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Partial purification and characterization of polyphenol oxidase from round brinjal (*S. melongena* var. depressum)

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Abstract

Polyphenol oxidase (PPO) catalyzes the conversion of phenolic compounds into *o*-quinones which will lead to food browning. This phenomenon causes huge implications on food industries, as it degrades food quality over time. By combining both ammonium sulphate precipitation and gel filtration chromatography, PPO was partially purified up to 5.26-fold with 11.23% yield. The enzyme activity was 5120 EU/mL using 4-methylcatechol as substrate. Maximal PPO activity was found at 30ºC, pH 5.0 for 4-methylcatechol and 40°C, pH 6.0 for catechol. The PPO showed a higher affinity towards 4-methylcatechol but higher thermal stability when reacting with catechol. The K_m and V_{max} values were 5.00 mM, 2000 EU/ml for 4-methylcatechol and 10.79 mM, 526.32 EU/ml for catechol. Energy for inactivation (E_a) obtained using 4-methylcatechol and catechol were 12.57 kJ/mol and 14.23 kJ/mol from respective substrates. Sodium disulfite was a better inhibitor where 79.17% of PPO inhibition was achieved. The isolation and characterization of round brinjal PPO serves as a guideline to predict the behavior of enzyme, leading to effective prevention of its browning during processing and storage.

Introduction

Round brinjal belongs to the family of Solanaceae and genus *Solanum*. This cultivar is categorized as *S. melongena* var. depressum, and is also known as dwarf brinjal (Choudhary, 1976). It is a perennial herbaceous plant with the height of 1.0 to 1.5 meter tall. The leaves have wool-like hairs underneath, and are broad and lobed, whereas the flowers are large and purple in color, despite vary morphologically depending on species (Bose *et al*., 2002).

Food browning embosses critical impact towards food processing industries as fruits and vegetables are susceptible to browning upon harvesting, followed by various postharvest activities (Gorny *et al*., 2002) This problem will lower the marketability, food value, and consumers' acceptability on the fruits and vegetables, affecting the economic situation of a country, especially to those rely on exporting unique and exotic harvest products that cannot be found elsewhere across the globe (Sapers and Miller, 1998; Gorny *et al*., 2000) Hence, it is crucial to understand the catalytic behavior of PPO using biochemical techniques in both enzyme extraction and characterization from this source so as to extend the shelf life of round brinjals.

Food browning may occur due to non-enzymatic browning or enzymatic browning. Non-enzymatic browning can occur because of rearrangement reaction between free sugars and amino acids, oxidation of natural organic acid, or the oxidation of reducing sugars, which all can be found in the food itself (Ajandouz *et al*., 2001; Martins *et al*., 2001; Fang, 2007) Conversely, enzymatic browning is caused by the oxidation reaction of endogenous phenolic compounds, catalyzed by PPO (Dogan *et al*., 2005).

PPO is a family of copper-containing oxidoreductases (Ding *et al*., 2002). It oxidizes o-diphenols to o-quinones, which will turn into brown pigments as the o-quinones polymerize immediately (Vamos-Vigyazo, 1981). This will produce a layer of brown to dark indentation on the surface of fruits and vegetables and the degree of browning depends on the amount of phenolic compounds, the presence of oxygen, metal ions, reducing substances, pH, temperature and the activity of PPO (Lee and Whitaker, 1995).

It is important to understand the catalytic behavior of the partially purified PPO from round brinjal, as well as its substrate specificity and other characteristics. Identification of suitable and acceptable inhibitor of round brinjal PPO will be possible, so as to increase its marketability and allow exporting round brinjals to other countries. This study will also expose the possibility of manufacturing processed round brinjal products, including canned

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round brinjal, and vegetable curry in a can.

Materials and Methods

Plant material and chemicals

Fresh round brinjals (*Solanum melongena* var. depressum) were purchased from Giant hypermarket, Taman Connaught, Cheras, Kuala Lumpur. They were originated from Cameron Highlands, Pahang. All chemicals were of analytical grade and used as obtained.

Enzyme extraction

Fresh round brinjals (100 g) were washed and chopped into small cubes and quickly homogenized with 5 g of polyvinylpyrrolidone dissolved in 300 mL of prechilled (4°C) 0.1 M phosphate buffer, pH 6.8 using a LB-8011ES Waring blender for 3 minutes at maximum speed. The homogenate was subjected to centrifugation at 6,600 g for 25 minutes at 4°C. Supernatant was then pooled and filtered using the Buchner filter and the filtrate was the crude enzyme extract, which was subjected to enzyme activity and protein determination assay. Excess crude enzyme extract was stored at 4°C prior to the partial purification.

Enzyme assay

PPO activity was determined using the spectrophotometric procedure by Zauberman *et al.* (1991) with slight modifications. First, 0.5 ml of 0.1 M substrate and 4.4 mL of 0.1 M phosphate buffer, pH 6.8 were added into a test tube and vortexed. Then, 0.1 ml of enzyme solution was added into the mixture and vortexed immediately for two seconds. This was followed by transferring 2 ml of the mixture to sample cuvette for absorbance reading at 15 seconds interval for 5 minutes, at 25°C. The blank consisted of 2 ml substrate solution in 0.1 M phosphate buffer. Wavelengths of 410 and 400 nm were set for substrates 4-methylcatechol and catechol, respectively. The initial velocity was calculated from the slope of the absorbance vs. time curve. One unit of PPO activity was defined as the amount of enzyme to cause a change of 0.001 absorbance per minute per mL of enzyme solution (Wong and Yu, 1999).

Protein determination

Protein content was determined according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Partial purification

Forty five mL of crude enzyme solution was fractionated using $(NH_4)2SO_4$ that were added slowly into the solution, while stirring on ice, until 50% saturation as accordance to Jiang (1999). Homogenization of mixture was allowed by stirring on magnetic hotplate stirrer for 10 minutes. The mixture was then incubated overnight at 4 and subjected to centrifugation at 12,000 g for 15 minutes after incubation. PPO was restored by dissolving the pellets in 5 mL of 0.1 M phosphate buffer, pH 6.8 whereas the supernatant was pooled and brought to 80% saturation. The fraction with the highest specific enzyme activity was dialyzed using dialysis tubing at 4°C in the same buffer for 48 hours with four changes of the buffer. In order to conduct further purification, the dialysate was transferred to a glass column filled with Sephadex G-100, equilibrated with 0.1 M phosphate buffer, pH 6.8. The column was eluted with the same buffer solution. Five-mL fractions were collected, until the 20th fraction. Only 20 fractions were collected because the total volume of the fractions (100 mL) was equivalent to the column volume. Thus, all of the fractions had represented the possible eluted amount from the column. Fractions with PPO activity were then combined and concentrated.

Characterization of PPO

Optimum pH

The PPO activity was determined in a pH range of 3.0 to 8.0. Two buffers were used, where 0.1 M citrate buffer was used in pH 3.0 to 6.0 while 0.1 M phosphate buffer was used in pH 7.0 and 8.0. Calculated PPO activity was expressed in the form of relative activity (%) at the optimum pH. The optimum pH obtained for this enzyme was used in all other experiments.

Optimum temperature

PPO activity was determined under a range of temperatures from 20 to 70°C at intervals of 10 . PPO activity was measured and calculated PPO activity was expressed in the form of relative activity (%) at the optimum temperature.

Substrate specificity and enzyme kinetics

Different concentrations (10 mM-100 mM) of 4-methylcatechol and catechol were used to determine the affinity of PPO towards each substrate. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) values of the enzyme were determined from a Lineweaver-Burk plot (Jiang, 1999).

Table 1. Purification of PPO from round brinjal

Purification step	Volume (mL)	Enzyme activity (EU/mL)	Protein concentration (mg/mL)	Specific enzyme activity (EU/mg)	Total activity (EU)	Purification fold	Yield (%)
Crude enzyme extract	344	7650.00 ± 611.01	1.423 ± 0.071	5375.97	2.632×10^6	1.00	100.00
50% $(NH_4)_2SO_4$	45	23640.00 ± 506.49	2.482 ± 0.115	9524.58	1.064×10^6	1.77	40.42
Gel filtration chromatography	25	11820.00 ± 244.40	0.418 ± 0.019	28277.51	2.955×10^5	5.26	11.23

Thermal inactivation

The procedure of thermal inactivation was adapted from Dogan *et al.* (2002) with modifications, where 0.5 mL of PPO solution was incubated in a temperature range of 50 – 90°C for 10, 20, 30, and 40 minutes at each designated temperature. After incubation, the heated PPO was immersed in ice bath immediately. Residual enzyme activity after incubation at each temperature and time interval was then determined. The initial enzyme activity was also determined using non-heated PPO, allowing the calculation of percentage residual activity. First-order inactivation was applied in this study and thus the rate constants *(k)* were determined from linear regression (Gundogmaz *et al*., 2003). The rate constants were also replotted in terms of Arrhenius plots, where energy required for inactivation, Ea, was determined. Decimal reduction time (D value) was calculated, and it denotes the time required to reduce the enzyme activity to 10% of its initial activity, at a given pressure and temperature.

Effect of inhibitors

Sodium disulfite and ascorbic acid were used as PPO inhibitors. The PPO activity was determined without inhibitor, and in the presence of inhibitors, at 1.0 and 3.0 mM by using 4-methylcatechol and catechol as the substrates.

Statistical analysis

All the characterizations of PPO from round brinjal (*S. melongena* var. depressum) were assayed in triplicates. The data collected were presented as means ± SD and also relative activity in percentage (%).

Results and Discussion

Extraction and partial purification of PPO

A 5.26-fold purification of PPO relative to protein a yield of 11.23% was achieved (Table 1). The elution profile of the PPO on Sephadex G-100 is shown in Figure 1, where the PPO activity peaked at fraction 6 with an activity of 5,500 EU/mL. Two significant peaks were formed in the elution pattern, where enzyme activity plunged at fraction 7 (1,333.33 EU/mL). This shows that the resolution of protein separation was low.

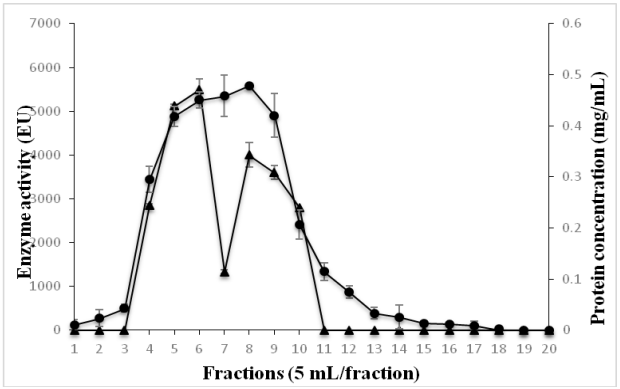

Figure 1. Elution profile of enzyme activity (▲) and protein concentration (●) of round brinjal PPO purified by Sephadex G-100

According to Table 1, there was an increase of enzyme activity after performing ammonium sulphate precipitation at 50% saturation. This explains the possibility of proteinaceous endogenous browning activators that present in round brinjals. Literatures reported that endogenous activators were identified in larvae of Sarcophaga bullata and are made up of lipid, playing major roles in activating the proenzyme of PPO which is prophenoloxidase (Anthon and Barrett, 2002; Dogan *et al*., 2002). Besides that, some endogenous proteases may also act on proenzymes in the activation of PPO.

Characterization of PPO

Optimum pH

The optimum pHs of the enzyme were found to be 5.0 using 4-methylcatechol and 6.0 using catechol. PPO optimum pH varies widely. For example, maximum activity was found to be at pH 5.0 for cabbage, pH 4.4-5.5 for strawberry, pH 6.0

Table 2. Substrate specificity for PPO from round brinjal

Substrate	Wavelength (nm)	V_{max} (EU/mL)	K_m (mM)	Specificity, K_m / V_{max} (EU/mL/mM)
4-Methylcatechol	410	2000.00	5.00	400.00
Catechol	400	526.32	10.79	48.78

for DeChaunac apple, pH 6.5 for apple, banana and medlar using different substrates (Sugumaran and Kanost, 1993; Chase *et al*., 2000; DeStefano *et al*., 2013). In general, fruits and vegetables show maximum activity at or near neutral pH values.

Optimum temperature

The enzyme had an optimum temperature of 30°C for 4-methylcatechol and 40°C for catechol. A sudden increase of enzyme activity at 60 for 4-methylcatechol reflected the possibility of thermal activation of latent PPOs that were purified along. This phenomenon suggested that round brinjal PPO to be thermally labile, as reported by Dogan *et al.* (2002). PPO activity has been found regarding to vary the substrate of the enzyme. Arslan *et al.* (2004) reported that the optimum temperatures for mulberry PPO were 20°C for 4-methylcatechol and 45°C for catechol. It has been reported that the optimum temperatures for PPO of peach, grape, banana, plum and artichoke were 20, 25, 30, 37 and 40°C, respectively (Thomas *et al*., 1985; DeStefano *et al*., 2013).

Enzyme kinetics and substrate specificity

The substrate specificity of PPO towards 4-methylcatechol is higher than that of catechol (Table 2). Thus, 4-methylcatechol is a better substrate. Substrate specificity reveals the best substrate for the enzyme, and its biochemical nature on catalyzing the reactions. Similar results were reported by Dogan *et al*. (2005) on artichoke PPO, where the V_{max}/K_m of catechol (697 EU/mL/mM) was lower as compared to 4-methylcatechol (1,393 EU/mL/mM). Besides that, PPOs from tea leaf (Halder *et al*., 1998) and field bean seed (Paul and Gowda, 2000) also share similar K_m value with catechol, which were 12.5 and 10.5 mM, respectively. Rocha *et al*. (1998) reported that variation in Km values may be due to PPO extracted from different sources, different assay methods used, and different pH used in extraction from plant sources.

Thermal inactivation

PPO activity dropped drastically from 96.43% to 51.42% at 50°C when 4-methylcatechol was used. At higher temperatures (60 to 90°C), the activity decreased to less than 45%. Decrease in the percentage residual activity at a higher temperature is due to the unfolding of the tertiary structure (Lu *et al*., 2007). Similarly, a sudden increase in enzyme activity was due to activation events that happened on the latent form of PPOs due to exposure to high temperatures (Dogan *et al*., 2002).

As for catechol, the residual activity dropped gradually as the temperature and heat treatment period were increased. The enzyme exhibited a sudden instability at 30 minutes at 90°C where a steep drop of PPO activity was observed. This shows that round brinjal PPO was thermally stable until the temperature of 90°C treated for 30 minutes.

The E_a of catechol (14.23 kJ/mol) was higher than that of 4-methylcatechol (12.57 kJ/mol). The D values of catechol (37.31-126.52 min) are generally higher than that of 4-methylcatechol (35.08- 125.01 min), where the log D values of catechol are lower than 4-methylcatechol. These relationships suggested that the PPO to be more thermally stable when reacting with catechol.

Effect of inhibitors

From Table 3, sodium disulfite (3.0 mM) exhibited the highest percentage inhibition of 79.17% on PPO activity when 4-methylcatechol was used as the substrate. Ascorbic acid did not cause any changes on percentage inhibition (16.67%) despite different concentrations (1.0 or 3.0 mM) were added, indicating that ascorbic acid was not a good inhibitor of PPO form round brinjal using 4-mathylcatechol. Conversely, the percentage inhibition of ascorbic acid (3.0 mM) decreased from 42.42% at 1.0 mM to 34.48% when reacted with catechol. This was due to the oxidation of endogenous and supplied ascorbic acid, which is another factor of food browning (Lu Valle, 1952).

Competitive inhibitions were observed for ascorbic acid and sodium sulfite when 4-methylcatechol and catechol were used as substrates, respectively (Table 3). Both inhibitors had relatively low K_i values, ranging from 0.134 to 1.526 mM. A competitive inhibitor is able to diminish the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate. However, competitive inhibition can be relieved by increasing substrate concentration (Embs and Markabis, 1965).

Non-competitive inhibition was exhibited by ascorbic acid in catechol, where K_m value was increased and Vmax value was reduced in the presence of the inhibitor. Similar result is obtained

Table 3. Effect of inhibitors on PPO from round brinjal

Substrate	Inhibitor	Concentration of inhibitor (mM)	Inhibition (%)	K_i (mM)	K_i' (mM)	Type of inhibition
4-Methyl catechol	Sodium disulfite	1.0	16.67	6.250	1.200	Mixed
		3.0	79.17	5.530	0.263	
	Ascorbic acid	1.0	16.67	0.134	-	Competitive
		3.0	16.67	0.298	-	
Catechol	Sodium disulfite	1.0	9.53	1.526	-	Competitive
		3.0	9.53	0.533	-	
	Ascorbic acid	1.0	42.42	0.060	-	Non-competitive
		3.0	34.48	5.535	-	

by Dogan *et al*. (2005) in investigations on PPO from artichoke.

Conclusions

Polyphenol oxidase (PPO) extracted from Solanum melongena var. depressum was purified 2.28-fold with 1.08% protein yield. The initial enzyme activity was 7650 EU/mL, under the presence of polyvinylpyrrolidone. Partially purified PPO from this source was characterized, and the optimum conditions for round brinjal PPO was 30 , pH 5.0 in the presence of the substrate 4-methylcatechol, and 40 , pH 6.0 for pyrocatechol. 4-Methylcatechol was more preferred by the enzyme, when compared to pyrocatechol, as shown by the Lineweaver-Burk analysis. Thermal inactivation studies suggested that the enzyme was heat-labile, where relatively high enzyme activities remain despite long exposure to high temperatures. Furthermore, sodium disulfite was found to inhibit PPO activity effectively than that of ascorbic acid, probably due to the oxidative properties of the latter chemical inhibitor.

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