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Purification and biochemical characterization of polyphenol oxidase from seeds of melon (*Citrullus colocynthis*)

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Abstract

Polyphenol oxidase (PPO) is an enzyme that is responsible for the enzymatic browning of fruits and vegetables. This is generally undesired process and need to be prevented in food technology. PPO from seeds of *Citrullus colocynthis* was purified, the physicochemical properties such as effects of pH and temperature, substrate specificity, effects of inhibitors and cations on PPO activity and the kinetic parameters for four substrates namely, catechol, L-DOPA, gallic acid and tyrosine, were determined. The purification steps resulted in 41-fold with 10 % yield, and the optima pH and temperature values for PPO from *C. colocynthis* were found to be pH 7.0 and 60 °C, respectively using catechol as substrate. About 9 % enzyme initial activity was retained after 60 min of incubation at 80 °C, and the apparent molecular weight was determined as 42 kDa by partially denaturing SDS-PAGE. PPO activity was inhibited by ascorbic acid, SDS and certain divalent (Ca^{2+}, Zn^{2+}, Mg^{2+} and, Fe^{2+}) and monovalent (Na^{+}) metal. Moreover, purified enzyme solution showed diphenolase activity toward catechol, gallic acid, L-DOPA and monophenolase activity toward tyrosine, therefore, tyrosinase was identified as the only one PPO in *C. colocynthis* seeds. This study revealed the use of temperature above 80 °C to inhibit PPO activity during processing and storage of melon seeds.

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Introduction

Cucurbits are edible crops found in the Cucurbitaceae family, they are distributed in the tropical and subtropical countries. A variety of plant belonging to this family with a lot o f nutritional benefits is *Citrullus colocynthis* (L.) (Benmoumou and Madidi 2019). The seeds of *C. colocynthis* contain proteins, essential oils, mineral, dietary fibre and other nutritionally important components that could be harnessed as alternatives for human diet (Kumar *et al.* 2008). *C. colocynthis* seeds can be obtained either in shelled or unshelled form in West African Markets and are used greatly in cookery.

The seed of the *C. colocynthis* is whitish in color, with oval-flat shape, consisting edible fatty acids such as linoleic and oleic acids (Teixeira da Silva and Hussain 2017). *C. colocynthis* seeds are part of the condiments used in the preparation of sauces consumed in most African nations, they are consumed crushed or grilled and served to thickening sauces and sometimes make into a cake for its delicacy (Benmoumou and Madidi 2019). Similar to other commercially-available crops, dehulled *C. colocynthis* is prone to browning due to post-harvest effect. In the course of dehulling and crushing or making into powder,

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the color changes from whitish to greyish, making it appears dull, and eventually turns brown, thereby loses its acceptability for market value. Fruits, vegetables and seeds are susceptible to enzymatic browning, which is typically catalyzed by polyphenol oxidase (PPO) (Liu *et al*. 2007; Guven *et al*. 2017; Adeseko *et al*. 2019).

PPOs catalyse mono- and *o*-diphenol conversion to *o*-quinones during ripening, postharvest handling, storage and processing of fruits and seeds (Spagna *et al.* 2005; Sélles-Marchart *et al.* 2006). Enzymatic browning is caused by the oxidation of phenolic compounds to quinones and their eventual (non-enzyme-catalysed) polymerization to melanin pigments (Jiang *et al*. 2003). The evidence of PPO is proved by the brownish colouration found in the tissue of numbers of fruits and vegetables, which eventually leads to discoloration observed in many plant food materials (Yang *et al.* 2004; Núñez- Delicado *et al.* 2005). Oxidative browning reactions in many foods of plant origin, generally cause deterioration in food quality by changing structural, nutritional and organoleptic properties and these reactions significantly diminish consumer acceptance, thereby reducing the economic values (Dincer *et al.* 2003). The aim of this study was to isolate and purify PPO from the seeds of *C. colocynthis* and investigate its intrinsic physicochemical properties, in order to ameliorate the adverse browning often experience during processing and storage of melon seeds, for consumer acceptability enhancement.

Experimental

Sample preparation

Fully matured fruits of *C. colocynthis* (Egusi Maga) were purchased from a local market in Akure (Ondo State, Nigeria) and identified at Department of Crop Science and Pest, School of Agriculture, Federal University of Technology (Akure, Ondo State, Nigeria). The seeds were removed, air dried for two weeks and later dehulled manually.

Preparation of crude enzyme extract

Melon seeds (400 g) of *C. colocynthis* were thoroughly homogenized in 1.2 L of ice cold 25 mM phosphate buffer (pH 6.8) containing 10 mM ascorbic acid using a warring blender. The homogenate was filtered using four layers of cheese cloth. The filtrate was again filtered using layers of glass wool to remove the floating lipid, followed by centrifugation in a centrifuge at 16,000 rpm for 30 min at 4 °C. The supernatant was kept at -4 °C for 45 min. The lipid layer was separated. The supernatant was stored in a refrigerator and used as crude enzyme for further experiments.

Determination of protein concentration

Protein concentration was determined according to the method described by Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

Determination of PPO activity

PPO activity was determined with a spectrophotometer by measuring an increase in absorbance at 420 nm at room temperature using catechol as substrate as described by Mayer (2006) with a slight modification. The reaction mixture consisted 0.2 mL freshly prepared enzyme solution and 2.8 mL of 10 mM catechol in 20 mM potassium phosphate buffer (pH 6.8) while the blank contained the buffer and the substrate. One unit (U) of PPO activity was defined as the amount of the enzyme that increased the absorbance by 0.001 per min.

Purification of PPO from C. colocynthis

The crude enzyme (250 mL) was brought to 80 % ammonium sulfate saturation, the precipitate was collected by centrifugation at 16,000 rpm for 10 min. The precipitate was dissolved in 5 mL of 0.1 M potassium phosphate buffer (pH 6.8) and dialyzed with the same buffer at 4 °C overnight. The dialysate was placed on a DEAE-A50 Sephadex column (3.5 × 13 cm). The column was pre-equilibrated with 0.1 M potassium phosphate buffer (pH 6.8) and the protein was eluted using the same buffer (flow rate: 10 $mL.h^{-1}$). The unbound proteins were eluted by a linear gradient of 0 to 0.5 M NaCl in 0.1 M potassium phosphate buffer (pH 6.8). The absorbance of the fraction was

monitored at 280 nm and fractions were assayed for PPO activity at 420 nm using catechol as substrate. The fractions with PPO activity were pooled and concentrated using 4 M sucrose. Gel filtration of the concentrated peak fractions exhibiting PPO activity was carried out on a Sephadex G-200 column (1.4 × 75 cm; flow rate: 5 $mL.h^{-1}$) using the same buffer. Absorbance of each fraction was taking at 280 nm while fractions exhibited enzyme activity were pooled together, concentrated and an aliquot was used for SDS- PAGE.

Determination of molecular weight of PPO

The molecular weight of the purified PPO was determined by SDS-PAGE using 10 % gel according to Laemmli *et al*. (1970) with standard protein markers (17 – 103 kDa) and were stained with Coomassie Brilliant Blue.

Determination of C. colocynthis PPO substrate specificity

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

Effect of pH on PPO activity in presence and absence of SDS

The enzyme pH optimum was determined with and without SDS according to the method of Sanni (2016) using various buffers at 0.1 M pH ranges from 2.0 – 9.0. The reaction mixture contained glycine-NaOH buffer (pH 2.0 – 3.0); 0.1 M sodium acetate buffer (pH 4.0 – 5.0); 0.1 M potassium phosphate buffer (pH 6.0 – 7.0) and 0.1 M Tris-HCl 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.

Effect of pH on stability and activity of PPO

The pH stability of the purified enzyme was determined according to the method of Sanni (2016), by preparing various buffers of pH 2.0 – 9.0 using 0.1 M glycine NaOH (pH 2.0 – 3.0), 0.1 M sodium acetate buffer (pH 4.0 – 5.0), 0.1 M potassium phosphate buffer (pH 6.0 – 7.0) and 0.1 M Tris-HCl buffer (pH 8.0 – 9.0), and then incubating the purified enzyme with each specified buffer solution for 6 h. The residual activity was determined by drawing 1 mL of aliquot enzyme at one-hour interval subsequently after initial 0-hour activity according to standard assay method.

Effect of temperature on stability and activity of PPO

The effect of temperature on PPO activity was investigated by varying the temperature conditions between 30 – 80 °C. The reacting mixture consisted purified enzyme and catechol was incubated at the stated temperatures while 1 mL of aliquot enzyme was withdrawn at an interval of 10 °C after 10 min of incubation. The activity was determined according to the standard assay procedure. The thermal stability was determined by incubating the enzyme at different temperature conditions: 30 – 80 °C. The initial activity was determined at the 0 min while the residual PPO activity was determined at 10-min interval for each temperature for 1 h according to the standard assay procedure.

Kinetic parameters of C. colocynthis *PPO*

The kinetic constants, K_m and V_{max}, of the purified enzyme was determined using Lineweaver-Burk plot with catechol, gallic acid, tyrosine, and L-DOPA as substrates, at varying concentrations (5 – 40 mM) in 0.1 M potassium phosphate buffer (pH 6.8).

Effect of inhibitors on C. colocynthis *PPO activity*

PPO activity was determined in the presence of ascorbic acid, EDTA, urea and SDS (5, 10 and 20 mM). The reaction mixture was incubated for 20 min and the change in absorbance was measured by spectrophotometer at 420 nm. Control tests for inhibitors plus substrate plus buffer were also run at the same time.

Table 1. Purification of PPO from *C. colocynthis.*

Step	Total volume [mL]	Protein concentration [mg.mL^{-1}]	Total protein [mg]	Activity [U.mL^{-1}]	Total activity [U]	Specific activity [U.mg^{-1}]	Purification fold	Yield [%]
Crude enzyme	1120	7.40	8,288	0.8	896	0.1	1	100
$[NH_4]_2SO_4$ precipitation	85	6.50	553	2.0	170	0.3	2.8	19
DEAE Sephadex A-50	40	2.75	100	2.8	112	1.1	10	13
Sephadex G-200	15	1.25	18.8	5.7	85.5	4.6	41	10

Total Protein (mg) = Protein concentration (mg.mL^{-1}) × Total volume (mL); Total Activity (U) = Activity in the fraction (U.mL^{-1}) × Total volume (mL); Specific Activity (U.mg^{-1}) = Total activity (U)/Total protein (mg); Yield (%) = (Total Activity of Purified step/Total Activity of the crude) × 100 Purification Fold = (Specific Activity of Purified Step/Specific Activity of the Crude).

Effect of cations on PPO activity

The effect of cations on PPO activity was determined using Cu^{2+}, Mg^{2+}, Fe^{2+} and Zn^{2+} salts at concentration of 5, 10, and 20 mM respectively dissolved in catechol solution containing 0.1 mM phosphate buffer (pH 6.8) for 10 min. Purified enzyme solution, 0.1 mL was added with 2.9 mL of the mixture of each metal ion and buffer solution were incubated for 20 min while the enzyme activity was determined according to the standard assay procedure.

Results and Discussion

Isolation and purification of C. colocynthis *PPO*

PPO from *C. colocynthis* was purified using ammonium sulfate precipitation, ion-exchange and size exclusion chromatography. The specific activity of the purified enzyme was 4.6 U.mg^{-1}; a 41-fold purification of the enzyme was achieved with 10 % yield. The summary of the purification procedure is given in Table 1. PAGE in the presence of SDS produced a single protein band as represented in Fig. 1. The subunit molecular weight of the purified enzyme was estimated as 42 kDa by SDS-PAGE, this result is consistent with a previous report from Marques *et al.* (1995), whose studies on apple PPO for the native (42 kDa) and proteolyzed (27 kDa) forms detected under partially denaturing conditions were found to have molecular weights of 64 and 42 kDa, respectively. Studies on broad bean PPO also showed that the 45 kDa was obtained as the molecular weight under partially denaturing conditions (Cary *et al.* 1992; Robinson and Dry 1992).

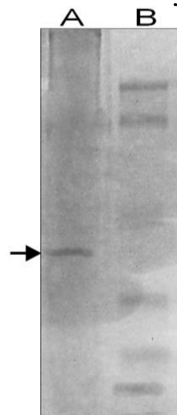

Fig. 1. SDS-PAGE of the purified PPO fraction after gel filtration. Lane A is the molecular weight of the purified PPO from *C. colocynthis* and Lane B represents molecular weight of protein markers.

In general, molecular weights of PPOs vary significantly from source. The isoforms of PPOs from many plant sources were reported to range in molecular mass from 32 to over 200 kDa, mostly within the range of 35 – 70 kDa (Flurkey 1986; Sherman *et al.* 1991; Steffens *et al.* 1994; Fraignier *et al.* 1995; Van Gelder *et al.* 1997; Yang *et al.* 2000).

Physicochemical and kinetic parameters of PPO

The pH optimum was observed at neutral pH 7.0 while about 14.3 – 28.6 % relative activity was observed at acidic region, pH 2.0 – 5.0 (Fig. 2). The pH optimum of PPO from plants also varies depending on the plant source. Yoruk and Marshall (2003) reported that pH optimum varies widely with plant source but is generally in the range of 4.0 – 8.0.

The influence of pH in presence of SDS on PPO activity is summarized in Fig. 3, the observed deactivation of *C. colocynthis* PPO by low

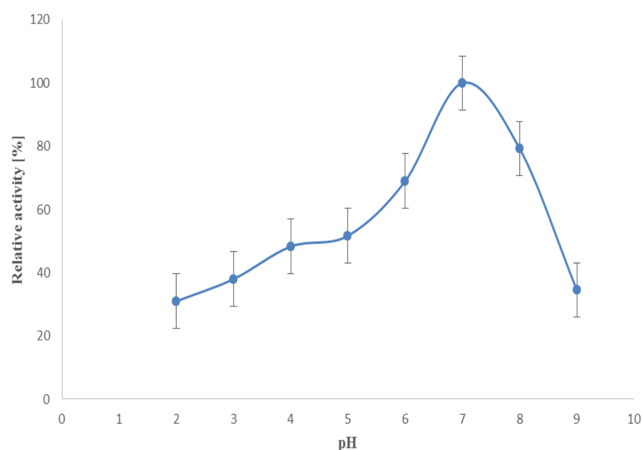

Fig. 2. Effect of pH on the activity of purified PPO. Data represent mean ± STD (n = 3).

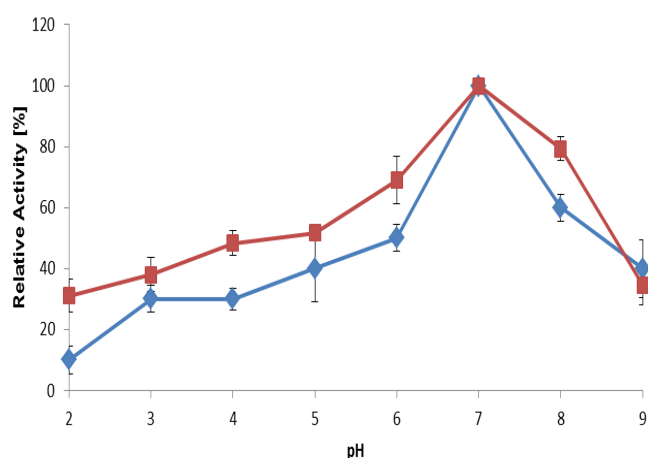

Fig. 3. Effect of pH on the activity of purified PPO in presence (diamonds) and absence (squares) of SDS. Data represent mean ± STD (n = 3).

concentration of SDS with pH is in agreement with the generally reported activation of PPO by extreme low concentration of SDS (Escribano *et al.* 1997). Kenten (1957) has reported the activation of crude bean leaf PPO by SDS below 1 mM SDS. Though some authors (Moore and Flurkey 1990; Jimenez and Garcia-Carmona 1996; Escribano *et al*. 1997; Laveda *et al.* 2000) revealed from their experiments, the joint effects of pH and SDS on PPO activity that the detergent causes a shift in 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 (Sanchez-Ferrer *et al.* 1993).

Subjecting *C. colocynthis* seeds to acidic medium could possibly control its browning effect owing to inhibition of PPO activity at this said pH, thereby increasing its quality.

The results of effect of pH on the stability of the purified PPO is presented in Table 2; there was a drastic reduction in enzyme activity at acidic pHs. The enzyme was able to retain about 17.0, 23.2, 33.2, 43.5, 46.0, and 69.6 % residual activity for pH 2 – 7 respectively. However, at pH 7.0, a high percentage relative activity of about 70 % was observed, while about 37 and 17 % residual activities were observed for pH 8.0 and 9.0, respectively.

The influence of temperature on PPO activity and stability are presented in Fig. 4 and Table 3 respectively. However, an optimum temperature at 60 °C was achieved using catechol as substrate. There was a gradual increase in activity of the enzyme with increase in temperature from 30 – 50 °C given 33 – 63.9 % relative activities but almost complete deactivation of PPO was observed at 80 °C. The observed temperature here compared well with the reported 60 °C as temperature optimum for strawberry (Serradell *et al.* 2000) and cucumber PPOs (Miller *et al.* 1990).

Table 2. Stability of pH of PPO from *C. colocynthis*.

pH	Residual activity [%]
2	17.4±2.5
3	23.2±4.4
4	33.2±8.7
5	43.5±2.5
6	46.0±9.1
7	69.6±8.6
8	37.9±5.0
9	17.4±5.0

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

Table 3. Thermostability of PPO after 60 min of incubation.

Temperature [°C]	Residual activity [%]
30	61.9±3.5
40	56.5±2.8
50	55.1±2.3
60	51.2±1.7
70	42.1±1.3
80	9.1±0.9

The experiment was repeated three times, and each value is given as the mean ± standard deviation.

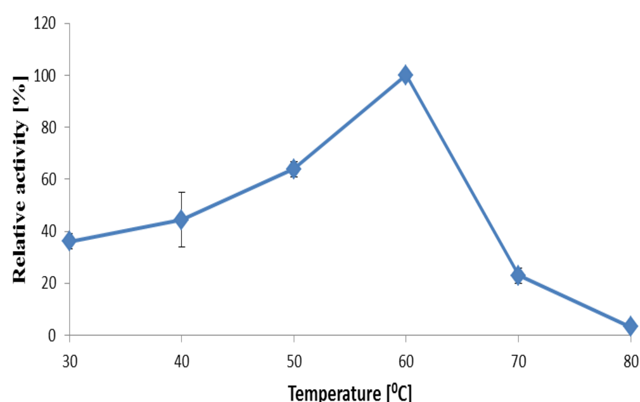

Fig. 4. Effect of temperature on the activity of PPO. The results were expressed as percentage relative to the maximum activity at 60 °C taken as 100 %. Data represent mean ± STD (n = 3).

Yang *et al.* (2000) had earlier ascertained that PPO from different plant sources has optima temperatures ranging from 30 – 60 °C. Nevertheless, at temperatures above 60 °C, a decrease in PPO activity was observed with 42 and 9.1 % residual activity at 70 and 80, respectively. An optimum temperature, 60 observed in this study is relatively higher than those from other plant sources such as apple (Zhou *et al.* 1993), banana (Yang *et al.* 2000) and mango and corn tassel (Robinson *et al.* 1993; Guven *et al.* 2016) with a temperature optimum of 30 °C; cocoa bean (Lee *et al.* 1991) and 45 °C; sunflower (Raymond *et al.* 1993). Variations in temperature may also alter the solubility of oxygen, one of the substrates required for PPO to perform its catalytic activity (Wang *et al.* 2007).

The thermal stability of PPO from *C. colocynthis* is presented in Table 3. About 62, 56, 55, and 54 % residual activity were observed at 30, 40, 50, and 60 °C respectively after 60 min of incubation. However, about 42 and 9.1 % residual activity were measured at 70 and 80 °C, respectively. The PPO of this was moderately stable with about 50 % activity recorded after 60 min of incubation at 60 °C. These results compared favourably with the earlier studies of Valero *et al.* (1988), who reported a complete inactivation of grape PPO at 75 °C after 15 min of incubation. Thermal stability of plant PPOs is influenced by the nature of phenolic substrate used during determination (Park and Luh 1985).

The results of the kinetic parameters of *C. colocynthis* PPO are summarized in Table 4, the K_m values of PPO using catechol, gallic acid, L-DOPA and tyrosine as substrates were: 5.04, 6.38, 7.48 and 7.89 mM respectively while the values for V_{max} were: 1.51, 0.78, 0.67 and 0.54 U.min^{-1}, respectively. Therefore, catechol having the low K_m with the highest value for V_{max} is the best substrate for *C. colocynthis* PPO.

The activity of the purified PPO *C. colocynthis* using four different substrates was in order of: catechol > gallic acid > L-DOPA > tyrosine as shown in Table 5.

The level of PPO activity towards phenolic substrates varies widely in the plant kingdom (Sherman *et al.* 1995). However, these differences may be due to the nature of the side chains, number of hydroxyl groups and their position in the benzene ring of the substrates (Oktay *et al.* 1995; Mueller *et al.* 1996).

Inhibition of C. colocynthis *PPO activity*

The effect of cations and inhibitors on PPO activity is presented in Table 6. Fe^{2+}, Zn^{2+}, Mg^{2+} and, Ca^{2+} were found to inhibit the activity of the enzyme while Cu^{2+} increases the enzyme activity at all concentrations investigated. However, there was increase in percentage inhibition as the concentration of the metal ions increases. Ascorbic acid, EDTA, SDS, and urea were observed to inhibit the activity of PPO from melon seeds.

Table 4. Kinetic parameters of PPO from C. colocynthis.

Substrate	K_m [mM]	V_{max} [U.min^{-1}]	V_{max}/K_m [U.mM^{-1}]
Catechol	5.40	1.51	3.0 ×10^{-1}
Gallic acid	6.38	0.78	1.2 ×10^{-1}
L-DOPA	7.48	0.67	0.9 ×10^{-1}
Tyrosine	7.48	0.54	0.7 ×10^{-1}

The experiment was repeated three times, and each value is given as the mean ± standard deviation.

Table 5. Substrate specificity of PPO from *C. colocynthis.*

Substrate	Relative activity [%]
Catechol	100±0
Gallic acid	74.1±9.9
L-DOPA	33.3±4.9
Tyrosine	22.2±5.6

The experiment was repeated three times, and each value is given as the mean ± standard deviation.

Table 6. Effect of cations on PPO activity.

Cation	Relative activity [%]		
	5 mM	**10 mM**	**20 mM**
$CuSO_4$	106±5.6	114±3.3	117±5.5
$FeSO_4$	36.1±2.0	44.4±0.8	66.7±2.6
$ZnSO_4$	66.7±2.0	56.6±2.9	33.3±2.6
$MgSO_4$	25.2±3.9	50.0±5.7	69.4±2.5
NaCl	44.4±5.6	13.9±1.7	6.68±2.46
$CaCl_2$	38.9±2.9	27.8±4.8	11.1±4.2

The experiment was repeated three times, and each value is given as the mean ± standard deviation.

Table 7. Effect of Inhibitors on PPO activity.

Inhibitor	Relative activity [%]		
	5 mM	**10 mM**	**20 Mm**
Ascorbic acid	79.2±4.0	37.2±5.7	20.8±1.3
EDTA	75.8±6.7	54.2±3.3	8.3±3.4
SDS	41.7±6.8	29.3±2.4	12.0±3.3
Urea	83.3±9.7	53.3±5.3	37.5±1.9

The experiment was repeated three times, and each value is given as the mean ± standard deviation.

There was increase in the percentage inhibition as the concentration of the inhibitors increases (Table 7). SDS was a stronger inhibitor with 58 % reduction in enzymatic activity at 5 mM and over 90 % reduction with increasing concentration above 10 mM. EDTA and ascorbic acid are strong inhibitors of PPO from *C. colocynthis*.

Earlier studies on PPO have revealed SDS as a potent inhibitor of tyrosinase activity and it was suggested that this compound might cause inhibition by forming complexes with copper atoms in the active site (Kong *et al.* 2000). SDS with a concentration of 5 mM and above inhibited the PPO activity of *C. colocynthis* (Liu *et al.* 2004). However, PPO from *C. colocynthis* showed 83.3 % inhibition in the presence of Urea at 5 mM concentration, this is in agreement with the earlier studies of Liu *et al.* (2004) and Endo *et al.* (2003). In this study, the presence of Zn^{2+}, Mg^{2+} and, Fe^{2+} showed no activating effect on PPO activity but an increase in enzyme activity was observed in the presence of Cu^{2+} at all concentrations investigated. The result is consistent with the earlier reports of Kong *et al.* (2000), Liu *et al.* (2004), Dalfard *et al.* (2006) and whose studies on plant PPOs confirmed increase in enzyme activity in the presence of copper ion.

Conclusion

This study revealed optimum temperature of the purified PPO from *C. colocynthis* for its enzyme activity to be 60, while there was almost total enzyme deactivation at 80 °C after 60 min of incubation. The enzyme molecular weight was 42 kDa. Nevertheless, the enzyme oxidized tyrosine, therefore, tyrosinase was identified as the only one PPO in *C. colocynthis* seed. The enzyme was deactivated at acidic pH and temperature above 70 °C. Therefore, subjecting processed melon seeds to temperatures above 80 °C could inhibit the browning effect of PPOs in *C. colocynthis* and its products.

Conflict of Interest

The authors declare no conflict of interest.

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