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 $V_{\text{max}}$ 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 diphensol 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 buffer (pH 5.5), containing 20 ml of 4% Triton X-114, 1 mM MgCl₂, 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) H₂O₂, 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 (Vₘₐₓ) and Michaelis–Menten constant (Kₘ) indexes were measured with variable substrate concentrations in the reaction mixture. Substrate specificity (Vₘₐₓ/Kₘ) 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 0.01M 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 IC₅₀ values, which show 50% inhibition of the enzyme.

**Effect of Metal Ions**

Na⁺, K⁺, Zn²⁺, Ni²⁺, Ca²⁺ and Fe³⁺ 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 isoenzymes 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  |
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 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.
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

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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$ (mM)</th>
<th>POD</th>
<th>PPO</th>
</tr>
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<tbody>
<tr>
<td>Ascorbic acid</td>
<td>0.19</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Sodium cyanide</td>
<td>10</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Sodium azide</td>
<td>26</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>2.1</td>
<td>0.95</td>
<td></td>
</tr>
</tbody>
</table>

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).
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 $V_{\text{max}}$ 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.

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دراست مطالعه خصوصیات پلی فنل اکسیداز و پراکسیداز میوه ازگیل ایرانی

م. پلشه، و.ع. صادقی ماهونته

چکیده

در این مطالعه، عصاره پروتئینی خام حاوی پلی فنل اکسیداز و پراکسیداز از میوه ازگیل (Mespilus germanica L.) رشد یافته در استان گلستان، استخراج شد. فعالیت پراکسیداز و پی-فیل اکسیداز به ترتیب با استفاده از سویسراهای گایکول و کاتکول بررسی شد. تاثیر pH دما و پایداری حرارتی، ممانعت کننده‌ها و کاتیون‌ها بر فعالیت آنزیم‌های پراکسیداز و پی-فیل اکسیداز بررسی شد. مطلق نتایج، پی-فیل اکسیداز ازگیل ایرانی شاخص های K_m و V_max توسط کاتون‌ها و pH بهبود یافته برابر فعالیت پراکسیداز و پی-فیل اکسیداز اثر می‌یابد. در غیر این موارد، استفاده بیشترین اثر ممانعت کننده‌های را بر فعالیت دو آنزیم نشان داد. کاتیون‌های کلسیم و روزبه‌دارتی بیشترین اثر کاهشی و افزایش را بر فعالیت دو آنزیم داشتند.