied. 5 mL fractions were collected in which the protein level and PPO activity towards catechol as substrate were monitored. The fractions which showed PPO activity were combined and were used as an enzyme source in the following experiments [12].

Protein determination

Protein contents of the enzyme extracts were determined according to lower my method using bovine serum albumin as a standard [13].

Assay of enzyme activity

Catecholase and cresolase activity were determined by measuring the absorbance at 420 nm using a

spectrophotometer (6305 JENWAY). To determine the best concentration of enzyme preparation corresponding to the highest enzyme activity, the activity was assayed in 3 mL of reaction mixture consisting of 2.5 mL substrate (15 mM catechol and 20 mM p-cresol separately) and different concentrations (0.1-0.3 mL) of the enzyme preparation (1mg/mL). This mixture was topped-up to 3.0 mL with the phosphate buffer (pH 6.7) in a 1 cm light path quartz cuvette. The blank consisted of 3.0 mL 0.1 M phosphate buffer (pH 6.7). Two controls were prepared: the cuvette of the first control contained 2.5 mL substrate and 0.5 mL buffer solution, whereas the second control cuvette contained 2.9 mL buffer and 0.1 mL enzyme preparation. Absorbance values of these controls were subtracted from that sample. PPO activity was calculated from the linear portion of the curve. The initial rate of PPO catalyzed oxidation reaction was calculated from the slope of the absorbance–time curve. An enzyme preparation of 0.175 mL showed the highest activity using catechol as a substrate which was used in all other experiments. One unit of PPO activity was defined as the amount of enzyme that produces 1 micromole of quinine per minute. Assays were carried out at room temperature and results were the averages of at least three assays and the mean and standard deviations were plotted.

PH optimum and stability

PPO activity as a function of pH was determined using catechol and p-cresol as substrates. Phosphate and phosphate-citrate buffer, ranging from pH 3.0 to 9.0 was used in the assays. The pH stability was determined by incubating the enzyme in the above buffer (pH 3.0 to 9.0) for 30 min and at the end of the incubation period, samples were taken and assayed under standard conditions as described above. All of the assays were performed in triplicate. PPO activity was calculated in the form of unit per mg protein at the optimum pH. The optimum pH value obtained from this assay was used in all the other experiments.

Substrate specificity

For determination of Michaelis constant (Km) and maximum velocity (Vmax) values of the enzyme, PPO activities were measured with two substrates at various concentrations. 1/V and 1/[S] values, obtained from these activity measurements, were used for drawing Line weaver– Burk graphs. In order to determine Michaelis constant (*K*m) and maximum velocity (Vm), Catecholase and cresolase activities were measured using catechol (0-100 mM), p-cresol (0-100 mM) as substrates. *K*m and *V*m values of the enzyme were calculated from a plot of hyperbolic of V vs. S.

Effect of temperature on PPO activity

To determine the optimum temperature for PPO, the activity of the enzyme was measured at different temperatures (25-90 °C) using 75 µL enzyme, 2.7 mL of 15 mM catechol as substrate and completed to 3 mL with 0.1 M sodium phosphate buffer (pH 6.7). The blank consisted of 3.0 mL of 0.1 M phosphate buffer. Controls were run under the same tested temperature. The tubes were pre-heated to the selected temperature to prevent temperature lag before the addition of 0.175 mL aliquot of enzyme solution. The enzyme samples were removed from the water bath after pre-set times and were immediately transferred to ice bath to stop thermal inactivation. After the sample was cooled in ice bath, the residual activity was determined spectrophotometrically using the standard reaction mixture. A non-heated enzyme sample was used as

blank. The percentage residual activity was calculated by comparison with the unheated sample.

Effects of inhibitors

The examined inhibitors were L-glycine, L-cysteine and kojic acid. The reaction mixture contained 2.7 mL of catechol at a final concentration of 20 mM in 0.1M phosphate buffer (pH 6.7), 0.1 mL inhibitor at a final concentration of 0.05 and 1 mM and 0.175 mL enzyme solution. The change in absorbance was measured spectrophotometrically at 420 nm. Control tests for inhibitors plus substrate plus buffer were also run at the same time. Percentage inhibition as calculated using the following equation: Inhibition (%) = [(*A*o – *A*i)/*A*o)].100, where, *A*o is the initial PPO activity (without inhibitor) and *A*i is the Catecholase activity with inhibitor.

Results and discussion

PPO was purified from Black mulberries using a DEAE-cellulose column. A summary of extraction and purification is given in Table 1. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to the DEAE-cellulose column, yielding three peaks with PPO activity (Figure 1). A 15-20-fold purification were achieved. Partial purification of Black mulberry PPO led to the isolation of three fractions that exhibited differential substrate utilization and SDS activation profiles, suggesting them to be three PPO is enzymes that we designated IsoPPOI and IsoPPOII and IsoPPOIII. At pH 5, IsoPPOI was much less active with catechol as substrate (486Unit.mg^{-1}.prot) than with p-cresol (959 Unit.mg^{-1}. Prot). IsoPPOII as the same as IsoPPOI was much less active with catechol (365 Unit.mg^{-1}.prot). Taking into consideration the pH activity profile obtained for morus nigra partial purified PPO with catechol as substrate and exhibiting peaks at pHs 5, 6.5 and 8. IsoPPOI was likely to be the main contributor to the peak in acidic pH, while IsoPPOII was likely to be the main contributor to the peak at neutral pH and IsoPPOIII was likely to be the main contributor to the peak at alkaline pH.

Table 1. Purification of PPO from Black mulberry

Entry	Purification step		Volume (mL)	Total Protein (mg)	Total activity (µM/min)	Specific activity (µM/min.mg protein)	Purification (Fold)
1	Crude extract		350	8.9	685	0.22	1
2	$(NH_4)_2SO_4$ precipitation (30%)		194	7.2	632	0.45	2
3	$(NH_4)_2SO_4$ precipitation (80%)		102	6.7	556	0.80	3.6
4	DEAE-cellulose	Fraction 1	23	4.5	345	3.3	15
		Fraction 2	12	3.9	236	5	22.7
		Fraction 3	15	2.8	185	4.4	20

PH optima

Optimum pH for Catecholase and cresolase activity with catechol and p-cresol as substrates were 5, 6.5 and 8 (Figure 2). As the pH increased from 3 to 9, the enzyme activity increased, with maximal activity occurring at pH 5, 6.5 and 8. Differences in optimum pH for PPO with distinct substrates have been reported for the enzyme from

various sources [14-18]. However, pH optima for PPO activity in presence of catechol and p-cresol in Black mulberry is the same (Figure 3).

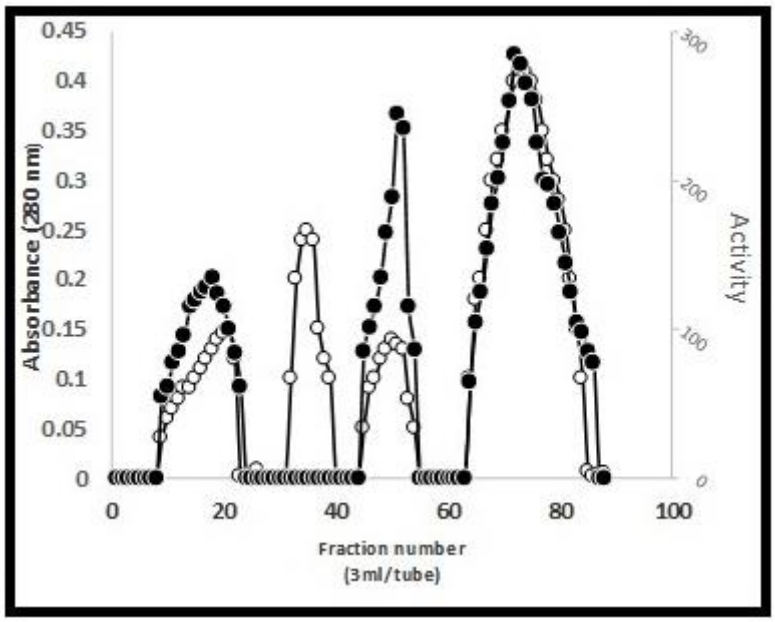

Figure 1. Elution pattern of Black mulberry PPO on DEAE-cellulose. Following ammonium sulphate precipitation, the dialyzed enzyme extract was applied to a 2.5 cm x 30 cm column, equilibrated and washed with 10 mM phosphate buffer, pH 6.7. Elution of adsorbed proteins was performed using a linear gradient of 10 to 200 mM phosphate buffer (pH 6.7) at a flow rate of 0.5 mL/min.

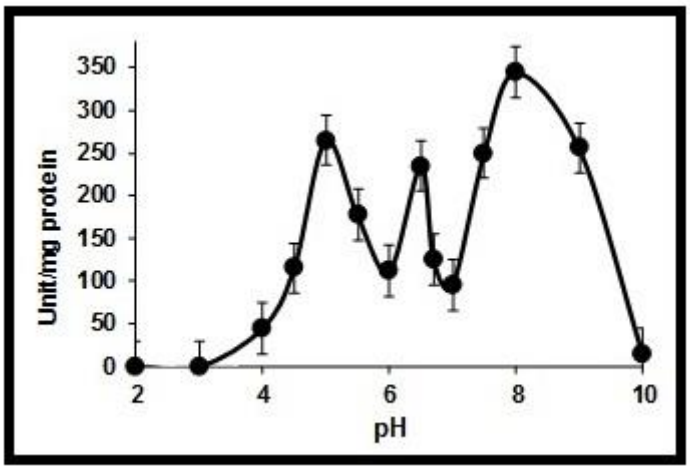

Figure 2. Activity of Black mulberry PPO as a function of pH. Each data point is the mean of three determinations. The vertical bars represent standard deviations

Kinetic parameters

Km and Vm values for Black mulberries PPO for different substrates at pH 5, 6.5 and 8 are presented in Table 2. The affinity of the enzyme varied depending on the substrate used. Black mulberrys PPO had a higher affinity for catechol, as evidenced by the lower *Km* value of pH 5, 6.5 and 8. In accord with Vm/Km ratio, the best substrate is cresolase so, catalytic efficiency of cresolase at pH 5, 6.5 and 8 were 83.3, 61 and 92.4. Of the substrates tested, the best substrate for

Black mulberrys PPO was p-cresol. In a study carried out by Gao jia et al. [22] *Km* value for PPO from the sour cherry pulp was found to be 3.5 mM, using catechol. Duangmal and Apenten [20] reported the following *Km* values for taro PPO: 9.0 mM for 4-methylcatechol, 67.9 mM for catechol and 89.9 mM for p-cresol. The same investigators reported the following *Km* values for potato PPO: 1.1 mM for 4-methylcatechol, 6.7 mM for catechol and 1.5 mM for p-cresol, but km value for Black mulberry catecholase and cresolase activity of PPO(IsoPPOI) is calculated 6.4 catechol and 11.5 mM for p-cresol, so km value for IsoPPOII is calculated 8.5 mM for catechol and 12 mM for p-cresol and km value for IsoPPOIII is calculated 7.5 mM for catechol and 9.5 mM for p-cresol (Table 2). As can be seen, the affinity of PPOs from various sources for various substrates varies widely.

Table 2. Kinetic parameters of Black mulberrys PPO

Entry	pH	Activity	Vmax (Unit.mg^{-1}.prot)	Km (mM)	Catalytic efficiency (units mg^{-1} prot mM^{-1})
1	5	catecholase	486	6.4	75.9
		cresolase	959	11.5	83.3
2	6.5	catecholase	365	8.5	42.9
		cresolase	732	12	61
3	8	catecholase	566	7.5	75.4
		cresolase	878	9.5	92.4

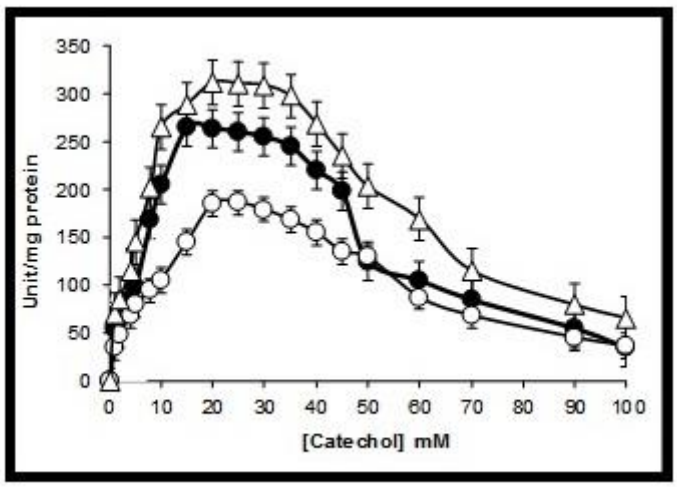

Figure 3. Activity of partial purified polyphenol oxidase in presence of catechol as substrate

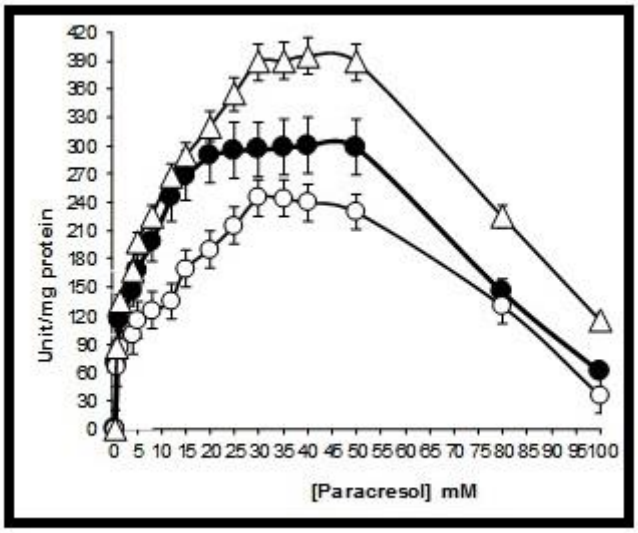

Figure 4. Activity of partial purified polyphenol oxidase in presence of p-cresol as substrate

The effect of temperature on PPO activity

Optimum temperature for cresolase activity at pH 5 with p-cresol as the same as catecholase activity with catechol was 40 °C (Figure 4). This behavior of the PPO enzyme with these substrates was confirmed after several repetitions. Heating for 30 min at 27 and 40 and 70 °C for p-cresol and catechol increases the activity; however, at the higher temperatures, the enzyme after 5-10-minute incubation was rapidly inactivated. Optimum temperatures for PPO activity in other sources were reported to be between 20 and 40 °C. The catecholase and cresolase was reasonably stable at 27 and 40 °C and, as expected, the rate of inactivation was higher with increasing temperature (Figure 5). When enzyme exposed to 40°C, a 150% residual activity was registered for 10 minutes. The times required for 50% inactivation of PPO activity at 70 °C was 7 minutes (Figures 5, 6 and 7). Black mulberry - PPO is a heat-stable enzyme at 40-55°C; so is more resistant to heat than PPO from peppermint [14,17,19].

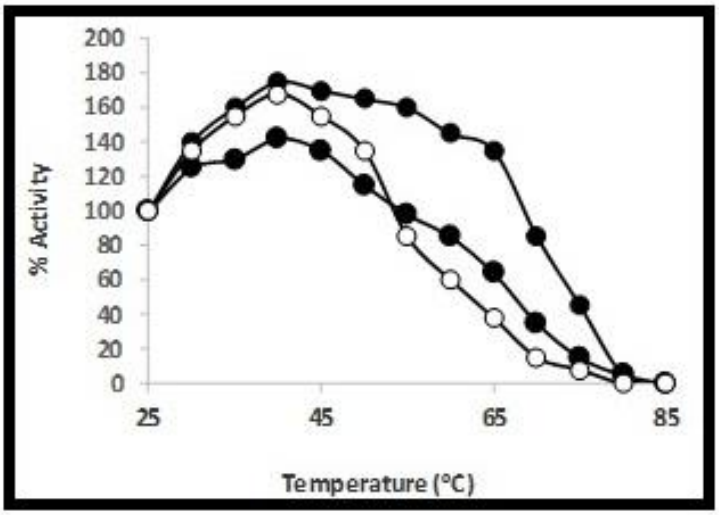

Figure 5. Activity of Black mulberry PPO as a function of temperature. Each data point is the mean of three determinations. The vertical bars represent standard deviations. [(●), catechol 20 mM]and [(○), p-cresol 8 mM].

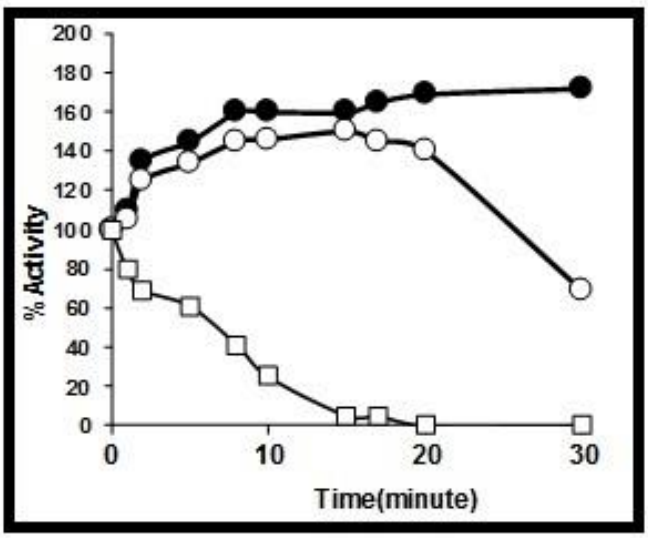

Figure 6. Heat inactivation of PPO at different temperatures. The enzyme was incubated at the temperatures 40, (●); 55, (○) and 70(□) °C) and the remaining activity was determined with catechol as substrate

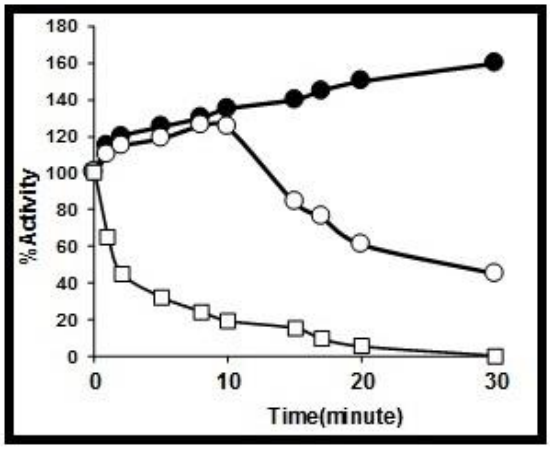

Figure 7. Heat inactivation of PPO at different temperatures. The enzyme was incubated at the temperatures 40, (●); 55, (○) and 70(□) °C) and the remaining activity was determined with catechol as substrate

Effect of inhibitor

The effects of cysteine, glycine and kojic acid on Black mulberrys catecholase and cresolase activity at pH 5, 6.5 and pH 8 were studied at various concentrations using catechol and p-cresol as substrates and the results were reported as percentage inhibition in Table 3. The inhibition degree variation in dose dependent manner. From the results, it can be concluded that the most potent inhibitors were kojic acid, because a higher degree of inhibition was achieved. Cysteine was the least potent inhibitor of IsoPPOI and IsoPPOII and IsoPPOIII at pH 5, 6.5 and 8 but the most potent inhibitor at pH 5, 6.5 and 8 was kojic acid. The most inhibitory effect of kojic acid, glycine and cysteine were PPO at pH 8 > PPO at pH 6.5 > PPO at pH 5. It has been reported that some plant PPOs are membrane-bound. Therefore, use of detergents is required to solubilize the enzyme. Phenol compounds interfere with purification of proteins from plants. They cross-link proteins by hydrogen bonds and covalent interactions. Furthermore, homogenization of the plant tissues initiates enzymatic browning which results in the formation of quinones. The quinones may also form covalent linkages that may not be reversible. The use of phenol-absorbing polymers, such as polyethylene glycol (PEG) or PVPP and use of reducing agents such as ascorbic acid is commonly applied in order to overcome these problems [20]. The pH optimum for PPO activity from Black mulberry was found to be 5, 6.5 and 8. It is noteworthy to mention that the pH optimum for a PPO is found to be dependent on the enzyme source, substrate and extraction methods used. Other reported values include 6.50 for banana peel PPO [21], 4.20 for grape PPO [22], 5.70 for broccoli PPO [23] and 7.5 for avocado PPO [24]. Halder et al. [23] reported an optimum pH value of 5.0 for tea PPO. In a study carried out by Dogan et al. [19] on different aubergine cultivars, the temperature optima varied between 40-45 °C using catechol and p-cresol as substrates. Ding et al. [12] reported an optimum temperature of 30 °C for loquat PPO using chlorogenic acid as substrate. Other reported values include 25 °C for grape PPO [20] and 30 °C for banana PPO [21]. The optimum temperature obtained in this study is 40 °C for catechol and p-cresol at pH 5 and 6.5 and 45-50°C for catecholase

and cresolase at pH 8 that are dependent on the substrate and pH. An optimum temperature (50 °C) for strawberry PPO was reported by Vamos-Vigyazo, L. [20] that is the same of optimum temperature of Black mulberry PPO at pH 7. PPO is generally considered as an enzyme of low thermostability. Heat stability was reported to differ among cultivars and multiple forms of PPO from the same source as well as between fruit tissue homogenates and their respective juices [24]. PPO from Black mulberrys showed high thermal stability at the temperatures studied. The mode of action of inhibitors differs from each other. The mode of inhibition of kojic acid is by reducing the enzyme Cu^{+2} to Cu^{+} rendering the enzyme inactive an unavailable for O_2 binding and by complexing with quinone compounds to prevent melanin formation via polymerization. L-cysteine is a thiol compound, which is a strong nucleophile and suppresses enzymatic browning mainly via formation of colourless addition products with *o*-quinones. At the concentrations tested, the inhibition degrees of the inhibitors were very different. L-cysteine being the least potent inhibitor and kojic acid showed a higher degree of inhibition at pH 5, 6.5 and 8. The least potent inhibitor of IsoPPOII (pH6.5) was cysteine. In a study carried out by Gomez-Lopez (2002), it was found that the most effective inhibitor for avocado PPO was cysteine. Rapeanu et al [19], found that most potent inhibitors for grape PPO were ascorbic acid, cysteine and sodium metabisulfite. In conclusion, after the final purification step, 15, 22.7 and 20-fold purification earned by three fractions. The enzyme showed a broad activity over a broad pH and temperature range. The thermal inactivation studies showed that the enzyme is heat resistant. The enzyme showed the highest activity toward p-cresol.

Table 3. Effect of inhibitors on Black mulberrys PPO activity

Entry	pH	Inhibitor	Concentration (mM)	% Inhibition
1	5	Cysteine	0.1	19 ± 1
			1	46 ± 2
		Glycine	0.1	22 ± 2
			1	51 ± 1
		Kojic acid	0.1	47 ± 2
			1	85 ± 3
2	6.5	Cysteine	0.1	33 ± 2
			1	72 ± 3
		Glycine	0.1	39 ± 2
			1	78 ± 3
		Kojic acid	0.1	59 ± 2
			1	92 ± 4
3	8	Cysteine	0.1	41 ± 2
			1	78 ± 3
		Glycine	0.1	46 ± 3
			1	79 ± 3
		Kojic acid	0.1	65 ± 3
			1	98 ± 4

*Each value is the mean of three determinations ± standard deviations

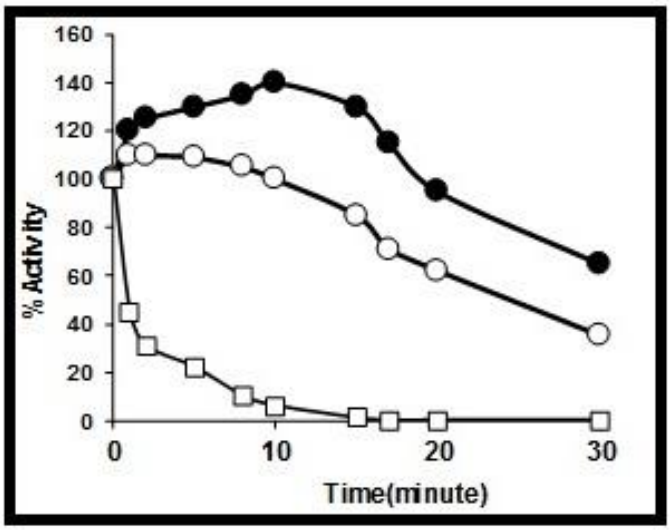

Figure 8. Heat inactivation of PPO at different temperatures. The enzyme was incubated at the temperatures 40, (●); 55, (○) and 70(□) °C) and the remaining activity was determined with catechol as substrate

Of the inhibitors tested, the most potent inhibitors were kojic acid. Different properties for PPO at pHs 5, 6.5 and 8 showed that there are probably at least three isoenzymes.

Conclusion

Partially purified polyphenol oxidase, from Black mulberrys, showes cresolase activity with a Km (11.5 mM) and catecholase activity with Km (6.4 mM) at pH 5. Km for cresolase and catecholase activity of IsoPPOII at pH 6.5 were 12 and 8.5 and for IsoPPOIII at pH 8 were 9.5 and 7.5 mM, respectively. The enzyme showed high activity over a broad pH range of 3 – 9, so the optimum pH for PPO activity was found to be 5, 6.5 and 8. The optimal temperature for catecholase was found to be 45°C for IsoPPOIII but 40°C for IsoPPOII and IsoPPOI. Affinity of PPOs for various substrates varies widely. The enzyme showed a broad activity over a broad pH and temperature range. The thermal inactivation studies showed that the IsoPPOIII is more heat-resistant than IsoPPOII and IsoPPOI. The most potent inhibitor was kojic acid. Kojic acid is a potent inhibitor of IsoPPOIII > IsoPPOII > IsoPPOI.

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References

[1] W. Broothaerts, J.LI.B. Mcpherson, E. Randall, W.D. Lane, P.A. Wierma. *J. Agric. Food Chem*., **2000**, *48*, 5924-5928.

[2] J. Mejri, A. Aydi, M. Mejri, *Asian Journal of Green Chemistry*, **2018**, *2*, 246-267.

[3] C.W. Van Gelder, W.H. Flurkey, H.J. Wichers. *Phytochemistry*., **1997**, *45*, 1309-1323.

[4] D.A. Robb. In: Contie R (ed), *CRC Press. Boca Raton.*, **1984**, *2*, 207-241.

[5] A. Sanchez-Amat, F. Solano, *Biochem.Biophys. Res. Comm*., **1997**, *240*, 787-792.

[6] M. Izadi, M. Fazilati, *Asian Journal of Green Chemistry*, **2018**, *2*, 346-379.

[7] F. Gandia-Herrero, F. Garcia-Carmona, J. Escribano. *J. Agric. Food Chem*., **2004**, *52*, 609-615.
[8] M. Okot-Kotber, A. Liavoga, K.J. Yong, K. Bagorogoza, *J. Agric. Food Chem*., **2002**, *50*, 2410-2417.
[9] M. Jimenezatienzar, M. Pedreno, F. Garciacarmona, *Biochem. Int.*, **1991**, *25*, 861-868.
[10] R.K. Santosh, P. Beena, A.G. Appu Rao, L.R. GOWDA, *Biochem. J*., **2006**, *395*, 551-562.
[11] J.H. Golbeck, K.V, Cammarata, *Plant Physiology*, **1981**, *67*, 977-984.
[12] CK. Ding, K. Chachin, Y. Ueda, Y. Imahori, J *Agric Food Chem*., **1998**, *46*, 4144-4149.
[13] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, *J. Biol. Chem*., **1951**, *193*, 265-27 .
[14] E.J Lourenço, J.S. Leão and V.A. Neves, *Journal of Science Food and Agriculture*, **1990**, *52*, 249-259.
[15] E.J. Lourenço, V.A. Neves and M.A. Da Silva, *Journal of Agricultural and Food Chemistry*, **1992**, *40*, 2369-2373.
[16] E.M. Gonzalez, B. Ancos and M.P. Cano, *Journal of Agriculture and Food Chemistry*, **2000**, *48*, 5459-5464.
[17] D. Kavrayan, T. Aydemir, *Food Chemistry*, **2001**, *74*, 147-154.
[18] S. Dogan, Y. Turan, H. Ertürk, O. Arslan, *Journal of Agriculture and Food Chemistry*., **2005**, *53*, 776-785.
[19] G. Rapeanu, A. Van Loey, C. Smout, M. Hendrickx, *Food Chemistry*, **2006**, *94*, 253-261.
[20] L. Vamos-Vigyazo, *Crit Rev Food Sci.*, **1981**, *15*, 49-127.
[21] C-P. Yang, S. Fujita, K. Kohno, A. Kusubayashi, MD. Ashrafuzzaman, N.H ayashi, *J Agric Food Chem*., **2001,** *49*, 1446-9.
[22] MÜ. Ünal, A. Fiener, *J Sci Food Agric.*, **2006**, *86*, 2374-2379.
[23] U. Gawlik-Dziki, U. Szymanowska, B. Baraniak, *Food Chem.*, **2007**, *105*, 1047-1053.
[24] VM. Gomez-Lopez, *Food Chem.*, **2002**, *77*,163-169.

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