Journal of Applied Biology & Biotechnology Vol. 12(2), pp. 191-197, Mar-Apr, 2024
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2024.160918

Thermal treatment and inhibition study with non-toxic product of polyphenol oxidase from Mediterranean Palm heart (*Chamaerops humilis* L.)

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ARTICLE INFO

Article history:
Received on: September 13, 2023
Accepted on: January 15, 2024
Available online: February 20, 2024

Key words:
Polyphenol oxidase,
Mediterranean palm heart
(*Chamaerops humilis* L.),
Nontoxic inhibitor,
Thermal inactivation,
Enzyme characterization.

ABSTRACT

This paper is the first study on the inhibition and thermal inactivation of polyphenol oxidases (PPO) from the heart of Mediterranean palm (*Chamaerops humilis L.*). PPO was extracted and purified, and its physicochemical properties, kinetic parameters of inhibition in the presence of a non-toxic sulfhydryl amino acid (L-cysteine), and parameters of thermal inactivation were determined. The PPO catalyzed the oxidation of 4-methylcatechol and pyrogallol as substrates but did not affect tyrosine. The best substrate was 4-methylcatechol. Enzyme activity decreases when pH decreases, and the optimal pH was 6.8. PPO enzyme activity was inhibited by citric acid, sodium floride, and L-cysteine, which was the most effective inhibitor and was a non-competitive type inhibitor. The optimal temperature of the enzyme was 35°C, and thermal stability was measured at 45°C, 55°C, 65°C, 70°C, 75°C, and 80°C. The half-life values of PPO were 92.4, 38.07, 25.16, 2.26, 0.43, and 0.42 min, respectively. The D value and activation energy values show that the PPO from the Mediterranean palm heart is very sensitive to temperature change. Its activity decreases rapidly above 40 and 70°C. At 75°C, 2 min is enough to reduce the enzyme activity to 10% of the initial value. The present study concludes that enzymatic browning of Mediterranean palm hearts can be reduced by thermal treatment, pH adjustment, and the use of non-toxic sulfhydryl amino acid (L-cysteine).

1. INTRODUCTION

A heart of palm is rich in essential minerals, antioxidants, carbohydrates, protein, lipids, and vitamins [1]. The Mediterranean palm (*Chamaerops humilis. L*), named Doom in North Africa, is an endemic palm of the Mediterranean basin. The "Doum" has shown anti-inflammatory, anabolic, antiseptic, urinary, antilithic and diuretic activities [2]. In Morocco, the heart of this palm is very appreciated by the consumer for its antioxydant content and anti-aging effect but it is marketed by the rural population in its raw state without any processing, which decreases its commercial value. This is because it is very sensitive to air contact and undergoes rapid browning when peeled due to the existence of the polyphenol oxidase (PPO) enzyme, which reacts with endogenous polyphenol [3].

PPOs are enzymes widely present in plants. They belong to a group of copper-containing metalloproteins. The presence of the copper atom allows the enzyme to transform o-diphenols into o-quinones. The reaction continues with the polymerization of the resulting quinine products to give colored substances [4]. Two types of polyphenols are involved in this reaction: monophenol, which gives cresolase activity, and o-diphenol, which gives catecholase activity [5]. This reaction plays a great role in the food industry's transformation. Thus, PPO from several plant tissues has been intensively studied, such as from apple [6], tadela (*Phoenix dactylifera* L.) [7], buriti [8], cucumber [9], sweet potato [10,11], blackberry [12], Kirmizi Kismis grape [13], and African bush mango [14].

The control of PPO activity of the vegetable from harvest to the

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consumer is necessary to minimize browning of the vegetable and maintain its economic value. Enzymatic browning of vegetables can be limited by several techniques, including the use of reaction inhibitors, physical treatments such as high pressure [15], microwave treatment [16], and the use of modified atmosphere packages [17], and the use of chemical agents such as phitic acid [18], ascorbic acid [19], essential oils [20], sulfiting agents, cysteine, glutathione, inorganic halides, acidulants such as citric, malic, and phosphoric acids [21], kojic acid [22], captopril [23], and thiopronine [24]. Finally, thermal treatment is an important physical technique used in the food industry. This process was finding able to inactivate PPO from mango slices and chili powder by heating at 80°C for 10 min [25,26].

In this study, PPO was extracted and purified from the heart of Mediterranean palm, and its kinetic parameters were determined for 4-methyl catechol, catechol, gallic acid, chlorogenic acid, pyrogallol, and tyrosine. Because the majority of the well-known PPO inhibitors have been shown to be toxic or possibly detrimental to biologic systems, their use in the food industry must be limited. The present study was the first on thermal treatment and kinetic inhibition of PPO from the heart of Mediterranean palms in the presence of a non-toxic sulfhydryl amino acid (L-cysteine). The effects of temperature, pH, and other chemical inhibitors on PPO activity, the inactivation rate constant (k), half-life time ($t_{1/2}$), reduction time (D), and some thermodynamic quantities were also determined.

2. MATERIALS AND METHODS

2.1. Materials

4-methyl catechol, catechol, galic acid, chlorogenic acid, pyrogallol, tyrosine, L-cysteine, sodium florid, citric acid, sodium dihydrogen phosphate, sodium hydrogen phosphate, poly (vinyl-pyrrolidone), ascorbic acid, ammonium sulfate, and triton X100 were purchased from Sigma-Aldrich Inc. PPO was extracted from the heart of the Mediterranean palm (*C. humilis* L.) by blender and centrifuge (Sigma 3–18 K). Absorbance was measured using an ultraviolet (UV)-MINI 1240 Shimadzu spectrophotometer.

2.2. Enzyme Extraction

PPO extraction was realized as described in the previous studies [6-8]. The used solutions are 100 mM phosphate buffer (pH = 6.8) containing 10 mM ascorbic acid, 0.5% polyvinylpyrrolidone, and 1% of Triton X100. The enzyme solution was precipitated with 80% saturation ammonium sulfate. The pellet was dissolved in 0.1 M sodium phosphate buffer, pH 6.8, and dialyzed at 4°C against phosphate buffer for 24 h.

2.3. Protein Quantity

The Bradford method, using bovine serum albumin as a standard, was used to determine the protein contents of the enzyme extracts [27].

2.4. Assay of PPO Activity

Spectrophotometry was used to determine PPO activity. The assay mixture was performed at room temperature with 0.2 mL of enzyme and 2.8 mL of substrate dissolved in a 100 mM phosphate buffer solution. 3 min after mixing the reagents, the change in absorbance was measured using a UV-MINI 1240 Shimadzu spectrophotometer. The linear part of the plot of absorbance (A) against time (t) was used to calculate PPO activity. The enzyme activity unit (U) was taken as an increase of 0.001 units of absorbance per minute per mL of enzyme [28,29].

2.5. Enzyme Kinetics and Substrate Specificity

A mixture containing 0.2 ml of enzyme and 2.8 ml of substrate (4-methyl catechol, catechol, galic acid, chlorogenic acid, pyrogallol, or tyrosine) was prepared at 25°C at the different finale concentrations of 5, 10, 20, 30, 40, and 50 mM C in a phosphate buffer solution (100 mM, pH = 6.8). Then, PPO activity was measured by spectrophotometry. The Lineweaver–Burk Method (plot of 1/V vs 1/[S]) was used to determine K_M and V_{max}, and the V_{max}/K_M ratio, referred to as "catalytic efficiency'', was calculated [28,29].

2.6. Effect of pH on PPO Activity

A reaction mixture containing 0.2 mL of enzyme, 0.5 mL of catechol, and 2.3 mL of buffer solution of 4.0–8.0 using 0.1M acetate (pH = 4–6) and 0.1 M phosphate (pH = 6–8) was prepared, and the enzyme activity was assayed as above. Then the optimal pH for PPO activity was determined [30].

2.7. Effect of Temperature on PPO Activity

The effect of temperature on enzyme activity was determined by increasing the temperature from 20°C to 80°C using a shaking water bath. The reaction mixture contained 0.2 mL of enzyme, 0.5 mL of catechol (30 mM), and 2.3 mL of phosphate buffer (100 mM). The phosphate buffer and PPO extract were heated in test tubes. The temperature of the reaction mixture was monitored until the predetermined temperature was reached. One minute later, catechol was added, and the enzyme activity was assayed as above [26,28].

2.8. Effect of Inhibitors on the Enzyme Activity

PPO activity of substrate was determined in the presence of inhibitors (L-cysteine, citric acid, and sodium floride) at the different final concentrations. A plot of 1/V vs. 1/[S] for different concentrations of inhibitor was used to estimate the inhibition constant (Ki) of each inhibitor [11].

2.9. Thermal Treatment of PPO

The thermal study of PPO was realized according to the Benaceur *et al.* [7] method in the range of 45–80°C for 0, 10, 20, 30, 40, and 50 min, respectively. A mixture containing 0.2 ml of enzyme, 0.5 ml of catechol (30 mM), and 2.3 ml of phosphate buffer was heated until the predetermined temperature and for the predetermined time. Then, residual enzyme activity was measured [7]. The slope of the natural logarithm (ln) of the At/A_0 vs. time graph was used to estimate the first-order inactivation constant (k), where At is the enzyme activity after thermal treatment and A_0 is the enzyme activity before thermal treatment [7]. The following equation, $t_{1/2}= \ln(2)/k$, was used to calculate the half-life of the enzyme ($t_{1/2}$). Decimal reduction time (D value) was calculated from the relationship between k and D value: $D = \ln(10)/k$ [7].

2.10. Thermodynamic Analysis

Arrhenius' law, expressed by $\ln(k) = \ln(A) - (Ea/R) * (1/T)$, was used to evaluate the relation between the values of k and the temperature [7], where R is the universal gas constant (8.314 J $mol^{-1}.K^{-1}$) and A is the Arrhenius constant. The slope of the line, obtained using the Arrhenius diagram, was used to estimate activation energy (Ea).

The following formula, $\Delta H = Ea - RT$, was used to calculate the enthalpy of activation (ΔH) for each temperature. Free energy of inactivation (ΔG) was calculated using the expression: $\Delta G = -R * T * \ln(k * h_p/K_B * T)$,

where K_B is the Boltzmann constant (1.38×10^{-23} J/K), h_p is the Planck constant (6.626×10^{-34} J s), T is the absolute temperature, and k (s−1) is the inactivation rate constant of each temperature [7].

2.11. Statistical Analyses

Statistical analyses of all experimental data on PPO activity for different parameters for all assays were performed in three replicates, and values were expressed as mean ± SD. The collected data were analyzed using the analysis of variance procedure. Statistical analyzes were performed using the statistical software Statistical Analysis System (SAS, version 9.1, 2002).

3. RESULTS AND DISCUSSION

3.1. Extraction and Purification of PPO

Ammonium sulfate precipitation was used to remove sugars and high-molecular-weight proteins and to concentrate the sample [18]. Protein concentrations of the crude extract estimated using the Bradford method were 17.2 µg (0.2 ml of enzyme) and 8.6 mg/100 g. At subsequent steps of purification, the PPO activity per ml increased and, as expected, the protein (PPO) content decreased. After ammonium sulfate fractionation, 44.6% of activity remained (1234 U/min), while 83.7% of proteins were removed. After dialysis, 20% of activity remained (571 U/min), while 95% of proteins were removed [Table 1].

3.2. PPO Kinetics Parameters

PPO from Mediterranean palm hearts showed no activity using mono-hydroxyl phenol, low activity using trihydroxy phenol, but showed much greater activity with the o-dihydroxy phenol [Table 2]. It can therefore be concluded that the Mediterranean palm heart PPO has diphenolase activity but does not exhibit monophenolase activity. Similar results were observed with PPO obtained from other plants such as mango [31], atemoya fruit [32], apple (Malus domestica) [33], and broccoli [34]. PPO from other sources has both mono and diphenol oxidase activities, as mushrooms [28,35]. The lowest Km value was observed using 4-methyl catechol as substrate (3.53 mM), followed by catechol 5.0 mM, and the highest value was observed with pyrogallol, 28.8 mM [Table 2]. This result is consistent with those reported in the literature, where 4-methylcatechol usually has the best affinity for PPOs [29,36,37].

3.3. Optimum pH

The relative activity of PPO from Mediterranean palm hearts was determined at different pH values ranging from 2 to 8 using catechol as substrates [Figure 1]. PPO activity decreases when pH decreases. The optimal pH value is about 6.5, and activity was 3132 ± 57 (Units/min). Using catechol as substrate, the optimum pH of PPO activity from plants is usually in a pH range of 4–7.4. The optimum pH is 6.5 for rooster potato (Solanum tuberosum cv. rooster) [38] PPO, 6.8 for prawns [7,9] for buriti [8], and 5 for potato tuber PPO [39].

3.4. Optimum Temperature

The optimal temperature of PPO activity was determined at different temperatures ranging from 10°C to 80°C using catechol as substrates [Figure 2]. PPO has the highest activity at 35°C and was found to increase from 25°C to 35°C and then decline up to 40°C. At 60°C, where activity was about 40% of the maximum value. Literature data show that the optimum temperature of PPO activity depends on several factors, such as the source of the enzyme, the method of its extraction, and the type of substrate used to measure its activity [5]. The optimal temperature activity of PPO is mostly obtained between 30°C and 45°C. Rooster potato have optimum temperature of 30°C [38], 35°C for Buriti [8] and it is 45°C for PPO from prawns [40].

3.5. Effects of Inhibitors on the Enzyme Activity

Among the inhibitors used, L-cysteine was the most effective for Mediterranean palm heart PPO. Catechol activity was 50% when the concentration of L-cystein was 10 mM (IC_{50}=10 mM), declined to 5% when the concentration was 25 mM, and fell to 1% when the concentration of the inhibitor was 75 mM [Figure 3]. Thus, we conclude that L-cysteine may be used to prevent browning by PPO and to control PPO action in the Mediterranean palm heart.

To determine the type of inhibition, Lineweaver and Burk graphs for three concentrations of each inhibitor were used [Figure 4]. Litterateur results showed that the type of inhibition is related to the source of PPOs [5]. The present study determined that L-cysteine and sodium fluoride were non-competitive type inhibitors for PPO of the Mediterranean palm heart [Figure 4a and b]. A similar result was obtained for PPO from apples [41] and from Whangkeumbae pear [42] using L-cysteine as an inhibitor. Citric acid is an uncompetitive inhibitor [Figure 4c]. A similar result was obtained for PPO extracted from purslane, where citric acid was found as uncompetitive inhibitor [43]. Citric acid gives mixed inhibition of eggplant PPO [29]. However, citric acid was a competitive inhibitor of PPO in Chinese parsley [44].

Lineweaver-Burk graphs were used to determine inhibition constants (Ki). Following Ki values, L-cysteine was the most effective inhibitor with the lowest Ki value of 56 µM, followed by citric acid with a Ki value of 60 µM and sodium fluoride with a Ki value of 79 µM. Several studies concerning the inhibition of PPO have shown different values of the inhibition constant, dependent on the PPO source and pH. For L-cysteine, the Ki value was 1.28 µM with PPO from Ispir sugar bean [45] and 1.13 mM with PPO from lettuce tissues [46]. L-cysteine at higher concentrations (≥1.0 %), reacted with the resulted quinone to give colorless products, and the formation of quinone-sulfite complexes prevents the quinones polymerization [47]. Citric acid has a Ki value of 2.67 µM with PPO from dill [48] and 2.074 mM with PPO from lettuce tissues [46]. Sodium fluoride has a Ki value of 98 µM with PPO from apples at pH = 5 and decreases to 14 µM at pH = 3.5 [41].

3.6. Effects of Heat Treatments on PPO Stability

The effect of heat treatment on PPO stability was investigated from 45°C to 80°C [Figure 5]. Enzyme activity decreased with increasing temperature and treatment time. At 45°C and after 5 min of incubation, it remains 85% of the enzyme activity, and after 20 min, it remains

Table 1: Partial purification of polyphenol oxidase from Mediterranean palm heart.

Steps	Total volume (ml)	Total protein (mg)	Total activity (U/min)	Specific activity (U/mg protein)	Yield (%)	Purification (fold)
H_2O extract	100	8.6±0.56	2763±123	321±23	100±0	1
80% $(NH4)_2SO_4$	35	1.4±0.16	1234±56	881±67	44.6±3.8	2.7
Fraction dialyzed	15	0.45±0.032	571±32	1268±73	20±2	3.9

55%. At 65°C, PPO activity decreases significantly. At 80°C, no activity is observed after 5 min of incubation. The resistance of PPO activity to thermal treatment depends on the source of PPO [49,50]. PPO from Guankou grape was completely inactivated at 75°C after 10 min of incubation [51], and thermal inactivation of "Prata" and "Cavendish" banana peel PPO was achieved at 90°C after 5 and 15 min, respectively [49, 52].

3.7. Inactivation Constants and Thermodynamic Quantities of the Mediterranean Palm Heart PPO

Rate constants k of inactivation were calculated from linear regression of experimental enzyme retention data of the slopes of log % residual activity against time [19]. The kinetic parameters k, $t_{1/2}$, and D are presented in Table 3. The activation energies, Ea, were calculated using the Arrhenius equation [7] [Figure 6]. Then ΔG, Gibbs free energy, and ΔH, the change in enthalpy, were calculated [Table 3].

Table 2: Substrate specificity of polyphenol oxidase from Mediterranean palm heart.

Substrats	K_m (mM)	Vmax (Unit/min)	Ks (Unit.min^{-1}.mM^{-1})
Catechol	5.0±0.4	2932±57	575±51.2
Chlorogenic acid	9.5±1.7	6200.6±656	658±73
4-methyl catechol	3.53±0.38	11402±973	3203±101
Galic acid	24±3.4	4184±521	182±21
Pyrogallol	28.8±4	7407±589	257±18.5
Tyrosine	--	--	--

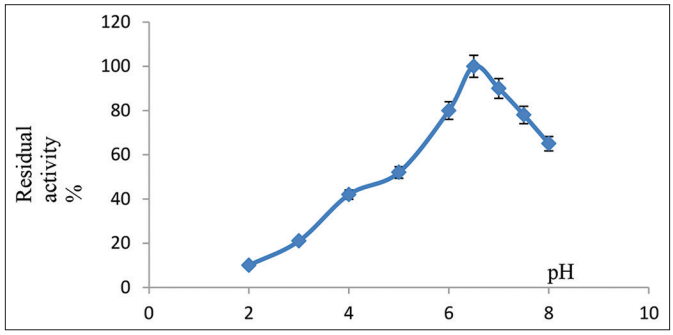

Figure 1: Effect of pH on Mediterranean palm heart polyphenol oxidase activity.

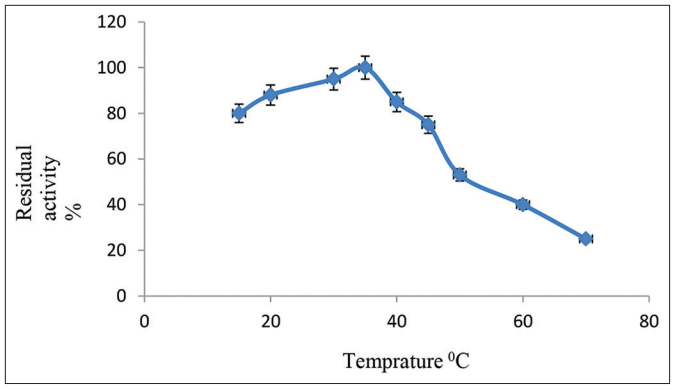

Figure 2: Effect of temperature on Mediterranean palm heart polyphenol oxidase activity.

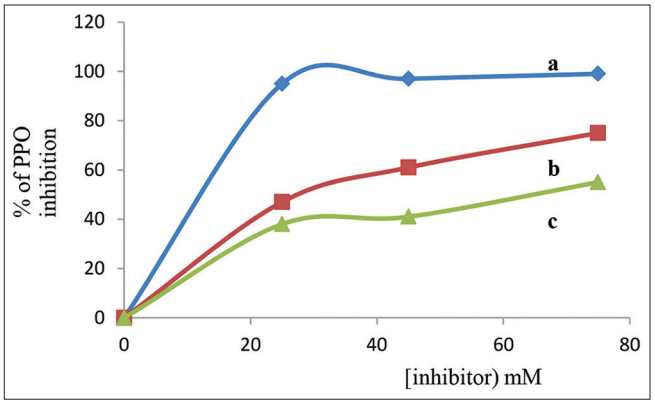

Figure 3: Percent inhibition of some chemical inhibitors on Mediterranean palm heart polyphenol oxidase activity: (a) L-cystein, (b) sodium florid, and (c) citric acid.

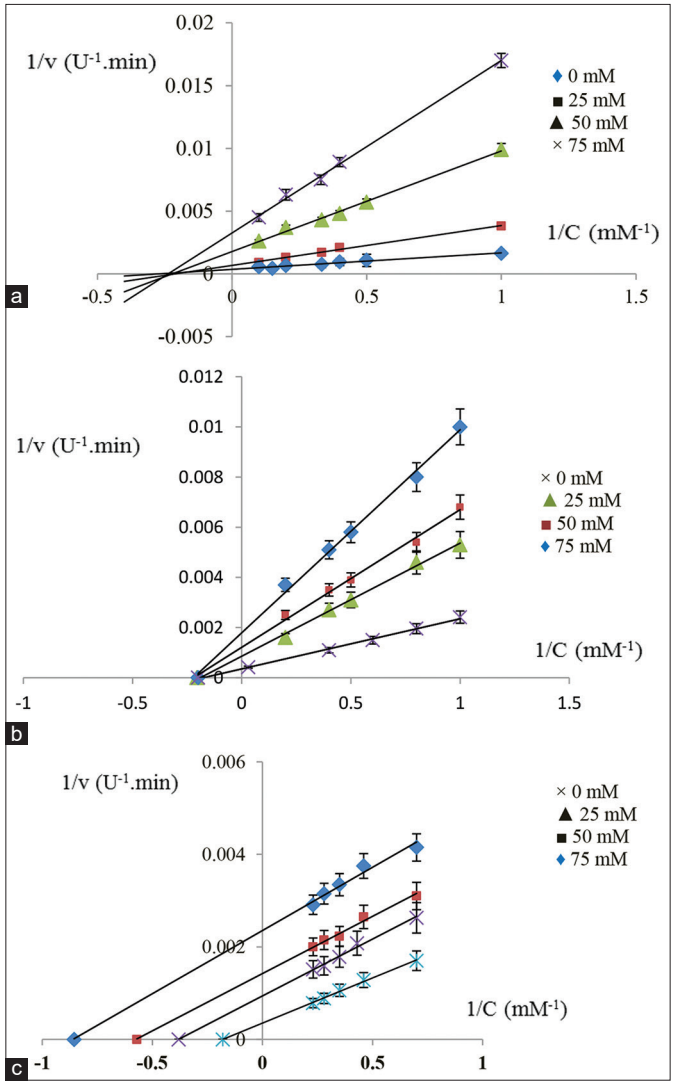

Figure 4: (a) Lineweaver-Burk plots using catechol as a substrate in the presence of L-cystein. (b) Lineweaver-Burk plots using catechol as a substrate in the presence of sodium florid (c) Lineweaver-Burk plots using catechol as a substrate in the presence of citric acid.

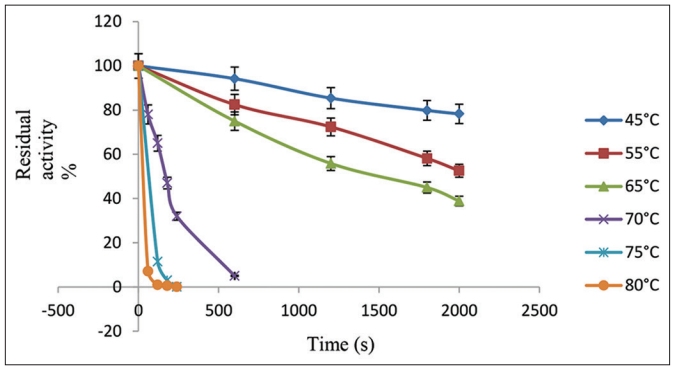

Figure 5: Effects of heat treatments on Mediterranean palm heart polyphenol oxidase residual activity.

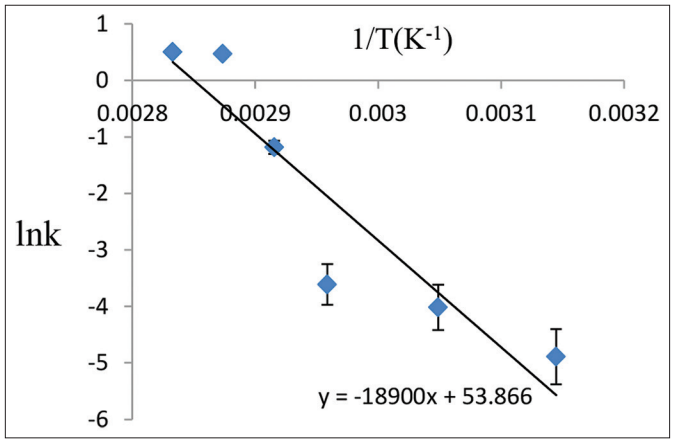

Figure 6: Arrhenius plot for catechol oxidation catalyzed by polyphenol oxidase (PPO) from Mediterranean palm heart PPO.

The results showed that k values increased with increasing temperature, indicating that PPO is less thermostable at higher temperatures. The enzyme showed a steep decrease in $t_{1/2}$ with increasing temperature, and D values declined with temperature [Table 3]. The D value, or time at a given temperature and pressure needed for 90% reduction of the initial activity, was <2 min at 80°C. The activation energy (Ea) value for heat-inactivation of PPO from Mediterranean palm heart was 157 kJ/mol, higher than those reported for rice (23.3 kJ/mol) [53], banana (155 kJ/mol) [54], but lower than that for Victoria grape (221.5 kJ/mol) [55], table grape (295.5 kJ/mol) [56], and cranberry (502 kJ/mol) [57]. High activation energy reflects a greater sensitivity of PPO to temperature [9]. Thus, we can conclude that the following k values, D, and activation energy show that the PPO is very sensitive to temperature change.

ΔG, Gibbs free energy, considered the energy barrier of the inactivation of the enzyme; ΔH, the change in enthalpy that measures the number of bonds destroyed during inactivation [19]. The results of ΔH show that the enthalpy is practically independent of temperature. So there is no change in the heat capacity of the enzyme. The mean value of ΔH found in this study (154 kJ/mol) is higher than those found for other PPOs, such as the PPO from vanilla bean (89 kJ/mol) [58]. The value of ΔG is directly related to the stability of a protein; a higher ΔG corresponds to great stability of the enzyme. When incubation temperature increases from 65°C to 80°C, there is a reduction in the ΔG values for the PPO from 104 kJ/mol to 91 kJ/mol [Table 3], indicating the destabilization of this protein. All ΔG values are in agreement with the value of 100 kJ/mol characteristic of the protein denaturation reaction as found for PPO from other plants [58].

Table 3: Inactivation parameters of Mediterranean palm heart PPO.

Temperature (°C)	K (min^{-1})	$t_{1/2}$ (min)	D (min)	ΔH (*Kj*)	ΔG (Kj)
45	0,0075±0.0007	92.4±6.7	307	154.3	101.83
55	0.018±0.008	38.07±0.65	127.9	154.2	102.66
65	0.027±0.005	25.16±0.023	85.2	154.18	104.7
70	0.306±0.07	2.26±0.19	7.5	154.14.	99.5
75	1.60±0.15	0.43±0.19	1.42	154.05	96.12
80	1.65±0.31	0.42±0.21	1.39	154.01	91.7

4. CONCLUSION

In this study, characterization, inhibition, and the effect of thermal treatment on the PPO enzyme from Mediterranean palm hearts were described. Our study has reported that the purified PPO enzyme has no activity with the phenol monohydroxy substrate. Kinetic parameters show that the PPO enzyme was very effective towards 4-methylcatechol as a substrate, followed by catechol. PPO activity decreases when pH decreases. The optimum pH and temperature values were determined, and the inhibition kinetics of the PPO show that the most effective inhibitor was L-Cysteine. The D, k values, and activation energies of the PPO enzyme were very sensitive to temperature change and had a rapid loss of its activity. We conclude from the present study that enzymatic browning of Mediterranean palm hearts can be reduced by thermal treatment, pH adjustment, and the use of non-toxic sulfhydryl amino acid (L-cysteine). However, further study can be performed in order to determine the optimal conditions for inactivation of the PPO enzyme and the effect of the residual enzyme on the stability of Mediterranean palm hearts during storage should also be carried.

5. ACKNOWLEDGMENT

We would like to thank Dr Gavira for the lab measurements in the Laboratorio de Estudios, Cristalogra´ficos, IACT (CSIC–UGR), Armilla, Granada, Spain.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

7. FUNDING

There is no funding to report.

8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

All the data obtained in the study are represented as table or figures.

11. PUBLISHER'S NOTE

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How to cite this article:
Bouchaib A, Ben Abdellah A, Chafik T, Laglaoui A, Arakrak A, Bakkali M, Benjouad A, Maurady A. Thermal treatment and inhibition study with non-toxic product of polyphenol oxidase from Mediterranean Palm heart (*Chamaerops humilis* L.). J App Biol Biotech. 2024;12(2):191-197.
DOI: 10.7324/JABB.2024.160918