Food Sci. Biotechnol. 25(S): 91-96 (2016)
DOI 10.1007/s10068-016-0103-x

Food Science
and
Biotechnology

Partial Purification and Characterization of Polyphenol Oxidase from Chinese Parsley (*Coriandrum sativum*)

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Received September 29, 2015
Revised January 30, 2016
Accepted February 23, 2016
Published online March 31, 2016

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pISSN 1226-7708
eISSN 2092-6456

Abstract Purification and characterization of polyphenol oxidase (PPO) from Chinese parsley (*Coriandrum sativum*) were achieved. Crude PPO exhibited an enzyme activity of 1,952.24 EU/mL. PPO was partially purified up to 6.52x with a 10.89% yield using gel filtration chromatography. Maximal PPO activity was found at 35°C, pH 8.0 for 4-methylcatechol and at 40°C, pH 7.0 for catechol. PPO showed a higher affinity towards 4-methylcatechol, but a higher thermal stability when reacting with catechol. L-Cysteine was a better inhibitor than citric acid for reducing PPO activity at concentrations of 1 and 3 mM in the presence of either substrate. Two 46 kDa isoenzymes were identified using SDS-PAGE. Isolation and characterization of Chinese parsley serves as a guideline for prediction of enzyme behavior leading to effective prevention of enzymatic browning during processing and storage, including inhibition and inactivation of PPO.

Keywords: polyphenol oxidase, Chinese parsley, characterization

Introduction

Chinese parsley, also known as cilantro, usually refers to leaves of *Coriandrum sativum*, which belongs to family Apiaceae. This plant originates from southern Europe, North Africa, and the southwestern regions of Asia (1) but has been cultivated worldwide. Chinese parsley is commonly used as a spice for culinary purposes and medicinal properties have been reported (2-4).

Similar to other commercially-available crops, Chinese parsley is prone to browning after harvest. Food browning has a critical impact on processed foods as fruits and vegetables are susceptible to enzymatic browning, which is typically catalyzed by polyphenol oxidase (PPO). After harvest, crops are usually subjected to serial post-harvest processing steps that include peeling, slicing, or cutting to allow efficient storage. Injury can destroy subcellular compartment alization in plant tissues, releasing endogenous phenolic substrates, and allow PPO to come into contact with substrates, causing browning (5).

PPO is a family of copper-containing oxidoreductases that is widely distributed among plants in chloroplasts and can catalyze hydroxylation of monophenols to *o*-diphenols, followed by oxidation of *o*-diphenols to *o*-quinones (6). The end product will then undergo further condensation reactions to become dark-colored melanins on the plant surface (7), leading to a high percentage yield loss due to degradation of food quality during post-harvest storage (8,9). Enzymatic browning also results in alteration of physical appearance due to changes in organoleptic characteristics, such as color, flavor, aroma, and texture (10), that further decrease the degree of consumer acceptability and indirectly lead to losses in the food industry. Thus, it is of great importance to study and control enzymatic browning of commercially processed crop-based foods.

The present study involves extraction and purification of PPO from Chinese parsley and storage conditions. Furthermore, pH and temperature optima, substrate specificity, enzyme kinetics, and inhibition characterization of PPO from Chinese parsley will lead to a thorough understanding of the catalytic behavior of parsley PPO. Therefore, this study was directed toward identification of suitable PPO inhibitory conditions and exploration of export possibilities for Chinese parsley, or application as a spice or flavor booster in processed food to increase market ability based on a distinctive aroma.

Materials and Methods

Plant material and chemicals Fresh Chinese parsley (*C. sativum*) was purchased from a local hypermarket in Kuala Lumpur in July 2015. Purchased herbs originated from the Cameron Highlands (Pahang, Malaysia). All chemicals were of analytical grade and were used as obtained.

Preparation of crude enzyme extracts The extraction method was

adopted from Ng and Wong (11) with slight modification. After washing with running tap water, 80 g of Chinese parsley was chopped into 1.0 cmx0.5 cm pieces that were immediately blended using an LB-8011ES industrial blender (Waring Laboratory, Torrington, CA, USA) along with 3 g of polyvinylpyrrolidone (Sigma-Aldrich, St. Louis, MO, USA) and 300 mL of 0.1 M phosphate buffer at pH 6.8 and 4°C under maximum speed (22,000 rpm) for 3 min. Resulting homogenates were then subjected to centrifugation using a Universal 320 R centrifuge (Hettich, Tuttlingen, Germany) at 6,000x*g* for 30 min at 4°C. Supernatants (50 mL) were pooled and filtered using a Buchner filter set. The resulting crude enzyme extract filtrate was stored at 4°C prior to partial purification. Extraction was repeated 3x to produce 3 batches of crude enzyme extract.

PPO assay PPO activity was determined via measurement of an increase in absorbance at 400 nm for catechol and 410 nm for 4-methylcatechol, respectively, using a PRIM Light spectrophotometer (Secomam, Champigny-sur-Marne, France). The reaction mixture contained 0.5 mL of enzyme solution, 0.5 mL of 0.1 M catechol or a 4-methylcatechol solution in 4.0 mL of a 0.1 M phosphate buffer at pH 6.8 at room temperature. Two milliliters of the reaction mixture was then transferred to a cuvette and absorbance readings were determined at 15 s intervals for 5 min using spectrophotometer (12). A blank consisted of 2 mL of substrate solution in a 0.1 M phosphate buffer at pH 6.8. The initial velocity was calculated from the slope of the absorbance *vs.* time curve, where a single unit of PPO activity was defined as the amount of enzyme that caused a 0.001 absorbance change per min (13).

Protein determination Protein concentrations were determined following the Lowry method (14). Bovine serum albumin was used as a standard for construction of a protein standard curve.

Partial purification of PPO A crude enzyme solution of 684 mL was subjected to ammonium sulfate precipitation via slow addition of solid ammonium sulfate crystals to the solution until 50% saturation was achieved while stirring on ice (15). The mixture was allowed to homogenize for 10 min during extended stirring on a magnetic hotplate stirrer, followed by overnight incubation at 4°C. The mixture was then subjected to centrifugation at 12,000x*g* for 15 min at 4°C. The enzyme was restored via dissolution of protein pellets in 5 mL of 0.1 M phosphate buffer at pH 6.8 and supernatants were pooled and subjected to repeat precipitation at 60, 70, and 80% saturation. The protein fraction with the highest specific enzyme activity was dialyzed against the same phosphate buffer at 4°C for 48 h. The buffer solution was changed 4x throughout the incubation period. Then, the dialysate was subjected to gel filtration chromatography using a Sephadex G-100 glass chromatography column (Sigma-Aldrich) packed with Sephadex G-100 that was first equilibrated with a 0.1 M phosphate bufferat pH 6.8 prior to loading of protein samples. Elution was done using a 0.1 M phosphate buffer at pH 6.8 and 5 mL fractions were collected until the 20th fraction, which represented the total column volume. Fractions that showed PPO activity were pooled for enzyme characterization.

SDS-PAGE One milligram of fully denatured protein sample was loaded into each well on the polyacrylamide gel. Protein samples were denatured via boiling with a buffer of 0.35 M Tris-HCl at pH 6.8, 10.3% (w/v) SDS, 36% (v/v) glycerol, 0.6 M DTT, and 0.01% (w/v) bromophenol blue, followed by loading into a 4% stacking gel and separation on a 12% resolving gel at 120 V until the dye front traveled to the bottom of the gel.

Characterization of PPO

***Optimum temperature*:** PPO activity was determined at 20, 25, 30, 35, 40, 45, 50, and 55°C. PPO activity was measured and calculated using the PPO assay method (12) and was expressed as a relative activity (%) to determine the optimum PPO temperature to be used as a working temperature in subsequent experimentation.

***Optimum pH*:** PPO activity was determined at pH 3, 4, 5, 6, 7, 8, and 9 with a 0.1 M citrate buffer used for pH 3 to 6 and a 0.1 M phosphate buffer used for pH 7 to 9. Similarly, enzyme activity was calculated and expressed as a relative activity (%) for determination of the optimum pH for PPO for use in subsequent experimentation.

***Substrate specificity and enzyme kinetics*:** A concentration range from 10 to 100 mM for catechol and 4-methylcatechol was used for determination of the substrate specificity of Chinese parsley PPO. Lineweaver-Burk plots were developed from kinetic data for determination of the Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) (16).

***Thermal inactivation*:** Partially purified PPO (0.5 mL) was incubated with water bath at 50, 60, 70, 80, and 90°C for 10, 20, 30, and 40 min to inactivate the enzyme (17) before proceeding with PPO assays. Heated PPO was immediately immersed in an ice bath for prevention of thermal degradation after incubation. The residual enzyme activity was determined for each time and temperature point and expressed as a percent residual activity (%) with respect to the activity of an initial non-heated PPO sample. The rate constant (k) was determined from a linear regression of first-order PPO inactivation data (18). The energy required for inactivation (E_a) was determined from an Arrhenius plot as:

$$\ln(k) = -\frac{E_a}{RT} + C$$

where R denotes the gas constant (8.31 J/mol/K), T is the inactivation temperature in Kelvin, and c denotes the constant. The inactivation energy was determined from the slope of an Arrhenius plot.

Decimal reduction times (D) and Z values were also determined. The D value denotes the time required for reduction of enzyme activity to 10% of the initial activity at a given pressure and temperature, while Z denotes the temperature increment required for a single logarithmic reduction or a 90% decrease in the D value (19).

Effect of inhibitors The 2 chemical inhibitors citric acid and L-cysteine were used in this study. Percentage inhibition (%) values for each inhibitor at concentrations of 1 and 3 mM in the presence of either catechol or 4-methylcatechol were calculated using the PPO activity without an inhibitor as the initial enzyme activity.

Statistical analysis All assays were performed in triplicate and data collected were expressed as mean±standard deviation (SD) (n=3) using Microsoft Excel and also as percent relative activity.

Results and Discussion

Partial purification of crude protein extracts PPO from Chinese parsley was purified 6.52x with a protein yield of 10.89% achieved via gel filtration chromatography (Table 1). The elution profile showed a distinctive enzyme activity peak in fraction 4 with an enzyme activity of 1,866.7 EU/mL (Fig. 1). High protein concentrations were also observed in fraction 4, indicating that this fraction contained highly purified PPO with high resolution separation because only a single peak was observed and other fractions did not exhibit enzyme activities, despite the presence of protein. Thus, fraction 4 contained partially purified PPO.

Loss of total enzyme activity and reduction in the percentage yield were unavoidable as many proteins, along with some PPO, were diminished in amount during the purification process. However, loss was accompanied by an increase in specific enzyme activity, which increased to 8,604.3 EU/mg from an initial value of 1,320.5 EU/mg. The overall degree of purification was low, compared with partial purification of pear PPO for which 13.3x purification was achieved using the same protocol (16). Parsley (*Petroselinum crispum*) PPO was purified 26.9x with 26.5% recovery (20) while 11.8x with 47.0% recovery was observed for potato PPO after 2 chromatography steps (21). Purification of PPO in this study can, thus, be improved based on application of different and additional chromatography steps before gel filtration.

SDS-PAGE Two distinctive protein bands appeared on lane 1 containing the crude extract and lane 2 containing a partially purified protein sample were near 46 kDa (Fig. 2). The presence of 2 bands suggested that the PPO from Chinese parsley consisted of isoenzymes of similar molecular weight (Mw) values. Due to yield loss, bands in lane 2 were less visible than bands in lane 1. However, most undesirable proteins were removed, indicated by removal of background smear, which resulted in a clearer lane. The presence of isoenzymes among PPO varieties from pears (22) and apple peels (23) has been reported. PPO varieties, both with Mw value of 41 kDa, were reported from cabbage and bananas (24).

Characterization of PPO

***Optimum temperature*:** Chinese parsley PPO activity peaked at 35°C for 4-methylcatechol and at 40°C in the presence of catechol,

Table 1. Purification of PPO from Chinese parsley

Step	Volume (mL)	Protein concentration (mg/mL)	TA (EU)[1)]	SEA (EU/mg)[2)]	Yield (%)	Purification fold
Crude extract	$6.8x10^2$	1.5±0.36	$1.3x10^6$	$1.3x10^3$	$1.0x10^2$	1.0
50% Ammonium sulfate	95	2.3±0.61	$7.1x10^5$	$3.2x10^3$	53	2.4
Sephadex G-100	25	0.68±0.16	$1.5x10^5$	$8.6x10^3$	11	6.5

[1)]TA, Total activity
[2)]SEA, Specific enzyme activity; EU, Enzyme unit

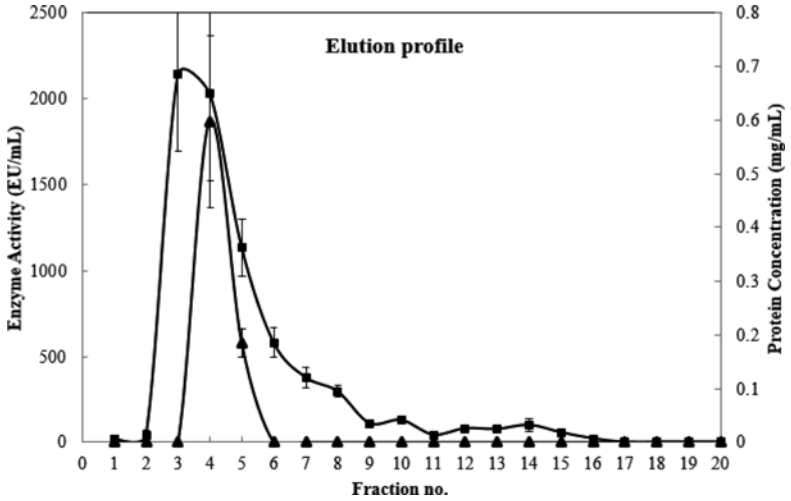

Fig. 1. Elution profile of enzyme activity (▲) and protein concentration (■) of Chinese parsley PPO by Sephadex G-100.

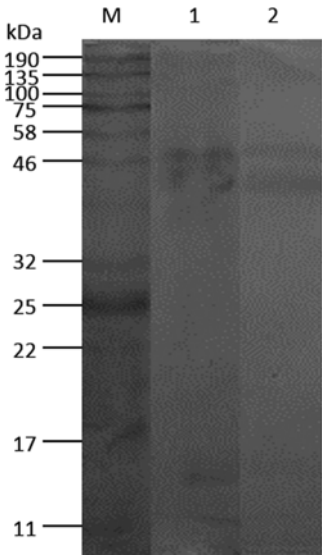

Fig. 2. SDS-PAGE of a crude protein extract (lane 1) and partially purified PPO (lane 2). Fully denatured samples run on 12% resolving gel. Lane M is the Mw marker in kDa.

consistent with the optimum temperature of medlar fruit (25) and Chinese cabbage (26) PPO varieties. Similar results were also reported where PPO exhibited a maximum activity at 40°C or both 4-methylcatechol and catechol (20). Despite variation of optimum PPO temperatures among sources, methods of extraction, and substrates used, PPO activity is generally the highest between 30 and 50°C. Temperatures lower than 30 or higher than 50°C had suggested low PPO activities from various sources such as round brinjal (11), Ankara pear (15), and Amasya apple (27).

***Optimum pH*:** Optimum enzyme pH values were 8.0 with 4-methylcatechol and 7.0 with catechol. Optimum PPO pH values have been reported to be between 4.0 and 8.5 (28,29). The activity of artichoke PPO was reported to be maximal at pH 5.0, 8.0, and 7.0 for 4-methylcatechol, pyrogallol, and catechol respectively (17) while a range of pH values from 4.8 up to 8.2 was reported for pear PPO varieties using 7 different substrate types (16). Typically, the optimum pH for PPO can be observed at or near neutral values.

***Enzyme kinetics and substrate specificity*:** PPO substrate specificity was higher for catechol at 64.5 EU/mL/mM than for 4-methylcatechol at 38.2 EU/mL/mM (Table 2). The K_m value of catechol of 31.0 mM was also lower than for 4-methylcatechol at 37.4 mM, suggesting a higher reaction affinity towards catechol. Similar results were reported for parsley (19) and durum wheat (30) PPO varieties. Substrate specificity varies depend on plant source and assay method. K_m values for taro PPO were reported to be 9.0 mM for 4-methylcatechol, 67.9 mM for catechol, and 89.9 mM for pyrogallol (31), whereas mango PPO exhibited the highest substrate specificity and affinity towards catechol with a K_m value of 8.2 mM, compared with other substrates (32).

***Thermal inactivation*:** PPO from Chinese parsley exhibited a reduction in catalytic activity as the temperature and duration of heat inactivation increased in the presence of both substrates, 4-methylcatechol and catechol reflected by an increment in *k* values (Table 3). The half-life of PPO ($T_{1/2}$) decreased as the incubation temperature increased due to instability of the enzyme at high temperatures. Both *D* and *Z* values were higher when enzyme reacted with catechol compared to 4-methylcatechol, suggesting that PPO had more thermal stability in the presence of catechol despite a lower E_a value in catechol (13.3 kJ/mol) than in 4-methylcatechol (18.1 kJ/mol).

An inactivation curve of 4-methylcatechol indicated that more than 50% of PPO activity was diminished after heating of PPO at 60°C for 30 min, and the activity was reduced to only 15% when heated at 90°C for 40 min. For catechol, 50% of the residual activity was observed after heated at 70°C for 30 min, a temperature higher than for 4-methylcatechol. A residual activity of 22% was observed with incubation at 90°C for 40 min.

Compared with PPO varieties from other sources, PPO from Chinese parsley was relatively thermally stable. The $T_{1/2}$ value of Yali pear PPO at 70°C was 6 min (29) with 8 min for avocado PPO (33).

Table 2. Substrate specificity and inhibition parameters of Chinese parsley PPO

Substrate	Inhibitor	Concentration of inhibitor (mM)	V_{max} (EU/mL)[1)]	K_m (mM)[2)]	K_i (mM)[3)]	Substrate specificity (EU/mL/mM)	Mode of inhibition
4-Methylcatechol	-	-	1,428.57	37.43	-	38.17	-
	Citric acid	1.00	1,428.57	77.71	0.93	18.38	Competitive
		3.00	1,428.57	126.42	1.26	11.30	
	L-Cysteine	1.00	588.23	38.59	0.70	15.24	Non-competitive
		3.00	370.37	42.37	1.05	8.74	
Catechol	-	-	2,000.00	31.00	-	64.52	-
	Citric acid	1.00	2,000.00	47.40	1.89	42.19	Competitive
		3.00	2,000.00	81.20	1.85	24.63	
	L-Cysteine	1.00	833.33	34.50	0.71	24.15	Non-competitive
		3.00	526.32	35.11	1.07	14.99	

[1)] V_{max}, Maximum velocity
[2)] K_m, Michealis-Menten constant; EU, Enzyme unit;
[3)] K_i, Affinity towards inhibitor

Table 3. Parameters for heat inactivation of Chinese parsley PPO

Substrate	Temperature (°C)	k (min^{-1})[1)]	$T_{1/2}$ (min)[2)]	D (min)[3)]	Z (°C)[4)]	E_a (kJ/mol)[5)]
4-Methylcatechol	50	$1.34x10^{-2}$	51.89	172.38	123.46	18.08
	60	$1.52x10^{-2}$	45.60	151.48		
	70	$1.54x10^{-2}$	44.92	149.23		
	80	$2.23x10^{-2}$	31.03	103.07		
	90	$2.81x10^{-2}$	24.67	81.95		
Catechol	50	$1.29x10^{-2}$	53.75	178.54	166.67	13.34
	60	$1.22x10^{-2}$	56.79	188.65		
	70	$1.52x10^{-2}$	45.60	151.49		
	80	$1.96x10^{-2}$	35.41	117.63		
	90	$2.03x10^{-2}$	34.20	113.62		

[1)] k, Inactivation rate
[2)] $T_{1/2}$, Half-life
[3)] D, Decimal reduction time
[4)] Z, Z-value
[5)] E_a, Inactivation energy

These reported values are low as $T_{1/2}$ values for Chinese parsley PPO at 70°C were 44.9 and 45.6 min for 4-methylcatechol and catechol, respectively. Thus, Chinese parsley must be treated at high temperatures for longer times in order to fully deactivate PPO.

Effects of inhibitors L-Cysteine was a good chemical inhibitor of PPO with up to 76.8 and 72.5% of activity inhibited in the presence of 4-methylcatechol and catechol, respectively. For citric acid, only 41.7% inhibition was observed in 4-methylcatechol and 25.0% in catechol at 3 mM. L-Cysteine reportedly serves as an effective PPO inhibitor via interference with production of the oxidation end product reaction catalyzed by PPO (34). L-Cysteine exhibited non-competitive inhibition for both substrates, 4-methylcatechol and catechol (Table 2). Inhibitor affinity (K_i) values obtained for L-cysteine were generally lower than for citric acid, suggesting that L-cysteine exhibited a stronger inhibition power.

For citric acid, a competitive inhibition mode was observed in both 4-methylcatechol and catechol as K_m values were highly altered due to substrate competition with the inhibitor for binding at the active site, leading to less availability of PPO binding sites for the substrate as the inhibitor concentration increases. Substrate specificities of PPO in the presence of either L-cysteine and citric acid in an enzyme assay mixture were lower, compared with an absence of inhibitors, indicating that inhibitors reduced the affinity of Chinese parsley PPO towards the substrate (Table 2).

Results from inhibitory studies were in agreement with results for L-cysteine on apple PPO, exhibiting non-competitive PPO inhibition (35). However, inhibition by L-cysteine was competitive for artichoke (17) and pear (16) PPO activities.

Disclosure The authors declare no conflict of interest.

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