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Characterization peroxidase from Prickly lettuce (*Lactuca serriola* L.) Leaves

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The molecular mass of peroxidase from Prickly lettuce (*Lactuca serriola* L.) leaves was 43 kDa as determined by gel filtration through Sephacryl-S200 column, the maximum POD activity at pH 7 and it was stable at pH range of 5.0-8.0 with retaining more than 80% of its original activity, the optimum temperature of POD activity and stability was 50°C, the inhibition (%) of POD activity was 100, 100, 95.72, 64.39, 48.65, 39.46 and 27.98% by using 1 mM of sodium floride, thio urea, EDTA, sodium di ethyl thio carbamate, sodium azide, magnesium sulphate and 2-Mercapto ethanol, respectively, the Km and Vmax of the POD for guaiacol as a substrate was 1.11mM and 0.71 EU/mL, respectively, the enzyme showed substrate specificity for guaiacol is used as variable substrate and H_2O_2 is used at saturated concentrations. Peroxidase was use for remove of phenolic compound from aqueous solution, the use of 20U\mg of POD lead to removed 17, 34, 58, 69, 81 and 93 of 4-chlorophenol; 36, 52, 81, 94, 110 and 100% of Phenol at 5, 10, 15, 20, 25 and 30 min, respectively.

Keywords: Peroxidase, molecular weight, characterization, inhibitors Km and Vmax, phenolic compound.

INTRODUCTION

Peroxidase (POD) belong to the oxidoreductase [EC 1.11.1.7] is heme and carbohydrate protein which a catalyzes the oxidation of various substrates at the expense of hydrogen peroxide as electron acceptor (Şişecioğlu et al. 2010; Goyal and Chugh, 2014), it's commonly used in wide range of applications such as food industry, pharmaceutical industry, bio-sensor construction, synthesis of various aromatic compounds, glucose identification, dyes decolorization, waste water treatment, phenolic compounds treatment, enzyme immunoassays and polymer synthesis (Hu et al. 2012).

Peroxidase generally found in plants such as Tree legume (*Leucaena leucocephala*) (Pandey and Dwivedi, 2011), Omb (*Phytolacca dioica* L.) (Guida et al. 2011), Papaya (*Carica papaya* L.)(Pandey et al. 2012), Sweet gourd (*Cucurbita moschata lam. poiret*) (Koksal et al. 2012) and Horseradish (*Armoracia rusticana* L.) (Sarika et al. 2015), its located in cell wall and participated in a wide types of physiological processes during life cycle of plants, as in controlling growth and ripening, ethylene biogenesis, phenol oxidation, cell metabolism, salt stress tolerance, organogenesis, seed germination, auxin oxidation, protection of tissue cells from damage as a results for infection by pathogenic microorganisms and wound healing. Is also related to quality of vegetables and fruits particularly flavor, ripening and enzymatic browning (Şişecioğlu et al. 2010; Pandey et al. 2012).

Several methods have been used to purified peroxidase from various plants and studied its characteristics such as precipitate by $(NH_4)2SO_4$, purification by ion exchange, gel filtration and affinity chromatography as Black radish(*Raphanus sativus* L.)(Şişecioğlu et al. 2010), Citron (*Citrus medica* L.)(Mall et al. 2013),

Pearl millet (*Pennisetum glaucum* L.) (Goyal and Chugh, 2014), Turnip roots (*Brassica rapa var. rapa* L.) (Kalin et al. 2014), White cabbage (*Brassica oleracea var capitata f. alba*) (Erdem et al. 2015) and Wheat (*Triticumae stivum ssp. vulgare*) (Altın et al. 2017).

Phenolic compounds are considered common pollutants in raw drinking, river and lake water which cause a danger effect to public health due to difficult biodegradable of these compounds through classical of water treatment (Bilal et al. 2018). Chemical and microbiological methods are very expensive, rise toxic chloro-organic compounds and potential risks of microorganisms (He et al. 2008). Therefore, the use of enzymes have become as a preference chosen for removal of these compounds from water sources (Lavery et al. 2010). Recently, plant peroxidase have obtained major important as a safe and effective method to the removal of phenolic compounds from nature and industrial sources of water (Ohore and Zhang, 2019), So, the this study was aimed to characterization of purified POD from Prickly lettuce leaves and application of it in removal phenol compounds.

MATERIALS AND METHODS

POD source

Peroxidase (EC 1.11.1.7) was obtained from a previous study by Aziz (2020), sodium phosphate buffer 0.1 M, pH 7 was used to dissolve of the enzyme.

POD assay

The activity of POD was estimated according to the method described by Whitaker and Bernhard (1972) using guaiacol as a substrate, enzyme activity (unit/ mL) was calculated according to the following equation:

$$\text{POD activity (unit/ mL)} = \frac{\Delta \text{A } 420\text{nm}}{\text{t} \times 0.1 \times 0.001}$$

Δ A 420nm = the amount of change in absorbance.

0.001 = The amount of change in the absorbance at a wavelength of 420nm per minute for one unit of peroxidase enzyme at a pH of 7 at 25°C for a volume of 3 mL of the reaction mixture.
0.1 = the amount of the enzyme solution used (mL).
t = reaction time in minutes.

Protein estimation

Protein was estimated through a method of Bradford (1976).

Molecular weight estimation

The molecular weight of POD was estimated by Sephacryl S-200 column 1.5×60 cm according to the method of Al-Soufi (2010) using lysozyme 14.4 kDa, trypsin 23 kDa, bovine serum albumin 67 kDa and canalbumin 76 kDa as standard proteins, the column was equilibrated and enzyme eluted by sodium phosphate buffer 0.3 M, pH 7 with flow rate 18 mL\h, 3 mL\fraction.

POD Characterization

Optimum pH of activity and stability was estimated by used 0.05 M buffer solution at pH 3-5.5 citrate phosphate, 6-7.5 sodium phosphate, 8-9 Tris-HCl (Al-Soufi, 2011); stability was estimated after incubation with buffer for 15 min with use guaiacol as substrates (Alsoufi, 2021). Optimum temperature for activity of POD was estimated at 30-70°C (Al-Soufi, 2013); stability was estimated after incubation at these temperature for 15 min with use guaiacol as substrates (Alsoufi, 2021). The effects of inhibitors on POD activity was estimated by used reaction solution that content 0.05 M sodium phosphate buffer pH 7, 0.01 M guaiacol and 0.1, 0.5 and 1.0 mM of sodium floride and thio urea; EDTA, sodium di ethyl thiocarbamate, sodium azide and magnesium sulphate as inhibitors (Al-Soufi, 2016). Km and Vmax values of POD were estimated by use 2-20 mM of guaiacol as substrates and 2 Mm hydrogen peroxide. Kinetic constants were calculated by the Michaelis-Menten method (Al-Soufi, 2016).

Application

Determination of total phenolic compounds

Phenolic compound (mg\mL) was estimated by use folin-ciocalteau method according use 10-100 mg\mL of gallic acid (3,4,5-trihydroxybenzoic acid) as a standard curve and absorbance at 765nm as a method of Alsoufi and Aziz (2020).

Removal of phenolic compounds

One mL of POD 20 U/mg in citrate buffer 20 mM pH 5 was add to the to the 500 mL of the 10 Mm of phenol and 4-chlorophenol, then incubation at 30°C and 50 rpm for 30 min, then estimation of remaining phenolic compounds concentration (%) as a method of Alsoufi (2018).

RESULTS AND DISCUSSION

Molecular weight

The molecular weight of POD was calculated as 43.267 kDa (Figure 1) according to gel filtration (Sephacryl S-200) method. This result is in the range of the results that have been reported in previous studies (35 to 95 kDa) of POD purified from the plants (Erdem et al. 2015), it was found to be 31 kDa in Pearl millet (Goyal and Chugh, 2014), 35 kDa in Lettuce stems (Hu et al. 2012), 38.8 kDa in Wheat (Altin et al. 2017), 40Kda in Horseradish peroxidase (Sarika et al. 2015), 66 kDa in Turkish black radish (Şişecioğlu et al. 2010), 73.2 kDa in White cabbage (Erdem et al. 2015) and 85 kDa in Sweet gourd (Koksal et al. 2012). The variance in the plant peroxidase molecular weight was attributed to content of amino acids and carbohydrate (Altin et al. 2017).

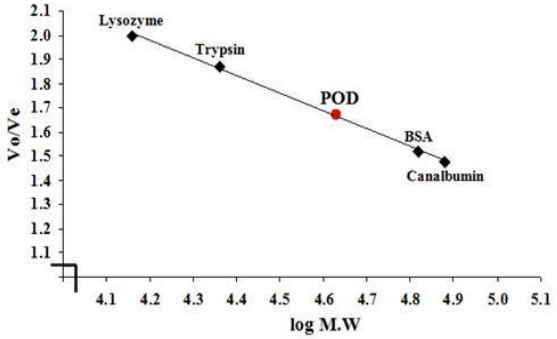

Figure 1: Standard Log MW of purified POD from Prickly lettuce by Sephacryl S-200 using standard proteins ranging from 14.4 to 76 kDa.

Effect of pH

The activity and stability was shown in (Figure 2) at a range from 3.0-9.0, maximum activity at pH 7, and its stable at pH range 5.0-8.0 with guaiacol as substrate, enzyme retaining more than 80% of its activity. Similar results were reported for different POD sources as in Papaya (Pandey et al. 2011), Sweet gourd (Koksal et al. 2012) and Chestnut kernel (Gong et al. 2015), while reported from others as a different value, the optimum pH activity of POD is 5.0 for a Tree legume (Pandey and Dwivedi, 2011) and Lettuce stems (Hu et al. 2012); 5.5 for Wheat (Altin et al. 2017); 5.6 for Pearl millet (Goyal and Chugh, 2014); 6.0 for Turkish black radish (Şişecioğlu et al. 2010), Citrus medica leaf (Mall et al. 2013) and Horseradish peroxidase (Sarika et al. 2015). The optimum pH for POD depending upon the source, substrate, amino acids, carbohydrate, nature of active site and optimum catalytic activity, therefore, pH is a main parameter for POD activity due to its effect on amino acids ionization state which lead to loss of heme group and activity (Mall et al. 2013; Altin et al. 2017).

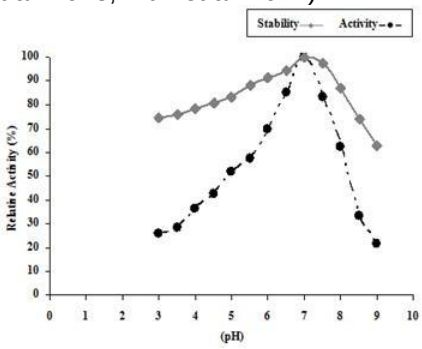

Figure 2: Optimum pH activity and stability of POD from Prickly lettuce leaves.

Effect of Temperature

The optimum temperature of activity and stability was found to be 50°C as shown in (Figure 3). The same results was found for Citrus medica leaf (Mall et al. 2013), Sweet gourd (Koksal et al. 2012) and Chestnut kernel (Gong et al. 2015), differently, optimum temperature of POD is 30°C for Turkish black radish (Şişecioğlu et al. 2010), Pearl millet (Goyal and Chugh, 2014) and White cabbage (Erdem et al. 2015); 40°C for Papaya (Pandey et al. 2011), Horseradish peroxidase (Sarika et al. 2015) and Wheat (Altin et al. 2017); 45°C for Lettuce stems (Hu et al. 2012) and 55°C for a Tree legume (Pandey and Dwivedi, 2011). Generally, plant peroxidases are consideration as a thermally stable enzyme, the increase of temperature will enhance of enzyme activity gradually up to reaching for maximum temperature, then activity will gradual decrease in the enzyme activity with increase of temperature that due to change in the enzyme structure (Hu et al. 2012; Goyal and Chugh, 2014).

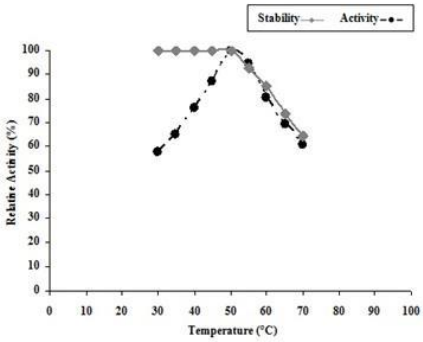

Figure 3: Optimum temperature (°C) activity and stability of POD from Prickly lettuce leaves.

Effect of inhibitors

The inhibition (%) of POD activity by various type of compounds were studied at (Table 1). The enzyme activity was complete inhibition using 1 mM of sodium floride and thio urea, while it was 95.72, 64.39, 48.65, 39.46 and 27.98% of EDTA, sodium di ethyl thiocarbamate, sodium azide and magnesium sulphate and 2-Mercapto ethanol, respectively.

In previous studies, various inhibitors are used to known its effect on POD activity, such as organic solvents and metal ions (Hu et al. 2012); L-cysteine and protocatechuic acid (Mall et al. 2013); sodium borohydride, DTT, b-mercaptoethanol, hydrazine, oxalic acid and sodium azide (Goyal and Chugh, 2014); citric acid and cetyltrimethylammonium bromide (Altin et al. 2017). The mechanisms of inhibitors agent on POD activity lead to its effect as a competitive inhibited agent which binding to the active site of POD as sodium azide (Pandey et al. 2012), or breaks disulfide bond in its structure as dithiothreitol and b-mercaptoethanol, or breaks the disulfide bond in protein as sodium borohydride, or acting as reducing agent as hydrazine (Goyal and Chugh, 2014), or metal chelator for Fe^{3+} ions from the active site as ethylenediaminetetra acetic acid (Ajila and Rao, 2009).

Table 1:Various inhibitors on activity of POD from Prickly lettuce leaves.

Inhibitors	Inhibition (%)		
	0.1 mM	0.5 mM	1 mM
Sodium floride	33.64	63.82	100
Thio urea	72.83	84.35	100
EDTA	68.35	79.06	95.72
Sodium di ethyl thio carbamate	39.18	55.14	64.39
Sodium azide	37.22	42.78	48.65
Magnesium sulphate	32.81	35.19	39.46
2-Mercapto ethanol	19.24	21.75	27.98

Enzyme Kinetics

The Km and Vmax of the POD was 1.11 mM and 0.71 EU/mL, respectively, as shown in (Figure 4), the enzyme showed substrate specificity for guaiacol is used as variable substrate and H_2O_2 is used at saturated concentrations.

The reported Km values using guaiacol as substrate for POD was 0.036 mM and 38728.17 EU ml^{-1} min^{-1}, respectively, from Turkish black radish (Şişecioğlu et al. 2010); 4.74 mM and 10585 U/ml min, respectively, from Lettuce stems (Hu et al. 2012); 17.1 mM and 15,500 EU/mL.min, respectively, from Sweet gourd (Koksal et al. 2012); 4.09 mM and 0.797 EU/mL, respectively, from Turnip roots (Kalin et al. 2014); 3.19 mM and 0.2 EU/mL, respectively, from White cabbage (Erdem et al. 2015).

POD can catalyse oxidation of a wide range of substrate, such as guaiacol, pyrogallol, *o*-phenylenediamine and *o*-dianisidine through a reaction with H_2O_2 (Altin et al. 2017).

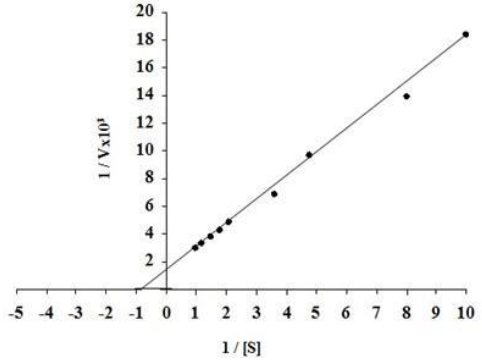

Figure 4: Km and Vmax values were 1.11 mM and 0.71 EU/mL respectively of POD from Prickly lettuce leaves.

Removal of phenolic compounds

The treatment by POD 20 U\mg lead to removed 17, 34, 58, 69, 81 and 93 of 4-chlorophenol and 36, 52, 81, 94, 110 and 100% of Phenol at 5, 10, 15, 20, 25 and 30 min respectively (Figure 5).

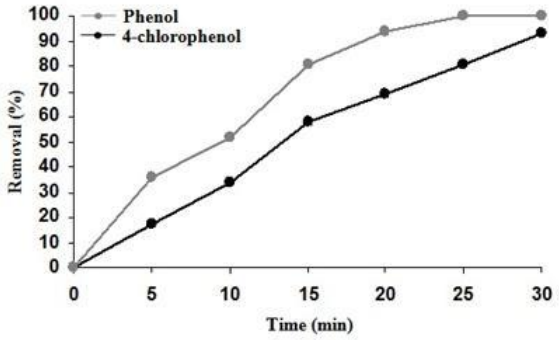

Figure 5: Remove of 4-chlorophenol and Phenol (%) from aqueous solutions by POD from Prickly lettuce leaves.

Plant peroxidases are used for treatment wastewater, industrial, medical, chemical, biological and natural water sources. In this context, it was use for removal of phenol and 2-chlorophenol form water treatment (Lavery et al.

2010), α-naphthol wastewater with 75% removal efficiency (Husain, 2010), bisphenol A with 98-100% removal efficiency, pH 6 at 30°C (Yamada et al. 2010), phenolic compounds form synthetic solution with 61.51% removal efficiency, pH 6 at 40°C for 45 min (Chagas et al. 2015).

The difference in removal of phenolic compounds is attributed to source of enzyme, pH, temperature, soluble or Immobilized, time, the mechanisms of removal (Husain, 2010; Chagas et al. 2015; Bilal et al. 2018)

CONCLUSION

Characterization of purified peroxidase from Prickly lettuce (*Lactucaserriola* L.) leaves was including pH, temperature, inhibitors and kinetics (Km and Vmax), the result of the study was encouraging to use peroxidase for removal of 4-chlorophenol and phenol from aqueous solutions with high efficiency.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Raghad A. Aziz: designed the study, collection of data, analysis, interpreted the data and drafted the manuscript.

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