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Isolation and Partial Purification of Polyphenol Oxidase from Seed of Melon (*Cucumeropsis edulis*)

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Abstract: Polyphenol oxidase (PPO) from *Cucumeropsis edulis* was extracted and partially purified through $(NH4)_2SO_4$ precipitation, dialysis, and ion-exchange chromatography on DEAE-Sephadex-A50. The spectrophotometric method was used to assay the enzyme activity in *C. edulis* using L-DOPA as substrate, the physicochemical properties such as the effect of pH and temperature, substrate specificity, kinetic constants - maximum enzyme velocity (V_{max}), and Michaelis - Menten constant (K_m) for three substrates namely, L-Dopa catechol and tyrosine were determined. The effects of inhibitors and metal ions on PPO activity were also investigated. The optimum pH and temperature values were found to be pH 6.5 and 50 °C, and the inhibitory effects of inhibitors such as ascorbic acid, EDTA, SDS, and metal ions were enhanced positively with increased concentration except with divalent metals such as Cu^{2+}, Fe^{2+}, and Zn^{2+} reflecting an activating effect on *C. edulis* PPO. Moreover, the enzyme solution showed both monophenolase and diphenolase activity with L-DOPA having the highest V_{max}/K_m value. However, the data obtained in this research provided a theoretical basis for the prevention of enzymatic browning of *C. edulis* during processing.

Keywords: Polyphenol oxidase; Cucumeropsis eduli; physicochemical properties; Enzyme activity.

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1. Introduction

Cucumeropsis edulis, a member of *Cucurbitaceae* family, is a variety of melon seeds, which is a creeping and an intercropping plant made use of in traditional farming practices, thrives well on rich light soil in the hot climatic regions of African nations [1-3]. Contrary to the other species of the family of the *Cucurbitaceae, C. edulis* plant is widely cultivated for their seeds, which have a high content of fat and protein [2]. Nasiru and Oluwasegun [4], Yasar [5], and Falade *et al.* [6] had earlier investigated the nutritional constituents as well as the effect of processing on the anti-nutrient quantities of *Curcubitaceae* seeds. The seeds of *C. edulis* contain proteins and other nutritionally important components that could be harnessed as alternatives for the human diet [3,7]. *C. edulis* seeds can be obtained either in shelled or unshelled forms in West African Markets and are used greatly in cookery. The seeds of the *C. edulis* present themselves under the whitish color of oval and flat shape. The seeds of the *C. edulis* are part of the condiments used in the preparation of sauces consumed in most African nations [2]. They are consumed crushed or grilled and served to thicken sauces and sometimes make into a cake for its delicacy. In the course of dehulling and crushing or making into

powder, the color is observed to change from whitish to greyish, making it appears dull and eventually lose its acceptability for market value. Similar to other commercially-available crops, dehulled *C. edulis*, is prone to browning due to post-harvest effect. Food browning has a critical impact on processed foods as fruits, vegetables, and seeds are susceptible to enzymatic browning, which is typically catalyzed by polyphenol oxidase (PPO) [8- 9]. After harvest, crops are usually subjected to serial post-harvest processing steps that include peeling, slicing, or cutting, crushing to allow efficient storage [9]. Injury can destroy subcellular compartmentalization in plant tissues, releasing endogenous phenolic substrates, and allow PPO to interact with its substrates, thereby causing browning [9-10].

PPO is a family of copper-containing oxidoreductases that are widely distributed among plants in chloroplasts and can catalyze hydroxylation of monophenols to O-diphenols, followed by oxidation of O-diphenols to O-quinones [11-12]. Oxidation products of phenols undergo further condensation reactions to become dark-colored (melanins) on plant surfaces, leading to a high percentage yield loss due to degradation of food quality during post-harvest storage [13-14]. Enzymatic browning also results in alteration of physical appearance, adversely affecting color, flavor, nutritional properties, thereby reducing the shelf life of processed food substances. Different preservation methods, such as freezing, canning, and drying are commonly applied to extend the availability of fruits and seeds [15-16]. However, browning remains a problem in *C. edulis* handling and preservation and is believed to be a major factor of quality loss during processing [9].

Characterization of PPO has widely been observed in various plant sources, cocoa bean seeds [17], potato [18], wheat [8], apple [19], African bush mango seeds [12], Sorghum [20], among many others. Information on the isolation of PPO from the seed of *C. edulis* and its physicochemical properties have not been established based on the available literature around. Therefore, it is necessary to isolate and characterize PPO from *C. edulis*, in order to proffer effective methods for controlling oxidative browning during the processing of *C. edulis*. In order to achieve this, a detailed biochemical analysis of polyphenol oxidase from *C. edulis* was conducted to determine its pH and temperature optimal reaction conditions, kinetic parameters, and the effects of various inhibitors and metal ions on the enzyme activity.

2. Materials and Methods

2.1. Sample preparation.

Fully matured fruits of *C. edulis* (*Egusi Ito*) were purchased from a local market in Akure, Ondo State, Nigeria, and identified at the Department of Crop Science and Pest, School of Agriculture, Federal University of Technology, Akure. The seeds were removed, air-dried for two weeks, and later dehulled manually. All chemicals and reagents were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of crude PPO extract.

Melon seeds (500 g) of *C. edulis* were thoroughly homogenized in 1.5 L of ice-cold 25 mM phosphate buffer (pH 6.8) containing 10 mM ascorbic acid using a Saisho warring blender. The homogenate was filtered using a four-layer of cheesecloth. The filtrate was again filtered using glass wool to remove the floating lipid, followed by centrifugation in a cold centrifuge at 16,000 rpm for 30 minutes at 4°C. The supernatant was kept at -4°C while the lipid layer was separated by removing the enzyme solution using a micropipette and further subjected to

centrifugation, after which the remaining lipid droplet was removed by passing the solution through glass wool. The supernatant was stored in a refrigerator and used as a crude enzyme for further experiments.

2.3. Purification of polyphenol oxidase from C. edulis.

2.3.1. Ammonium sulfate precipitation.

The crude enzyme (125 mL) was brought to 80% ammonium sulfate saturation by slowly adding solid ammonium sulfate at a temperature of 4 ºC using a magnetic stirrer by stirring continuously until all was dissolved. The precipitate was collected by centrifugation at 16,000 rpm for 10 minutes. The precipitate was dissolved in 5mL 0.1M phosphate buffer (pH 6.8) and dialyzed with the same buffer at 4 °C overnight with changes of buffer.

2.3.2. Ion - exchange chromatography.

The dialysate was placed on a DEAE - A50 Sephadex column (3.5 × 13 cm). The column was pre-equilibrated with 0.1M Potassium phosphate buffer, pH 6.8, and the protein was eluted using the same buffer (flow rate: 20 mL/hr) were passed through the column. The unbound protein was eluted from the column with starting buffer followed by releasing the bound protein with the use of a linear gradient of 0 to 0.5 M NaCl in 0.1M potassium phosphate buffer. The absorbance of the fractions was read at 280 nm, and each fraction was tested for polyphenol oxidase activity while the fractions exhibited polyphenol oxidase activity were pooled together and concentrated with 4 M sucrose and used for characterization.

2.3.3. Determination of protein concentration.

Protein concentration was determined according to the method described by Lowry *et al.* [21] using Bovine serum albumin (BSA) as a standard.

2.3.4. Determination of polyphenol oxidase activity.

PPO activity was determined by measuring the increase in absorbance at 420 nm using catechol as a substrate. The reaction mixture consisted of a 0.2 mL enzyme solution and 2.8 mL of 10 mM substrate solution in 25 mM phosphate buffer (pH 6.8), at 25°C [22]. One unit of PPO activity was defined as the amount of enzyme that caused an increase in absorbance of 0.001 per min.

2.4. Physicochemical properties of polyphenol oxidase.

2.4.1. Determination of *C. edulis* PPO substrate specificity.

Four different substrates (catechol, gallic acid, L-DOPA, and tyrosine) at 10 mM concentration were prepared in 0.1M phosphate buffer (pH 6.8). PPO activity was determined according to the standard assay procedure at a corresponding wavelength 420 nm (catechol), 270 nm (gallic acid), 475nm (L-DOPA), and 300 nm (tyrosine).

2.4.2. Effect of pH in the presence and absence of SDS on *C. edulis* PPO.

The enzyme pH optimum was determined with and without SDS, according to Escribano *et al.* [23], using various buffers at pH ranges from 2.0 - 9.0. 0.1 M. The reaction

mixture contained glycine NaOH buffer (pH 2.0 - 3.0); 0.1M sodium acetate buffer (pH 4.0 - 5.0); 0.1M potassium phosphate buffer (pH 6.0 - 7.0) and 0.1M tris- HCI buffer (pH 8.0 - 9.0) in the presence and absence of 0.69 mM SDS. Enzymatic activity was determined according to the standard assay procedure.

2.4.3. pH stability of *C. edulis* PPO.

The pH stability of the purified PPO was determined according to the method of Escribano *et al*. [23[by preparing various buffers of pH 2.0 - 9.0 using 0.1M glycine NaOH (pH 2.0 - 3.0), sodium acetate buffer (pH 4.0 - 5.0), potassium phosphate buffer (pH 6.0 - 7.0) and Tris- HCI buffer (pH 8.0 - 9.0), and then incubating the purified enzyme with the same buffer solutions for 6 hours. The residual activity was determined by drawing 1 mL of an aliquot enzyme at a one-hour interval subsequently after initial (0 hours) activity according to the standard assay procedure.

2.4.4. Thermo-stability of *C. edulis* PPO.

The effect of temperature on the enzyme activity was investigated by varying the temperature condition between 30 to 80 °C. The reacting mixture consisted of the purified enzyme, and L-DOPA was incubated at the above temperature range, while 1mL of the aliquot enzyme was withdrawn at an interval of 10 °C after 10 minutes. The activity was determined according to the standard assay procedure. The thermal stability was determined by incubating the enzyme at different temperature range (30 – 80 ºC). The initial activity was determined at the 0 minutes, while the residual polyphenol oxidase activity was determined at 10-minute intervals for each temperature according to the standard assay procedure.

2.4.5. Kinetic parameters of *C. edulis* PPO.

The kinetic parameters, K_m and V_{max} of the partially purified enzyme was determined using Lineweaver-Burk [24] plot with catechol, gallic acid, tyrosine, and L-DOPA as substrates, at varying concentrations (40, 35, 30, 25, 20, 15, 10, and 5 mM) in 0.1 M potassium phosphate buffer (pH 6.8).

2.4.6. Effect of inhibitors and activators on *C. edulis* PPO Activity.

Polyphenol oxidase activity was determined in the presence of ascorbic acid, EDTA, urea, and SDS. The assays were carried out at different final concentrations: 5, 10, and 20 mM of the reaction mixture. The activity was measured according to the standard assay procedure.

2.4.7. Effect of metal ions on *C. edulis* PPO activity.

The effect of metal ion at varying concentrations was determined using Ca^{2+} Cu^{2+}, Mg^{2+}, Fe^{2+} Zn^{2+} and Na^{+} salts at concentration of 5, 10, and 20 mM respectively in 0.1M potassium phosphate buffer (pH 6.8). PPO activity was determined according to the standard assay procedure.

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3. Results and Discussion

3.1. Purification profile of polyphenol oxidase of melon seed (C. edulis).

Although PPO has been purified and characterized from many plants, there is no any report describing PPO from melon seeds (*C. eduli*). In order to overcome the negative effects of its phenolic substrates on the ionic and hydrophobic characteristics, Polyvinylpolypyrrolidone (PVPP) (5%) and ascorbic acid (10 mM) were used to bind the phenols and to reduce quinones to phenolic substrates during extraction, respectively. There were several studies on the PPO purification and characterization using only the precipitated fractions of ammonium sulfate without using chromatographic purification methods [25]. The best extraction fraction here was achieved with 80% $(NH_4)_2SO_4$ saturation at 4 °C, followed by ion-exchange chromatography. Polyphenol oxidase activity was eluted in the fractions with the activity peak from the ion-exchange column, as shown in Figure 1. The specific activity of the partially purified enzyme was 1.64 U/mg; a 16-fold purification of the enzyme was achieved with a 0.4% yield. The summary of the purification procedure is given in Table 1.

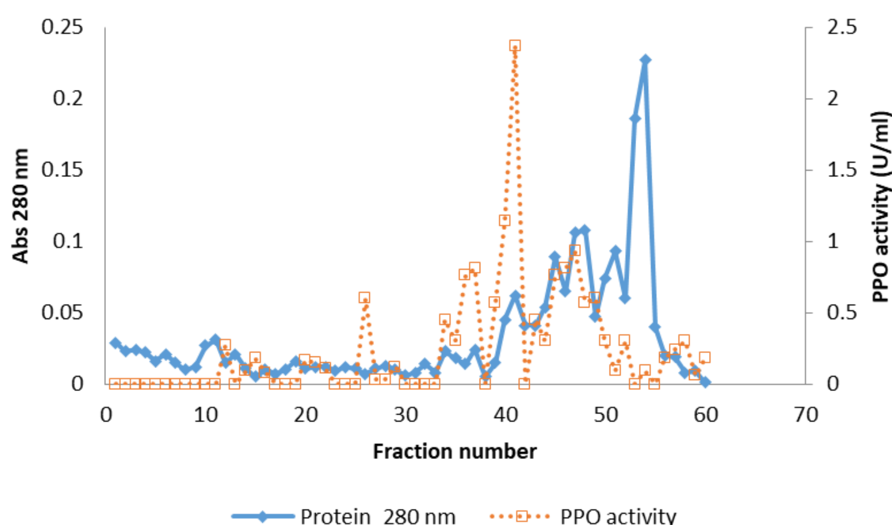

Figure 1. Elution profile of polyphenol oxidase on an ion-exchange column (3.5 × 13cm) of DEAE Sephadex A-50.

Table 1. Purification of polyphenol oxidase from *C. edulis.*

Step	Total Vol. (mL)	Protein concentration (mg/mL)	Total protein(mg)	Activity (U/mL)	Total Activity (U)	Specific Activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	750	6.5	4875	0.65	487.5	0.1	1	100
$(NH_4)_2SO_4$ precipitation	125	3.2	400	1.82	227.5	0.57	5.7	0.5
DEAE Sephadex A -50	40	2.95	118	4.85	194	1.64	16.4	0.4

Total Protein (mg) = Protein concentration (mg/ml) x Total volume (mL)
Total Activity (U) = Activity in the fraction (U/ml) x Total volume (mL)
Specific Activity (U/mg) = Total activity (U) / Total protein (mg)
Yield (%) = (Total Activity of Purified step / Total Activity of the crude) x 100
Purification Fold = (Specific Activity of Purified Step / Specific Activity of the Crude

3.2. Effect of pH on C. edulis PPO activity and stability.

The effect of pH on the *C. edulis* PPO activity was studied by using 10 mM L-DOPA as substrate at various pH values at room temperature. The pH activity profile (Figure 2a)

indicated that the optimum pH value for *C. edulis* PPO activity was 6.5. This result is similar to Bello *et al.* [26], who reported optimum pH 6.5 for pumpkin PPO but higher than that of Ferula sp. (6.0) [27] and medlar fruit [28]. The result of *C. edulis* PPO pH stability indicated that the enzyme was stable within pH 6–7 (Figure 2b), retaining more than 70% of its activity. On the other hand, the enzyme was almost unstable and inactive at pHs 2.0 and 9.0, with less than 25% of its residual activity. Collectively, proper pH value is essential for PPO to achieve maximum activity. It can be concluded that improper pH value is an efficient way to prevent enzymatic browning [29]. The observed inhibition of PPO in this study by low concentration of SDS is not consistent with generally reported activation of PPO by low concentration of SDS [23]. Kenten [30] has reported that the activation of crude bean leaf PPO by SDS occurred below 1 mM SDS. Though some authors, Moore [31], Jimenez and Garcia- Carmona [32], Escribano *et al*. [23], Laveda *et al.* [33], revealed from their experiments the joint effects of pH and SDS on PPO activity that the detergent causes a shift in the pH optimum of the enzyme from low to higher pH values but their reports is at variance with the same optimum pH observed in the presence and absence of SDS in this study. However, this behavior of a shift in pH does not seem ubiquitous as similar pH optimum profiles with and without SDS were obtained for latent potato leaf PPO [34]. Subjecting *C. edulis* seed to acidic pH in the presence and absence of SDS could possibly ameliorate its browning effect owing to inhibition PPO activity at this said pH, thereby increasing its quality.

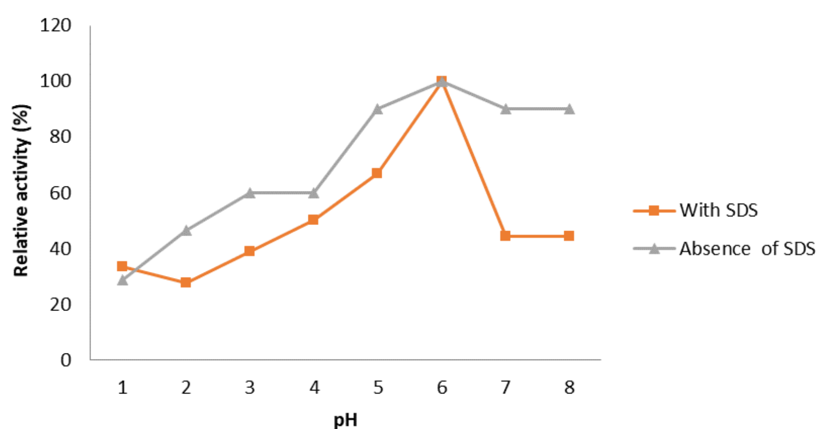

Figure 2(a). Effect of pH with SDS and without SDS on *C. edulis* PPO activity.

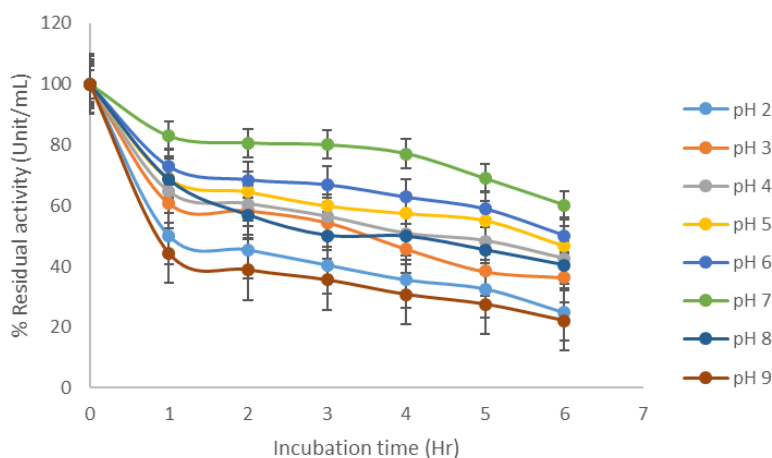

Figure 2(b). pH stability of *C. edulis* PPO. The activity of the enzyme was expressed relative to the activity of the enzyme at 0 min at 100%

3.3. Effect of temperature on C. edulis PPO activity and stability.

The temperature effect on the *C. edulis* PPO activity and stability was studied over a range from 30 to 80 °C using L-DOPA as substrate. The results are depicted in Figures 3a and 3b. The enzyme is active from 30 to 55 °C, showing maximum activity at 50 °C. A decrease of *C. edulis* PPO activity was observed at temperature > 60 °C, and the enzyme lost 30%, 50%, and 87% of its maximal activity at 60, 70, and 80 °C, respectively, until all activity was lost at temperature > 80 °C, due to thermal inactivation.

It has been previously reported that different plants exhibited different optimum temperatures. The optimum temperature of 40 ºC had earlier been reported for dog rose and artichoke PPOs [35,36], respectively, 35 ºC for mamey [37], 25 ºC for *Lonicera japonica* PPO and 20 ºC for lotus seed [38] using catechol as the substrate. The reported optimum value (50 °C) in this study is higher than 35 ºC optimum temperature for *Capsicum Annuum* seeds PPO, which was reported by Guven *et al*. [39], lower than 60 ºC temperature optima observed in two varieties of *Irvingia* seed PPOs as reported by Sanni [12]. An optimum temperature 20 ºC – 60 ºC generally observed for various plants using different substrates has been reported by Yoruk and Marshall [40] and Mayer [22] in their views of plant PPOs. However, it should be noted that the optimum temperature of PPO varies in different plant sources and that nature of the substrate influences the optimum temperature.

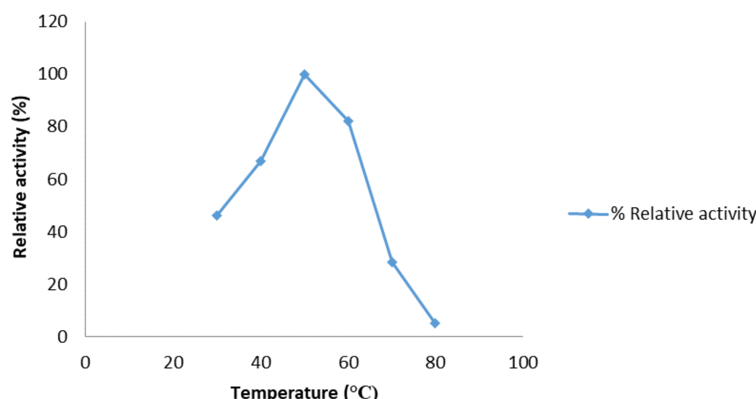

Figure 3(a). Effect of temperature on *C. edulis* PPO activity.

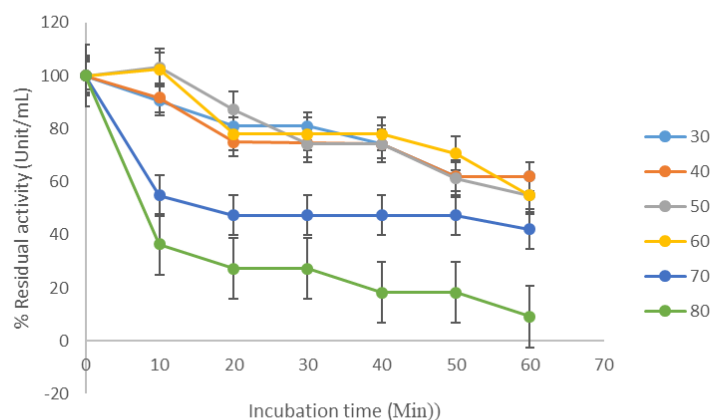

Figure 3(b). Thermal stability of *C. edulis* PPO. The activity of the enzyme was expressed relative to the activity of the enzyme at 0 min taken at 100%.

Insightfully, an appropriate temperature is necessary for the enzyme to achieve the maximum activity. In industrial production, avoiding the proper temperature of the reaction is

an efficient way to inhibit enzyme activity. The results (Figures 3a and 3b) showed that the appropriate temperatures to process *C. edulis* were above 60 °C in view of its PPO activity. Moreover, the curve of temperature-stability indicated that thermal treatment to enzyme inactivation was an essential procedure to prevent enzymatic browning. In summary, the data indicated that heating at high temperature is an effective method to ameliorate the enzymatic browning caused by PPO in *C. edulis.*

3.4. Substrate specificity and enzyme kinetics.

Substrate specificity for *C. edulis* PPO was investigated using 4 different substrates (Table 2) at the enzyme's pH optimum for L-DOPA as the substrate (pH 7.0). Activities were compared with the activity observed in the presence of L-DOPA (set as 100%). The highest activity was found using L-DOPA, followed by gallic acid, catechol, and tyrosine. *C. edulis* showed activity in all the substrate investigated, but a lower activity toward tyrosine (monophenol) was recorded. The slight monophenolase activity reported for *C. edulis* PPO suggests that *C. edulis* PPO has a weak activity on monophenols compared to the strong activity on diphenols. Furthermore, a reliable determination of monophenolase activity requires the use of a large amount of enzyme and a substantially extended monitoring time [41]. The results obtained in this study are in agreement with Sanni [12], who reported a higher activity of *Irvingia gaboneniss* and *Irvingia wombolu* seed PPOs using L-DOPA as the substrate.

Table 2. Substrate specificity of *C. edulis* PPO.

Substrate	% Relative activity
L-DOPA	100 ± 0
Gallic acid	66.5 ± 9.87
Catechol	63.3 ± 4.85
Tyrosine	19.1± 5.56

Data represent the mean ± standard deviation of replicate readings (n= 3)

3.5. Kinetic parameters.

K_m and V_{max} values for *C. edulis* PPO for three substrates are presented in Table 3. The affinity of the enzyme varied depending on the substrate used. *C. edulis* PPO had a higher affinity for L-DOPA, as evidenced by lower K_m value and higher V_{max}. The criterion for the best substrate is the V_{max}/K_m ratio, which was recorded higher in L-DOPA. Of the substrates tested, the best substrate for *C. edulis* PPO was L-DOPA. The result of this study compared well with Saedian [42], who reported K_m value (8.5 mM) for *Solanum lycopersicum* PPO using L-DOPA as the substrate.

Table 3. Kinetic parameters of *C. edulis* PPO.

Substrate	K_m (mM)	V_{max} (Unit/min)	V_{max}/K_m(Unit/min.mM^{-1})
L-DOPA	3.64	0.92	2.53 x10^{-1}
Gallic acid	5.03	0.58	1.15x10^{-1}
Catechol	5.06	0.55	1.09 x10^{-1}
Tyrosine	6.68	0.37	0.55 x10^{-1}

Data represent the mean ± standard deviation of replicate readings (n= 3)

3.6. Effect of inhibitors and metal ions on enzyme activity.

The effects of inhibitors such as ascorbic acid, EDTA, SDS, and urea (5−20 mM) on *C. edulis* PPO activity in a concentration-dependent manner were studied. The effects of

various metal ions ($CuSO_4$, $ZnSO_4$, $MgSO_4$, $CaCl_2$, $FeSO_4$, $CaCl_2$, and NaCl) on enzyme activity were also investigated with varying concentrations of metal ions, 5 mM, 10 mM, and 20 mM respectively. The enzymatic activity of the control mixture without inhibitors and metal ions was taken as 100% and then compared to the other treatments.

The presence of chemicals on *C. edulis* PPO activity assay showed diverse effects. As presented in Tables 4 and 5, the percentage inhibition was compared with that of the control. The percentage (%) residual enzyme activity with NaCl and $CaCl_2$ suggests that Na^+, Ca^{2+} Cl^- showed an inhibitory effect on *C. edulis* PPO activity. Similar results were reported for Victoria grape PPO [43]. Among the divalent cations tested, Cu^{2+}, had a positive effect on *C. edulis* PPO activity at a salt concentration above 10 mM. Enzyme activity increases with an increase in the concentration of metals. The effect of ions is highly variable according to enzyme sources [40]. For example, $Zn^{2+}$and Fe^{2+} and Mg^{2+} at the concentration of 5 - 10 mM showed a slightly negative effect on *C. edulis* PPO. In contrast, for higher concentrations (20 mM) it stimulated *C. edulis* PPO activity. Several common PPO inhibitors had an inhibitory effect on the *C. edulis* PPO. Among these inhibitors, Urea, SDS, EDTA, and Ascorbic acid presented no potent effect at concentrations of 5 mM. However, their deactivating effects increased with an increased concentration of 10 mM and above.

Table 4. Effect of Inhibitors on the activity of *C. edulis* PPO.

Inhibitors	Relative activity (%)		
	5 mM	**10 mM**	**20 mM**
Ascorbic acid	70.0 ± 6.9	32.5 ± 2.12	17.5 ± 1.25
EDTA	75.0 ± 9.04	55.0 ± 3.39	17.5 ± 0.95
SDS	38.3 ± 2.75	17.5 ± 1.65	7.5 ± 0.29
Urea	95.0 ± 9.71	67.5 ± 6.92	22.5 ± 0.87

EDTA= Ethylenediaminetetraacetate, **SDS**= Sodium dodecyl sulphate.
Data represent the mean ± standard deviation of replicate readings (n= 3)

Table 5. Effect of metal ions on activity *C. edulis* PPO.

Salt	Relative activity (%)		
	5 mM	**10 mM**	**20 mM**
$CuSO_4$	35.2 ± 1.62	63.5 ± 3.62	77.8 ± 6.35
$FeSO_4$	33.3 ± 1.96	53.7 ± 1.89	58.2 ± 1.69
$ZnSO_4$	29.6 ± 0.95	46.3 ± 1.89	70.4 ± 2.68
$MgSO_4$	42.6 ± 2.95	50.0 ± 6.73	68.5 ± 4.55
NaCl	25.9 ± 5.6	16.7 ± 0.65	5.62 ± 0.75
$CaCl_2$	38.9 ± 3.65	25.7 ± 1.15	18.5 ± 1.29

Data represent the mean ± standard deviation of replicate readings (n= 3)

The results here are in agreement with Guven *et al.* [39], who reported EDTA, SDS, and Ascorbic acid showing a deactivating effect on *Capsicum annuum* seeds PPO. Ascorbic acid exhibited nearly complete inhibition of *C. edulis* PPO at 20 mM, and it may act more as an antioxidant than as an enzyme inhibitor. This is consistent with the earlier studies of Gonzalez *et al.* [44] and Shrestha *et al.* [45], who reported ascorbic acid as the most potent natural anti-browning agent. Ascorbic acid reduced the initial quinone formation before it undergoes secondary reactions leading to browning [46-47].

4. Conclusions

This study reported the partial purification and the characterization of PPO from the seeds of melon for the first time. The optimal pH and temperature values of 6.5 and 50 °C were obtained for *C. edulis* PPO. The result of the kinetic parameters indicated PPO had the highest affinity for L-DOPA, and it was found that L-DOPA was the most efficient phenolic substrate

for *C. edulis* PPO when considering the ratio of V_{max}/K_m. The result was consistent with the previous reports that most plant PPOs exhibit a high affinity for L-DOPA as substrate [40; 48]. The PPO activity was strongly inactivated by ascorbic acid and SDS. This study reveals possible treatments that can be implemented to ameliorate the browning effect of PPO during the processing of melon seeds for human consumption and market values. However, further research is needed to determine inherent enzyme characteristics and propose more edible inhibitors applicable at the industrial scale.

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Conflicts of Interest

The authors declare no conflict of interest.

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