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Characterization of Polyphenol Oxidase from *Eruca sativa*

Negin SHABNAM[1], Sibel KAHRAMAN*[1]

Abstract

Enzymatic browning reactions by polyphenol oxidases cause alteration of appearance, flavor and nutritive value of vegetables and fruits. It is one of the important problems for vegetables used as salads and causes lots of economic losses. In this study, polyphenol oxidase (PPO) from *Eruca sativa* was extracted and characterization studies were carried out. Substrate specificity for variable substrates, optimum pH, optimum temperature, effect of different metal ions were assayed. K_m and V_{max} values were determined as for pyrocatechol K_m = 10.24 mM, V_{max} = 0.0018 U min^{-1}, catechin; K_m = 12.57 mM, V_{max} = 0.0012 U min^{-1}, gallic acid; K_m = 23.07 mM, V_{max} = 0.0001 U min^{-1}. Optimum pH and temperature were determined as pH:7.0 and 20 ^oC respectively. Effect of various metal ions such as, K^+, Fe^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+} on enzyme activity were measured. K^+, Mg^{2+}, Zn^{2+}, Cu^{2+} ions inhibited PPO activity significantly. However Fe^{2+} ion did not inhibit PPO activity.

Keywords: Characterization, *Eruca sativa*, enzyme, polyphenol oxidase

1. INTRODUCTION

Polyphenol oxidases (PPO, EC 1.14.18.1) are a group of copper-containing enzymes which catalyses two basic reactions, in the presence of molecular oxygen, the o-hydroxylation of monophenols to give o-diphenols and the subsequent oxidation of o-diphenols to o-quinones [1, 2]. O-quinones are the precursors of the browning products [3]. Enzymatic browning reaction is one of the important problems for vegetables especially for salads and causes lots of economic loss. Because, enzymatic browning poses a significant issue for vegetables used in salads since it can lead to undesirable changes in their appearance, flavor, and nutritive value, which can directly affect consumer acceptance and marketability.

Purification and characterization studies were carried out for many different plants, vegetables and fruits because of the role of PPO in enzymatic browning reactions. Since many studies aimed to suppress the activity of PPO for protecting its quality.

Eruca sativa is an edible annual plant, also known as salad rocket, roquette [4]. *Eruca sativa*, which is widely popular as salad, is one of the species of *Eruca* native to the Mediterranean region; Morocco, Portugal, Syria, Lebanon and Turkey [5, 6]. It is a good source of vitamin A, vitamin C, calcium, iron and minerals and antioxidants like flavonoids.

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Hydroxycinnamic and kaempferol derivatives are the main phenolic compounds in *Eruca sativa* [7]. After harvesting the plant these phenolics are subjected to oxidation which leads to enzymatic browning. This affects nutritive values and also organoleptic properties of the plant like appearance, flavor and acceptance of the consumers. Browning reactions are also sign for injury.

In our study, biochemical characterization of *Eruca sativa* PPO was aimed by determining pH and temperature effect on enzyme activity, substrate specificity, effect of metal ions and inhibitors and also kinetic determinations like K_m and V_{max} values for different substrates. The novelty and benefit of this study lie in the detailed analysis of the PPO enzyme in *Eruca sativa*, for which limited research has been previously conducted. We determined the optimum conditions for enzyme activity, such as pH, temperature, substrate specificity, and the effect of metal ions and inhibitors. This in-depth analysis offers valuable insights into the factors affecting PPO activity, which may potentially lead to innovative strategies for the prevention of enzymatic browning in *Eruca sativa* and the enhancement of its overall quality and consumer acceptance. These findings could contribute to developing strategies for preventing quality loss in *Eruca sativa*, in terms of appearance, taste, and nutritional value.

2. EXPERIMENTAL

2.1. Materials

Pyrocatechol, catechin and gallic acid were obtained from Sigma Aldrich, Merck (St. Louis, MO, USA). All the chemicals used in the study were analytical grade.

2.2. Plant Material

The identified *Eruca sativa* seeds were used from İstanbul tohumculuk. They had been cultivated in Ulus Organic Garden (İstanbul) between March and June 2015. They were stored at +4 ^{o}C until further use. All of the studies were performed within 24 hours after the plants were collected.

2.3. Preparation of Crude Enzyme Extract

E. sativa (5 g) was homogenized by using Waring blender (Waring commercial, USA) in 40 mL of 50 mM phosphate buffer (pH=7.0). The homogenate was filtered before centrifugation with a NF 400R centrifuge at 4100 rpm for 20 min at 4 ^{o}C. The supernatant was used as the enzyme extract.

2.4. Enzyme Activity Assay

PPO activity was determined with spectrophotometer (Optizen POP, KLAB, Daejeon, Republic of Korea) by using pyrocatechol as substrate, measuring the increase in absorbance at 420 nm [8]. The reaction mixture contained 1.9 mL of 50 mM substrate solution prepared in 50 mM phosphate buffer at pH: 7.0 and 0.1 mL of enzyme solution. For enzyme activity, one unit was described as change 0.001 in absorbance per minute.

2.5. Evaluation of Enzyme Properties

2.5.1. Substrate specificity

Substrate specificity was tested by using 50 mM solutions of pyrocatechol, catechin and gallic acid. The reaction of substrates with PPO was measured spectrophotometrically at the particular wavelength of each substrate in 50 mM phosphate buffer (pH 7.0).

2.5.2. Effect of pH on PPO activity

Optimum pH for *E. sativa* PPO activity was assayed by monitoring its activity at pH range of 4.0-10.0 with 50 mM buffer solutions. Acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–7.0), and Tris-HCl buffer (pH 8.0–9.0) were used for different pH degrees. PPO activity was determined under the standard assay condition.

2.5.3. Effect of temperature on PPO activity

Optimum temperature for *E. sativa* PPO activity was assayed by measuring the enzyme activity at different temperatures. Enzyme was incubated at water bath for 45 min at the temperatures from 20 to 70 ^{o}C with 10 ^{o}C increases, prior to the addition of substrate. PPO activity was determined with pyrocatechol substrate.

2.5.4. Enzyme kinetic parameters

The specificity of *E. sativa* PPO activity was distinguished by mixing the crude extract with three different substrates: pyrocatechol, catechin and gallic acid at 420 nm at various concentrations; 5, 10, 15, 20, 25 and 30 mM. The kinetic data was plotted as 1/V versus 1/[S]. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) parameters were obtained with variable substrate concentrations in the standard reaction mixture. Substrate specificity (V_{max}/K_m) was determined by using Lineweaver-Burk plot [9].

2.5.5. Effect of metal ions on PPO activity

The effect of 5 mM solution of each metal ions such as K^+, Fe^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+} and Hg^{2+} on enzyme activity was evaluated and then percentage residual activities were found out in comparison with standard assay mixture in the absence of metal ion.

3. CONCLUSIONS AND DISCUSSION

3.1. Substrate Specificity

The varied number of phenolic compounds were reported as substrates for PPO. In this study, pyrocatechol, catechin and gallic acid were used for comparing substrate specificity. Pyrocatechol was used as main substrate because the enzyme showed high affinity (Table 1). It was followed by gallic acid and catechin respectively. The relative activities of the other substrates calculated regarding the pyrocatechol activity. *E. sativa* PPO showed low affinity towards catechin and gallic acid. The affinity order of the enzyme towards the substrates pyrocatechol > catechin> gallic acid. In a separate study on lentil sprout PPOs [10], it was demonstrated that the enzyme had the greatest affinity for catechol (K_m = 1.32, 1.76, and 0.94 mM, respectively), which is consistent with our findings.

Table 1 Substrate specificity of *Eruca sativa* PPO

Substrates (50 mM)	Relative activity (%)
Pyrocatechol	100
Catechin	14
Gallic acid	21

3.2. Kinetic Parameters

K_m value is showing the affinity of the enzyme towards the tested substrate. Lower values are indicatives of high affinity, however higher values are indicatives of low affinity. For *E. sativa* PPO activity, Michaelis-Menten constant (K_m), the maximum reaction velocity (V_{max}), and V_{max} / K_m values were calculated using Lineweaver and Burk [9] method (Table 2). K_m and V_{max} values for pyrocatechol, catechin and gallic acid substrates were calculated and can be seen from the Table 2. *E. sativa* PPO had the highest affinity to pyrocatechol substrate due to smallest K_m value (10.24 mM). All the determination studies were done using same substrate concentrations. K_m value was reported for mamey fruit PPO K_m = 44 mM [11], 682.5 mM in cabbage [12] and 20 mM in Stanley plum [13] for catechol substrate. The blueberry PPO exhibited a K_m of 15 mM and V_{max} of 2.57 DA420 nm/min [14], for blackberry PPO, K_m of 17 mM and V_{max} of 2.02 DA420nm/min, with catechol [15]. Zhao et al., [16] reported 10.17 mM K_m value with catechol for sugar cane similar to our findings. Catechin was reported as the best substrate with a K_m of 0.49 mM for Indian tea leaf PPO [17].

Table 2 K_m and V_{max} values of *E. sativa* PPO for different substrates

Substrates (50 mM)	K_m (mM)	V_{max} (μM min^{-1})	V_{max}/K_m
Pyrocatechol	10.24	$18x10^{-5}$	$1x10^{-5}$
Catechin	46.84	$32x10^{-5}$	$6.8x10^{-6}$
Gallic acid	23.07	$1x10^{-4}$	$4.3x10^{-6}$

These differences for K_m value caused by substrate, type of extraction, purity fold of the enzyme, and location of the enzyme [18].

3.2.1. Effect of pH

The activity of *E. sativa* PPO was determined at different pH values, changing from 4.0 to 10, using pyrocatechol as substrate. All procedure was done at room temperature.

The results show that highest activity peak was obtained at pH 7.0 (Figure 1). When the pH of the media changes, also the charge of the enzyme's surface which affecting the solubility and conformation of it. That also affects the binding of the enzyme with various substrates and inhibitors [19]. Generally, PPO enzyme from different sources like plants, vegetables and fruits show its maximum activity near to neutral pHs [1, 5] therefore our result is consistent with the literature. It is worth noting that PPO reached its maximum activity near pH 7.5 for *Acetes chinensis* [20]. Altunkaya [5] found out optimum pH as 6.0 for fresh cut rocket PPO. It was also reported that optimum pH was 7.0 for borage PPO [21], red chard PPO [22], mamey fruit PPO [11].

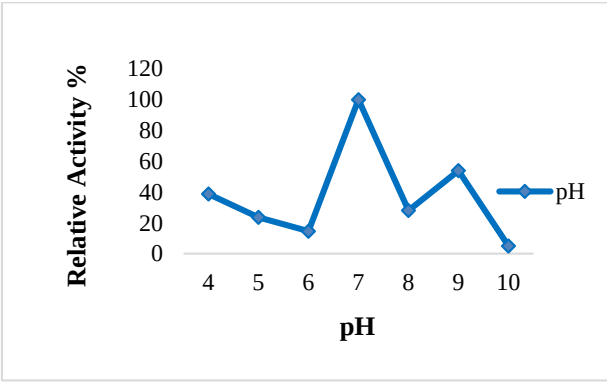

Figure 1 pH stability of *E. sativa* PPO

E. sativa PPO activity was decreased either alkaline or acidic pH's. Enzyme was almost fully inactivated at extreme alkaline conditions like pH:10 and also activity loss was observed for acidic pH's (4.0-6.0). *E. sativa* PPO activity at pH: 5.0 was lost 80%.

3.2.2. Effect of temperature

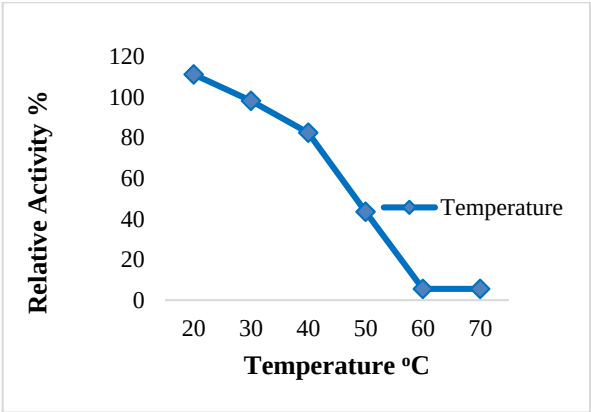

Figure 2 Temperature effect on *E. sativa* PPO

The effect of temperature between 20 and 70 ºC were determined using pyrocatechol as substrate (Figure 2). Enzyme was incubated prior to the substrate addition. The results show that optimum temperature for *E. sativa* PPO was 20 ºC. The enzyme activity was decreased in significant proportions higher than 50 ºC. Moreover when the enzyme incubated at higher temperatures like 50-70 ºC it was almost totally inactivated (5.5% residual activity).

Temperature is one of the parameters which modulate the catalytic activity of enzymes either increasing or decreasing the activity. Optimum temperature for PPO was reported as 10 °C to 60 °C for different sources and substrates [23]. Moreover PPO was known as stable at lower temperatures but unstable at higher temperatures [5]. *E. sativa* PPO activity was decreased with increasing temperatures and its activity almost completely lost when the assay temperature reached 60 ºC. Palma-Orozco et al. [11] found that the mamey PPO activity decreased between 50–60 ºC and after 65 ºC no activity remained. Optimum temperature was reported as 30 ºC for fresh cut rocket PPO [5].

Sikora *et al.* [10] reported the maximum activity of PPO from lentil sprouts occured at 35 ^{o}C when using catechol as the substrate.

3.2.3. Effect of metal ions

The effects of various metal ions on the *E. sativa* PPO activity were studied at a concentration of 5 mM using pyrocatechol substrate at standard assay conditions. The results are presented as percentages of residual activity of PPO in Table 3.

Table 3 Effect of metal ions on *E. sativa* PPO activity

Metal ions	Relative activity (%)
K+	21.78
Fe^{2+}	100
Mg^{2+}	24.81
Zn^{2+}	23.48
Cu^{2+}	32.57
Hg^{2+}	62.50

K^{+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+} ions decreased the PPO activity. The minimum residual activity was calculated as 21.78 % in the presence of K^{+} ions. However, enzyme kept its activity in 100% after the addition of Fe^{2+} ion. Zhao et al. [16] reported inhibitory effect of K^{+}, $Mg^{2+,}$ Zn^{2+}, Cu^{2+} ions on sugar cane PPO. In contrast to that Mg^{2+} and Cu^{2+} increased Thomson seedless grape PPO activity, while Zn^{2+} and K^{+} had an inhibitory effect [24]. Jang et al. [25] found out that PPO activity was inhibited by Mg^{2+} and Cu^{2+} and activated by Fe^{2+} and Zn^{2+} for *Solanum tuberosum* Jasim. Metal ions play important roles in maintaining substrate binding in the active site of metalloenzymes and in controlling the redox activity of metalloenzymes in enzymatic reaction [26], and the influence of metal ions on the activity of PPO is more complicated, with the degree of influence varying across different sources [27]. For instance, the activity of PPOs from flower buds of *Lonicera japonica* increased in the presence of Zn^{2+} and Mg^{2+} (10 mM) [28].

4. CONCLUSION

In this study, we aimed to provide a comprehensive investigation of PPO in *Eruca sativa*, focusing on its biochemical properties and the effects of different factors on its activity. Our findings revealed the enzyme's highest affinity for pyrocatechol (K_m = 10.24 mM) among the three tested substrates. The optimal pH for enzyme activity was found to be 7.0, with a decline observed at acidic pH values between 4.0 and 6.0 and alkaline pH values between 8.0 and 10.0. The optimal temperature was determined to be 20 °C, and the enzyme was found to be unstable at high temperatures.

Among the tested metal ions, the maximum decrease in enzyme activity occurred in the presence of K^{+}, while enzyme activity remained intact in the presence of Fe^{2+}. These findings contribute to our understanding of the optimal conditions for *Eruca sativa* PPO activity and the potential use of inhibitors for preventing quality loss. This study presents valuable insights into the potential applications of *Eruca sativa* PPO and paves the way for future research on preserving the appearance, taste, and nutritional value of the plant.

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Authors' Contribution
The authors contributed equally to the study.

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No conflict of interest or common interest has been declared by the authors.

The Declaration of Ethics Committee Approval
This study does not require ethics committee permission or any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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