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Characterization of Polyphenol Oxidase and Peroxidase From Iranian Medlar (*Mespilus germanica* L.) Fruit

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ABSTRACT

In this study, the crude protein extract containing PolyPhenolOxidase (PPO) and Peroxidases (POD) were extracted from medlar fruit (*Mespilus germanica* L.) grown in Golestan Province, Iran. POD and PPO activities were studied using guaiacol and catechol as substrates, respectively. The effect of pH, temperature and thermal stability, inhibitors and cations were investigated. Results showed that V_{max} was higher for PPO compared to the POD. The optimum pHs for POD and PPO were obtained at 6.5 and 5.5, respectively. The optimum temperature for both enzymes was 35°C. The Iranian medlar POD was more thermal stable than the PPO. Ascorbic acid had the highest inhibitory effect on both enzymes. Ca^{2+} and Zn^{2+} had the highest decreasing and increasing effect on both enzymes.

Keywords: Characterization, Medlar, Peroxidase, Polyphenoloxidase.

INTRODUCTION

Peroxidase (POD, EC 1.11.1.7) are plant hemoproteins and oxidoreductase which catalyze a reaction in which hydrogen peroxide dose is used as the acceptor and another substance dose as the donor of hydrogen atom. POD is directly involved in many plant functions such as hormone regulation, defense mechanisms, indolacetic degradation and lignin biosynthesis (Serrano- Martínez *et al*., 2008). Moreover, POD is concerned with enzymatic browning because diphenols may function as reducing substrate in enzymatic browning (Chisari *et al*., 2007).

Plant polyphenoloxidases (PPO, EC 1.14.18.1) are a group of copper-containing enzymes that catalyze oxidation of polyphenolic compounds which are responsible for enzymatic browning reactions occurring during various stages of processing plant materials (Sheptovitsky and Brudwig, 1996).

Enzymatic browning is a main problem in a number of fruits and vegetables such as potato (Lee and Park, 2007), lettuce (Gawlik-Dziki *et al*., 2007) and strawberry (Chisari *et al.,* 2007) which leads to rejection by the consumer. This fact is caused by conversion of phenolic compounds to *o*-quinones, which subsequently polymerize to be a brown pigment (Jiang *et al.,* 2004). In addition, enzymatic browning may alter plant proteins and be more toxic to potential phytopathogens (Aydemir, 2004). POD and PPO are involved enzymes in the reaction, therefore, they have been characterized in several plants such as the Chinese cabbage (Nagai and Suzuki, 2001), hot pepper (Arnnok *et al*., 2010), potato (Marri *et al*., 2003), broccoli (Gawlik-Dziki *et al*., 2007), hot chilli pepper (Schweiggert *et al*., 2006), thymus (Dogan and Dogan, 2004), and pear (Hwang *et al*., 1996).

Medlar, *Mespilus germanica* L., is a member of Rosaceae, known with local

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names “Conos” or “Condos” in North of Iran. The fruit is brown, ranging from 1.5 to 3 cm in diameter and consumed fresh as well as in jams, jellies and marmalades (Bignami, 2000). The native form of medlar is widely found at forests in Golestan and Mazandaran Provinces. The Iranian medlar fruits are harvested every year through December and January. The fruit has several medical benefits such as elimination of kidney and bladder stones, constipation therapy and as a diuretic (Ayaz *et al*., 2008).

Aydin and Kadioglu (2001) reported total soluble sugar, ascorbic acid contents and PPO activity of Turkish medlar and Dincer *et al*. (2002) studied characterization of PPO in Turkish medlar. However, there is not enough study about the chemical composition of the Iranian medlar fruits. Thus in this paper characterization of POD and PPO from Iranian medlar fruit was studied in terms of optimum pH and stability, optimum temperature and stability, substrate specificities, degrees of inhibition by general enzyme inhibitors and effect of metal ions in order to help predict the behavior of Iranian medlar fruit POD and PPO.

MATERIALS AND METHODS

Medlar fruits (*Mespilus germanica* L) were harvested from forested regions of Gorgan, Iran. The fruits were immediately brought to the laboratory and stored at -20°C. All chemicals and reagents used were analytical grade and purchased from Merck, Germany.

Enzyme Extraction

The enzymes from Iranian medlar fruits were extracted using the method described by Ayaz *et al*. (2008) with some modifications. Briefly, the samples were powdered by a grinder (Model 160 Specimen Grinder, 18 mm diameter) and followed by 50 g the powdered medlar fruit was homogenized in 100 ml of 50 mM cold acetate baffer (pH 5.5), containing 20 ml of 4% Triton X-114, 1 mM $MgCl_2$, 1 mM PhenylMethylSulfonylFuoride (PMSF) as protease inhibitor, 2 mM EDTA, for 2 minutes. The homogenate was filtered through Whatman No. 42 filter paper and kept for 1 hour. to complete separation and followed by centrifuging at 20,000×g for 30 minutes. The supernatant was filtered through Whatman No. 42 filter paper and the supernatant containing protein extract was collected and stored at –20°C until use. It was used as a crude enzyme extract for POD and PPO analyses. All steps of enzyme extraction were performed at 4°C (Ayaz *et al*., 2008).

Protein Determination

Soluble protein content of the extracts was measured according the method depicted by Gawlik-Dziki *et al*. (2007), with bovine serum albumin as the standard.

Enzyme Assays

PPO activity was spectrophotometrically measured using the method described by Dincer *et al*. (2002) with some modifications and using an ultraviolet-visible UV-160A spectrophotometer (Shimadzu, Japan) equipped with a quartz cell of 1 cm length. The activity was measured by calculating the increase in absorbance at 500 nm and pH 7. The reaction mixture consisted of phosphate buffer solution pH 7 (0.1M, 1.95 mL), 1 mL of 0.1M catechol as a substrate and 50 µL of the enzyme extract. The absorbance at 500 nm was recorded continuously at 25°C for 5 minutes. The blank sample was also considered containing the same mixture solution without the enzyme extract (Dincer *et al*., 2002).

POD activity was spectrophotometrically determined at 470 nm using guaiacol as a phenolic substrate with hydrogen peroxide. The reaction mixture contained 2.66 mL of

0.1 M phosphate buffer pH 7, 0.15 mL of 4% (v/v) guaiacol, 0.15 mL of 1% (v/v) H_2O_2, and 40 µL of the enzyme extract. The blank sample was considered as well (Sahhafi *et al*., 2012).

One unit of PPO and POD activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 min^{-1} (Galeazzi and Sgarbieri, 1981). All the experiments were carried out in triplicates and corresponding means were plotted.

Effect of Substrate Concentration

The POD and PPO activity were assayed by mixing the enzymatic extract with their substrate at the different final concentrations, 2, 5, 10, 20, 30, 40, 50, and 60 mM. The kinetic results were plotted as 1/specific activity (1/V) *versus* 1/substrate concentration (1/[S]). The maximum Velocity (V_{max}) and Michaelis–Menten constant (K_m) indexes were measured with variable substrate concentrations in the reaction mixture. Substrate specificity (V_{max}/K_m) was measured using the data obtained on a Lineweaver– Burk plot (Guo *et al*., 2009).

Effect of pH

The activity of PPO and POD was measured using 0.1M citrate buffer (pH 3.5-5), 0.1M phosphate buffer (pH 6-8) and 0.01 M Tris-HCl (pH 8-9.5). The optimum pH for the PPO and POD was obtained using catechol and guaiacol as substrates, respectively (Ayaz *et al*., 2008).

Effect of Temperature

The PPO and POD activity was measured at various temperatures controlled by a water bath (Memmert, Germany). The mixtures of substrate solution and buffer were incubated for 5 min. at various temperatures over the range of 20-65°C at the optimum pH values of the substrates, before the addition of the enzyme solution. The relative activity of PPO and POD were spectrophotometrically measured for each temperature by rapid addition of enzyme extract to the mixture. In order to determine the thermal stability of the enzymes, the enzyme solution in 50 mM phosphate buffer, pH 6.5 (because at this pH, the enzymes had high activity), was incubated in a water bath at temperatures of 30, 40, 50, 60 and 70°C for 10, 20, 30, and 40 minutes. After the mixture was cooled to room ambient temperature, 0.05 ml heated enzyme extract was mixed with the mixtures of substrate solution, and the enzymes activity residuals were spectrophotometrically measured. The residual percentage of PPO and POD activity was calculated by comparison with unheated respective enzyme (Gawlik-Dziki *et al*., 2007).

Effect of Inhibitors

Inhibition of enzyme by sodium azide (0-10 mM), sodium cyanide (0-10 mM) and ascorbic acid (0-5 mM) were measured. Since in pretests was found that ascorbic acid have high inhibitory activity than the other compounds and therefore a lower concentration was used from it. Percent activity graphs were drawn from these triplicate results for each inhibitor to find IC_{50} values, which show 50% inhibition of the enzyme.

Effect of Metal Ions

Na^+, K^+, Zn^{2+}, Ni^{2+}, Ca^{2+} and Fe^{3+} were used as metal ions to measure POD and PPO activity. The concentrations of each metal ion in the enzyme assay were 0.2 and 0.5 mM. The concentrations were used giving the performed pretests. The percentage of relative activities were measured by comparison with standard assay mixture with no metal ion added (Ayaz *et al*., 2008).

Statistical Analysis

All statistical analyses were performed using Minitab® version 16.1.1 (Minitab Inc. USA. 2010). Data from the experiments were subjected to student *t*-test. Values $P< 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Effect of Substrate Concentration

The maximum reaction Velocity (V_{max}), Michaelis–Menten constant (K_m) and V_{max}/K_m values of the Iranian medlar fruit POD and PPO activity, were calculated by Lineweaver and Burk graph and using catechol and guaiacol as substrates for the PPO and POD, respectively. According to Table 1, PPO shows higher V_{max} and K_m compared to POD. Saturation curves can be extracted from the substrate for each enzyme, the optimum activity values were obtained at 30 mM catechol and 40 mM guaiacol concentrations for PPO and POD, respectively.

Effect of pH

The pH activity profile for crude enzyme extract of Iranian medlar is shown in Figure 1 (a and b). The medlar PPO and POD were significantly affected by pH ($P< 0.05$). Figure 1 shows that both enzymes had very low activity in extreme acidic (pH< 4) and alkaline conditions (pH> 9). The optimum activity of the medlar PPO was achieved at pH 5.5. However, there are two peaks (the points with high enzyme activity) in the pH activity profile for POD, pH 6.5 and 8.5. According to Fullbrook (1996) theory, having several optimum pHs for a type enzymatic activity in a solution indicates presence of distinctly isoenzymes of a specific enzyme, there are two izoenzymes for Iranian medlar POD with optimum pH 6.5.

The optimum pH for any enzyme relies on plant materials and type of substrate in the activity assay (Fortea *et al*., 2011). Generally, most herbal enzymes show maximum activity at or near neutral pH. The optimum pH is 6.0- 8.5 for kiwifruit POD applying *p*-phenyllenediamine as a substrate (Fang *et al*., 2008), 6.5 for Turkish medlar PPO using 4-methycatechol as a substrate (Dincer *et al*., 2002), the optimum pHs are 6.8 and 5.5 for butter lettuce PPO by 4-methycatechol and catechol as substrates, respectively (Gawlik-Dziki *et al*., 2007). The optimum pH was reported 6.0 for spring cabbage POD using guaiacol as substrate (Belcarz *et al.,* 2008), and pH 6.5 for longan fruit PPO using 4-methycatechol as substrate (Jiang *et al*., 1999).

Effect of Temperature

The effects of assay temperature between 5 and 75˚C were determined using guaiacol with hydrogen peroxide and catechol as a substrate for the POD and PPO, respectively [Figure 2 (a and b)]. Figure 2 shows the optimum temperature is 35˚C for both enzymes; so that both enzymes have 100% of relative activity at this temperature. Arnnok *et al.,* (2010) reported that the optimum temperatures for PPO and POD of hot pepper (*Capsicum annuum* L.) were 30 and 40˚C, respectively. Moreover, it has

Table 1. Effect of substrate concentration of Iranian medlar POD and PPO**.**

	POD	PPO
V_{max} (U mg^{-1})	83	118
K_m (mM)	4.7	6.3
V_{max}/ K_m	0.017	0.018
Optimum pH	6.5	5.5
Optimum temperature (°C)	35	35

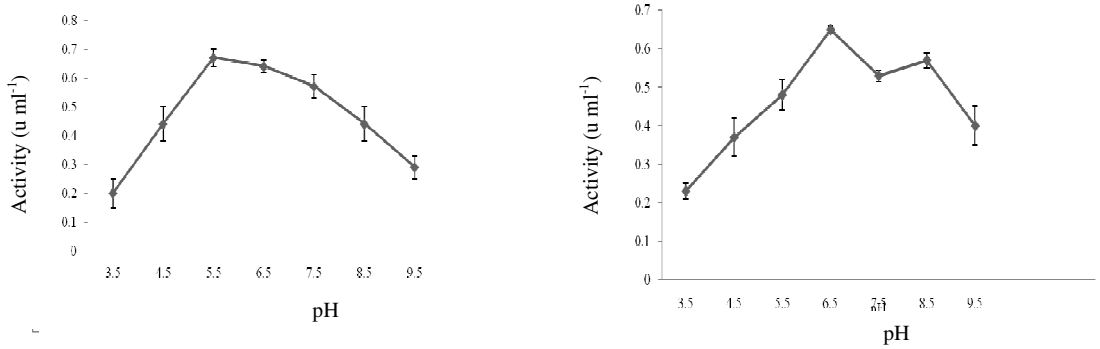
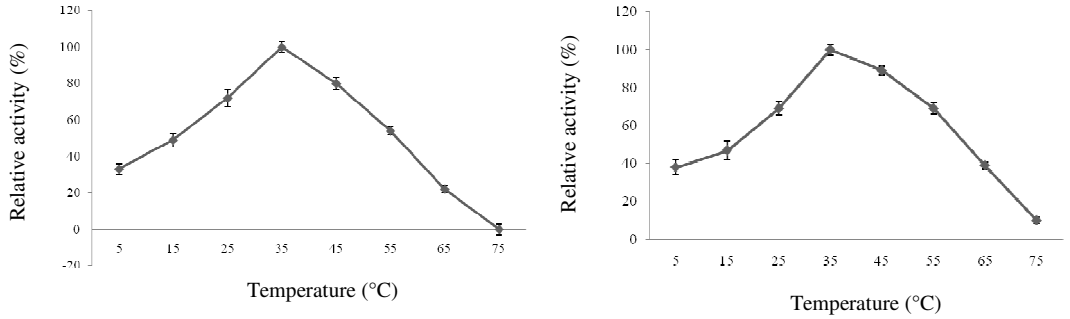


Figure 1. pH-activity profiles for Iranian medlar PPO (a) and POD (b).

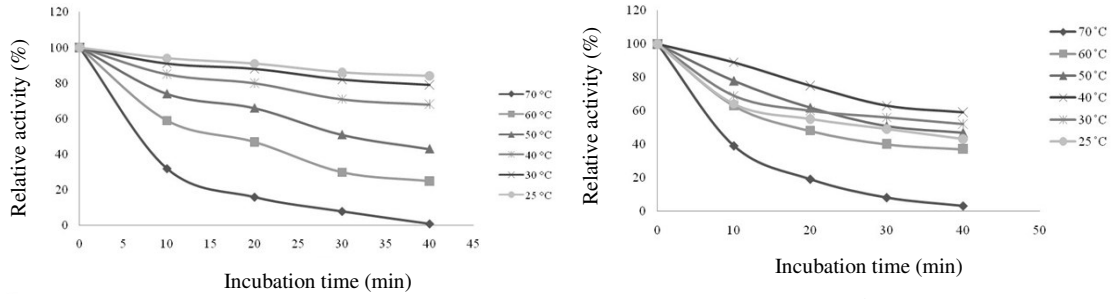


(a) (b)

Figure 2. Determination of optimum temperatures for Iranian medlar PPO (a) and POD (b) activities.

been reported that the optimum temperatures for POD of red alga (Fortea *et al*., 2011), PPO of butter lettuce (Gawlik-Dziki *et al*., 2008), PPO of Turkish medlar (Dincer *et al*., 2002) and PPO of plum (Siddiq *et al*., 1992) were 25, 35, 35 and 37˚C, respectively.

Figures 3-a and -b present the thermal stability profile for the crude PPO and POD, in the form of the residual percentage activity. Figure 3 indicates that Iranian medlar POD had more thermal stability/p'] PP than the PPO. Arnnok *et al*. (2010) evaluated the thermal stable of POD and PPO from hot pepper (*Capsicum annuum* L.) and observed similar results (more thermal stability of POD compared to PPO); so that the POD showed 37% relative activity after heating at 60°C for 40 minutes, however this value was 25% for the PPO. The times required for 50% inactivation of relative activity for the POD at 50, 60 and 70°C were achieved to be 32, 18 and 7 minutes, respectively. However, these values were found at 30, 16, 6 minutes for the PPO,



(a) (b)

Figure 3. Thermal stabilities of Iranian medlar PPO (a) and POD (b).

Table 2. Inhibitory activity of each the inhibitors against the medlar POD and PPO.

Inhibitor	IC_{50} (mM)	
	POD	PPO
Ascorbic acid	0.19	0.11
Sodium cyanide	10	6
Sodium azide	26	14
Benzoic acid	2.1	0.95

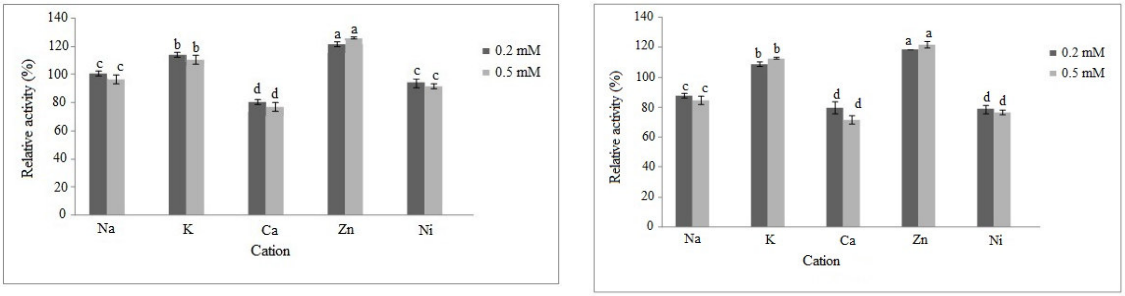
respectively. For Turkish medlar PPO, the times required for 50% inactivation of relative activity at 60 and 80°C were 15 and 6 minutes, respectively. However, it was not inactive at 50°C (Dincer *et al*., 2002). It has been observed that Stanley plum PPO at 70°C, banana PPO, at 70°C and Jerusalem artichoke PPO at 60°C were stable for 30 minutes and *Allium* sp. PPO was stable at 40°C for 30 minutes (Aydemir, 2004).

Effect of Inhibitors

Enzymatic browning of fruits can be reduced by using appropriate inhibitors and generally, the mechanism of this inhibition is different depending on the compound use. In this study, four inhibitors (ascorbic acid, sodium cyanide, sodium azide and benzoic acid) were used to prevent enzymatic browning. Table 2 shows inhibitory activity of each inhibitor against the medlar POD and PPO as IC_{50} values. Among all the inhibitors used, ascorbic acid was the most effective inhibitor for medlar POD and PPO, followed by benzoic acid. The results were consistent with green bean obtained by Guo *et al*. (2009), Victoria grape (Rapeanu *et al*., 2006), *Leucaena leucocephala* (Pandey *et al*., 2011), Turkish black radish (Şişecioğlu *et al*., 2010) and cherry laurel (Colak *et al*., 2005). Dincer *et al*. (2002) reported that cysteine showed the highest inhibitory effect on Turkish medlar PPO.

Effect of Various Metal Ions

Generally, enzymes need cations for increasing their structural stability and activity. In fact, cations can cause changes in the structure and function of enzymes by absorbing water from the surrounding environment of enzyme (Bisswanger, 2004). Thus, the medlar POD and PPO activity was assayed in the presence of various cations at 0.2 and 0.5 mM concentrations from Figures 4-a and -b, K^+ significantly increased the medlar POD and PPO activity ($P< 0.05$). However, Na^+ insignificantly decreased this activity at both concentrations ($P> 0.05$). Seen in Figure 4, Zn^{2+} showed the most increasing effect on the activity of both enzymes among divalent cations, so that these values were 22 and 26% for POD and PPO at 0.5 mM concentrations, respectively. Ca^{2+} had the most decreasing effect on the



(a) (b)

Figure 4. Effect of various metal ions on Iranian medlar fruit PPO (a) and POD (b). Different letters for cations indicate significant difference ($P< 0.05$).

activity of both enzymes (Figure 4). Ayaz *et al*. (2008) reported that Zn^{2+} and Cu^{2+} have the most increasing and decreasing effect on Turkish medlar PPO, respectively.

CONCLUSIONS

The results of this paper revealed that the Iranian medlar PPO has more V_{max} and K_m than the POD. The medlar POD and PPO have high enzymatic activity and they act at wide range of pH and temperature. The optimum pH for the PPO and POD activity were obtained as 5.5 and 6.5, respectively. The optimum temperature for the activity of both enzymes was obtained at 35°C. According to the results, the POD showed more thermal stability compared to the PPO. Ascorbic acid showed the highest inhibitory effect on both enzymes among all the other inhibitors used. Zn^{2+} and Ca^{2+} had the highest increasing and decreasing effect on both enzymes. Results of this study revealed characteristics of Iranian medlar POD and PPO, which can be used in the inactivation of enzymes processes, like blanching.

ACKNOWLEDGEMENTS

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مطالعه خصوصیات پلی‌فنل‌اکسیداز و پراکسیداز میوه ازگیل ایرانی

م. یلمه، و ع. صادقی ماهونک

چکیده

در این مطالعه، عصاره‌ی پروتئینی خام حاوی پلی‌فنل‌اکسیداز و پراکسیداز از میوه ازگیل (*Mespilus germanica* L.) رشد یافته در استان گلستان، استخراج شد. فعالیت پراکسیداز و پلی-فنل‌اکسیداز به ترتیب با استفاده از سوبستراهای گایکول و کاتکول بررسی شد. تاثیر pH، دما و پایداری حرارتی، ممانعت‌کننده‌ها و کاتیون‌ها بر فعالیت آنزیم‌های پراکسیداز و پلی‌فنل‌اکسیداز بررسی شد. مطابق نتایج، پلی‌فنل‌اکسیداز ازگیل ایرانی شاخص‌های V_{max} و K_m بیشتری را در مقایسه با پراکسیداز این میوه دارد. pH بهینه برای فعالیت پراکسیداز و پلی‌فنل‌اکسیداز این میوه به ترتیب ۶/۵ و ۵/۵ اندازه-گیری شد. دمای بهینه برای فعالیت این دو آنزیم $^{\circ}C$ ۳۵ مشاهده شد. پراکسیداز ازگیل ایرانی پایداری حرارتی بیشتری نسبت به پلی‌فنل‌اکسیداز این میوه نشان داد. اسید آسکوربیک در میان ممانعت‌کننده‌های مورد استفاده بیشترین اثر ممانعت‌کنندگی را بر فعالیت هر دو آنزیم نشان داد. کاتیون‌های کلسیم و روی به‌ترتیب بیشترین اثر کاهشی و افزایشی را بر فعالیت هر دو آنزیم داشتند.