International Food Research Journal 26(6): 1699-1706 (December 2019)

Journal homepage: *http://www.ifrj.upm.edu.my*

Aqueous two-phase separation (ATPS) of polyphenol oxidase from lotus root (*Nelumbo nucifera*) and its characterisation

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Article history

Received: 21 March, 2018
Received in revised form: 6 July, 2019
Accepted: 5 September, 2019

Keywords

Aqueous two-phase separation
Polyphenol oxidase
Lotus root
Characterisation
Inhibition

Abstract

Polyphenol oxidase (PPO) was extracted from lotus (*Nelumbo nucifera*) root and partially purified up to 11.51× with a 43.6% yield using aqueous two-phase separation system. The optimum pH was at 8.0 for both substrates; 4-methylcatechol and pyrogallol. PPO activities were maximally achieved at 50°C for pyrogallol, and 20°C for 4-methylcatechol. 84% and 71% loss of relative PPO activities were obtained for pyrogallol and 4-methylcatechol, respectively following heat inactivation at 90°C for 40 min. Inactivation rate constant (k) values ranged between 0.88×10^{-2} min^{-1} to 2.97×10^{-2} min^{-1} for pyrogallol, and 0.69×10^{-2} min^{-1} to 2.44×10^{-2} min^{-1} for 4-methylcatechol. The activation energy (E_a) were 75.32 kJ mol^{-1} and 73.14 kJ mol^{-1} for pyrogallol and 4-methylcatechol, respectively. PPO activity was strongly inhibited (> 79%) by ascorbic acid. SDS exhibited the greatest efficiency in activating lotus root PPO.

Introduction

Enzymatic browning often occurs in fruits and vegetables during processing and storage which consequently impacts on the quality, nutritional value and sensory properties (Queiroz *et al*., 2008). Enzymatic browning is mainly caused by the oxidation reaction of endogenous phenolic compounds catalysed by polyphenol oxidase (PPO). PPO (EC 1.14.18.1) is a common copper-containing enzyme that is widely distributed in the plant kingdom and it is also known as tyrosinase, catechol oxidase and monooxygenase. PPO catalyses hydroxylation of monophenols to o-diphenols, followed by oxidation of o-diphenols to o-quinones in the presence of oxygen, which then polymerise to brown products (Waliszewski *et al*., 2009).

Traditionally, PPO is purified from plant sources using ammonium sulphate precipitation followed by a series of chromatographic procedures. However, these techniques for separation and purification of enzymes are expensive, time consuming, and might cause denaturation of enzyme during the extraction process. Thus, the use of aqueous two-phase system (ATPS) has gained an interest for enzyme extraction, separation, purification and enrichment of proteins nowadays (Iqbal *et al*., 2016). The most common biphasic systems are formed by two polymers (usually polyethylene glycol (PEG) and dextran) or a polymer and a salt (e.g., phosphate, sulphate or citrate). ATPS has several advantages over conventional techniques such as simplicity of scaling up, high capacity, high selectivity, low cost, and environmental friendly (Babu *et al*., 2008).

Lotus (*Nelumbo nucifera*) is an aquatic crop which can be found throughout Asia. It is widely cultivated in China, Japan, India, Iran and other sub-tropical region, both as dietary supplement and medicinal herb. Through the ages, the rhizomes (lotus root) have been employed in cooking; stir-fried, soup, pickled, and even salads (Small *et al*., 2002). Lotus root itself is very crunchy, starchy and a little sticky. It is favoured not only in Asian but also Western cuisines. It has been known to be rich in dietary fibres, vitamins, phenolic compounds and antioxidants (Wang *et al*., 2011).

Lotus root has short shelf life as the white flesh easily turns brown and deteriorates when its peel

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is damaged, resulting in colour and flavour change which will reduce its commercial value. Prior to cooking, lotus roots are often kept in cold water to prevent discoloration (Jiang *et al*., 2012). However, this method can only delay the browning effect instead of completely inhibiting the PPO activity. Thus, the aim of the present work was to use the aqueous two-phase separation system for purification of lotus root PPO. Characterisation of lotus root PPO was also performed in order to predict the behaviour of the enzyme which can then serve as a guideline to effectively prevent the browning of lotus root during processing and storage, thus improving the quality of lotus root and increasing its marketability.

Materials and methods

Plant materials and chemicals

Lotus roots were purchased from a local market (Giant hypermarket, Taman Connaught, Cheras, Kuala Lumpur). They were originated from Sungai Siput, Perak, Malaysia. Triton X-114, ascorbic acid, sodium disulphite, catechin, catechol and manganese (II) chloride ($MnCl_2$) were purchased from Sigma-Aldrich (USA). 4-methylcatechol (Merck, Germany) and pyrogallol (Fluka Analytical, Germany) were also used. Sodium dodecylsulphate (SDS) and bovine serum albumin (BSA) were obtained from Fisher Scientific (UK).

Crude enzyme extraction

The extraction method was adopted from Lin *et al*. (2016) with slight modification. Lotus root (150 g) were washed and chopped into small pieces (1.5 × 1.5 × 1.5 cm). These root samples were then homogenised in 300 mL of pre-chilled (4°C) 0.1 M phosphate buffer at pH 6.8 by using an LB-8011ES industrial blender (Waring Laboratory, Torrington, CA, USA) for 1 min at maximum speed (22,000 rpm). The resulting homogenates were then subjected to centrifugation using a Universal 320R centrifuge (Hettich Zentrifugen; Hettich, Tuttlingen, Germany) at 4,000 rpm for 30 min at 4°C. Supernatants were pooled and filtered using a Buchner filter set. The collected crude enzyme filtrate was stored at 4°C prior to partial purification.

Assay of PPO activity

PPO activity was determined using a PRIM Light spectrophotometer (Secomam, Champigny-sur-Marne, France) by measuring an increase in absorbance at 410 nm for catechol and 320 nm for pyrogallol, respectively. The reaction mixture contained 0.1 mL of enzyme solution, 2.9 mL of 0.1 M 4-methylcatechol or pyrogallol solution in 0.1 M phosphate buffer at pH 6.8 at room temperature. Then, 2 mL of the reaction mixture was transferred to a cuvette and absorbance readings were determined at 15 s intervals for 5 min using a spectrophotometer. 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 one unit of PPO activity was defined as the amount of enzyme that caused a 0.001 absorbance change per min (Kumar *et al*., 2008).

Protein determination

Protein concentrations were determined following the Lowry method (Lowry *et al*., 1951). Bovine serum albumin (BSA) was used as a standard for the construction of a protein standard curve.

Partial purification using Triton X-114 and aqueous two-phase separation (ATPS)

Partial purification of PPO from lotus root was performed following the method of Sánchez-Ferrer *et al*. (1989) and Babu *et al*. (2008) with slight modifications. The supernatant of the PPO extract was subjected to phase partitioning by adding Triton X-114 at 4°C until the final concentration was 4% (v/v). The mixture was incubated at 4°C for 15 min and followed by warming up to 35°C using a water bath for 10 min. At this time, the solution became spontaneously turbid due to the formation, aggregation and precipitation of large mixed micelles of detergent. The turbid solution was then centrifuged at 10,000 g for 15 min at 25°C.

The collected supernatant was then subjected to an aqueous two-phase separation (ATPS) based on PEG 8000 - phosphate buffer system. The 10 g samples of ATPS composed of 5% (w/w) PEG 8000, 28.5% (w/w) phosphate buffer solution, 56.5% (w/w) deionised water and 10% (w/w) enzyme solution. The sample mixture was stirred for 15 min at room temperature prior to phase separation by centrifugation at 2,000 g for 10 min. The upper PEG-rich phase was discarded and the phosphate-rich phase containing PPO was collected for enzyme characterisation.

Characterization of PPO

Optimum pH

PPO activity was determined in a pH range of 4.0 - 9.0 at 1 unit intervals with a 0.1 M citrate buffer used for pH 4.0 to 6.0 and a 0.1 M phosphate buffer used for pH 7.0 to 9.0. PPO activity was measured, calculated and expressed as a relative activity (%) to

determine the optimum pH for PPO for use in the subsequent experimentation.

Optimum temperature

PPO activity was determined at 10, 20, 30, 40, 50 and 60°C. 0.1 M phosphate buffer of pH 8.0 was pre-treated to the respective temperature. 1.9 mL buffer was immersed in water bath (Memmert Lab Companion, Germany) with temperature ranging from 30 to 60°C, while 20°C assay was done by immersing the reaction mixture in ice bath. After the buffer has reached equilibration at selected temperature, 1 mL substrate was added. PPO extract was added to the solution after the mixture has reached the selected temperature. Enzyme activity was calculated and expressed as a relative activity (%) for determination of the optimum PPO temperature to be used in subsequent experimentation.

Enzyme kinetics and substrate specificity

PPO activity was assayed using catechol, 4-methylcatechol, pyrogallol and catechin at various concentrations (10 mM to 100 mM) based on absorption at a specific wavelength of each substrate under standard conditions. Lineweaver-Burk plots were developed from kinetic data for the determination of the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) (Lineweaver and Burk, 1934).

Thermal inactivation

Briefly, 1 mL of PPO was incubated in a water bath (Memmert Lab Companion, Germany) at 40, 50, 60, 70, 80 and 90°C for 10, 20, 30 and 40 min. 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 (Gundogmaz *et al*., 2003). The energy required for inactivation (E_a) was determined from the slope of an Arrhenius plot obtained from Eq. 1:

$$\ln k = -\frac{E_a}{RT} + c \qquad \text{(Eq. 1)}$$

where R = gas constant (8.31 J 1/mol 1/K), T = inactivation temperature in Kelvin, and c = constant. The inactivation energy was determined

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 (Doğru and Erat, 2012).

Effect of inhibitors

Ascorbic acid and sodium disulphite were used as PPO inhibitors in the present work. Percentage inhibition (%) values for each inhibitor at concentrations of 10 mM and 30 mM in the presence of either 4-methylcatechol or pyrogallol were calculated using the PPO activity without an inhibitor as the initial enzyme activity.

Effect of activators

Sodium dodecylsulphate (SDS) and manganese (II) chloride ($MnCl_2$) were used as PPO activators in the present work. Percentage activation (%) values for each activator at concentrations of 0.05 M and 0.10 M in the presence of either 4-methylcatechol or pyrogallol were calculated using the PPO activity without an activator as the initial enzyme activity.

Statistical analysis

All experiments were conducted in triplicates (n = 3) and data were expressed as means ± standard deviations (SD). A one-way analysis of variance (ANOVA) was performed using Minitab software version 17.0 (Minitab 17, Pennsylvania, USA). Differences were considered significant at 95% ($p < 0.05$).

Results and discussion

Partial purification using Triton X-114 and aqueous two-phase separation (ATPS)

The purification steps and results are summarised in Table 1. The crude PPO extract displayed maximum enzymatic activity (21,000 EU) on a protein basis. The Triton X-114 step removed most of the unwanted proteins with a loss of 55.8% of the total enzyme activity and the yield obtained was 44.2%. 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. This resulted in an increase in specific activity to 2205.82 EU/mg after ATPS from an initial value of 1915.71 EU/mg (Table 1).

PPO from lotus root was purified 11.51× with a protein yield of 43.6% achieved via ATPS (Table 1). The overall degree of purification obtained was high as compared to the partial purification of

Table 1. Purification of PPO from lotus root.

Step	Volume (mL)	Protein concentration (mg/mL)	TA (EU)	SEA (EU/mg)	Yield (%)	Purification fold
Crude extract	42.00 ± 0.80[a]	0.26 ± 0.01[a]	21000 ± 20.50[a]	1916 ± 10.20[c]	100.00 ± 0.00[a]	1.00 ± 0.00[c]
Triton X-114	30.00 ± 1.10[b]	0.11 ± 0.00[b]	9273 ± 12.40[b]	2760 ± 21.10[b]	44.20 ± 1.00[b]	1.44 ± 0.01[b]
PEG 8000	6.10 ± 0.70[c]	0.07 ± 0.01[c]	9150 ± 15.30[c]	22059 ± 30.20[a]	43.60 ± 0.80[b]	11.51 ± 0.05[a]

TA: Total activity; SEA: Specific enzyme activity; EU: Enzyme unit. Values are means ± standard deviations of triplicate analysis (n = 3). Different letters (a-c) in the same column are significantly different at $p < 0.05$.

Chinese parsley (Lin *et al*., 2016) and round brinjal (Ng and Wong, 2015), for which 5.26× with a 11.23% yield and 6.52× with a 10.89% yield were achieved, respectively using the combination of ammonium sulphate precipitation and gel filtration chromatography. Thus, partitioning in ATPS provides an efficient method for separation and purification of mixture of proteins or enzymes.

Characterisation of PPO

Optimum pH

Optimum PPO pH value was 8.0 for both 4-methylcatechol and pyrogallol. The optimum pH of lotus root PPO is similar to that of Chinese parsley PPO with 4-methylcatechol (Lin *et al*., 2016) and artichoke PPO with pyrogallol (Doğan *et al*., 2005), however, it was higher than that of round brinjal (pH 5.0; Ng and Wong, 2015); mamey (pH 7.0; Palma-Orozco *et al*., 2011); and cassava leaves (pH 7.5; Wong and Lee, 2014). The optimum pH obtained in the present work was slightly lower than that of Lonicera confusa (pH 8.5; Feng *et al*., 2014). These wide variations can be explained by the fact that optimum pH depends on the nature of phenolic substrates, temperature and extraction methods (Dincer *et al*., 2002).

Optimum temperature

Lotus root PPO had an optimum temperature of 20°C for 4-methylcatechol and 50°C for pyrogallol. Similar results were also reported where PPO exhibited a maximum activity at 20°C for cassava leaves, water caltrop and sweet basil (Doğan *et al*., 2005; Zhu and Zhan, 2010; Wong and Lee, 2014), while optimum temperature for strawberry (Serradell *et al*., 2000) and Chinese cabbage was 50°C (Nagai and Suzuki, 2001). The optimum temperatures of PPO are dependent on methods of extraction, substrates used, plant origin and also the plant growing conditions.

Enzyme kinetics and substrate specificity

PPO substrate specificity (V_{max} / K_m) was tested using four different substrates (Table 2). Pyrogallol exhibited the highest V_{max} / K_m value, followed by catechin, 4-methylcatechol and catechol. Substrate specificity reveals the most effective substrate for the enzyme, and its biochemical nature on catalysing the reactions (Zhu and Zhan, 2010). Some authors suggest that the most common substrates for PPO are 4-methylcatechol, catechol, catechins and chlorogenic acid (Todaro *et al*., 2010). In the present work, pyrogallol showed to be the best substrate for PPO from lotus root. This result is in agreement with those reported by Doğan *et al*. (2005) and Nagai and Suzuki (2001) for PPO from artichoke and cabbage, respectively.

PPO has affinity for various substrates and thus it shows variations in K_m values. Lotus root PPO displayed a K_m value of 20.0 mM when using pyrogallol as substrate (Table 2). This value was higher than other sources with the same substrate; PPO from mamey had a K_m of 1.3 mM (Palma-Orozco *et al*., 2011), 5.2 mM in artichoke (Doğan *et al*., 2005), 10.64 mM in cassava leaves (Wong and Lee, 2014) and 15.4 mM in cabbage (Nagai and Suzuki, 2001). Catechin was not chosen as the best substrate although it showed the lowest K_m value because it had a relative low catalytic rate, V_{max} as compared to pyrogallol (Table 2). In addition, criterion for choosing the best substrate is based on the highest catalytic efficiency (V_{max} / K_m).

Table 2. Kinetic parameter for different substrates on lotus root PPO.

Substrate	Wavelength (nm)	Vmax (EU/min mL)	Km (mM)	Vmax / Km (min^{-1})
Pyrogallol	320	5000.00 ± 45.10[a]	20.00 ± 1.20[a]	250.00 ± 2.00[a]
4-methylcatechol	410	303.03 ± 22.40[b]	21.20 ± 0.50[a]	14.29 ± 0.40[c]
Catechol	410	58.82 ± 3.80[c]	18.00 ± 0.70[b]	3.27 ± 0.20[d]
Catechin	410	51.28 ± 2.30[d]	2.56 ± 0.30[c]	20.03 ± 1.20[b]

Values are means ± standard deviations of triplicate analysis (n = 3). Different letters (a-d) in the same column are significantly different at $p < 0.05$.

Table 3. Thermal inactivation parameters of lotus root PPO.

Substrate	Temperature (°C)	k (min^{-1})	$t_{1/2}$ (min)	D (min)	Z (°C)	E_a (kJ mol^{-1})
Pyrogallol	40	0.88 × 10-2	78.75	261.70	-	-
	50	1.01×10^{-2}	68.61	228.00	-	-
	60	1.24×10^{-2}	55.89	185.70	-	-
	70	1.46×10^{-2}	47.47	157.70	-	-
	80	2.14×10^{-2}	32.38	107.60	-	-
	90	2.97×10^{-2}	23.33	77.50	-	-
	-	-	-	-	95.20	75.30
4-methylcatechol	40	0.69×10^{-2}	100.43	333.70	-	-
	50	0.92×10^{-2}	75.32	250.30	-	-
	60	1.29×10^{-2}	53.72	178.50	-	-
	70	1.30×10^{-2}	53.31	177.20	-	-
	80	1.47×10^{-2}	47.14	156.70	-	-
	90	2.44×10^{-2}	28.40	94.40	-	-
	-	-	-	-	104.20	73.10

Thermal inactivation

PPO from lotus root exhibited a reduction in catalytic activity as the duration and temperature of heat inactivation increased in the presence of both 4-methylcatechol and pyrogallol as reflected by an increment in k values (Table 3). The half-life of PPO ($t_{1/2}$) decreased as the incubation temperature increased from 40 to 90°C due to the unfolding of tertiary structure of enzyme. The Z and D values were lower when PPO reacted with pyrogallol than that of 4-methylcatechol. It can be concluded that the lotus root PPO was more thermal stable towards 4-methylcatechol.

The PPO was stable at 40°C, and the residual PPO activity remained above 80% for 30 min as obtained from the inactivation curve. The activity rapidly diminished when the temperature reached 80°C, with only 40% activity being retained after 10 min. Residual PPO activity was reduced to 29% for 4-methylcatechol and 16% for pyrogallol, respectively after heating at 90°C for 40 min. This suggests that high temperatures for longer time processes are effective for inhibiting the enzymatic browning caused by PPO in lotus root.

Effect of inhibitors

The inhibition was enhanced with the increase in concentrations of inhibitors (Table 4). Ascorbic acid was a better inhibitor of PPO with up to 79% of activity being inhibited in the presence of 4-methylcatechol and pyrogallol at a concentration of 10 mM. 92.68% inhibition was observed in pyrogallol and 88.68% in 4-methylcatechol at 30 mM. Ascorbic acid has been reported to be the most effective inhibitor for PPO, where it could inhibit the PPO activity up to 90% in various fruits and vegetables sources such as artichoke (Todaro *et al*., 2010) and quince (Yagar and Sagiroglu, 2002). As ascorbic acid is a non-toxic and naturally occurring substance, it may be useful for preventing enzymatic browning in lotus root. It has the ability to reduce the initial o-quinone formed to the original diphenol before it undergoes the secondary reactions leading to browning.

The inhibitory effect of 10 mM sodium disulphite was moderate (49.89%) in the presence of pyrogallol but was high (86.41%) with 4-methylcatechol. Ascorbic acid and sodium disulphite exhibited mixed inhibition for both substrates, pyrogallol and 4-methylcatechol (Table 4). Results indicated that both inhibitors, ascorbic acid and sodium disulphite could bind to the free form of PPO (oxy and met forms) and integrate with the enzyme-substrate complex (Valero *et al*., 2007). According to Lim and Wong (2018), sodium metabisulphite non-competitively inhibited ginger PPO using 4-methylcatechol.

However, the findings from the present work is contrary to those reported by Núñez-Delicado *et al*. (2007) in which 100% inhibition of PPO activity by sodium metabisulphite was attained while only 49% inhibition by ascorbic acid were observed for Napoleon grape. According to Jiang *et al*. (1997), ascorbic acid was a medium inhibitor for longan PPO and litchi PPO with 53% and 69% inhibition, respectively. Wong and Angel Lee (2014) reported that ascorbic acid inhibited cassava leaves PPO up to 80%. The effect of inhibitors on PPO activity differs depending on the compound used, concentrations and sources.

Table 4. Effects of inhibitors on lotus root PPO.

Substrate	Inhibitor	Concentration of inhibitors (mM)	Percentage Inhibition (%)	Type of inhibition
Pyrogallol	Ascorbic acid	10	79.05 ± 0.90^{e}	Mixed
		30	92.68 ± 1.20^{a}	
	Sodium disulphite	10	49.89 ± 0.60^{f}	
		30	88.80 ± 1.10^{b}	
4-methylcatechol	Ascorbic acid	10	79.04 ± 0.30^{d}	Mixed
		30	88.67 ± 0.40^{b}	
	Sodium disulphite	10	86.41 ± 0.20^{c}	
		30	89.13 ± 0.70^{b}	

Values are means ± standard deviations of triplicate analysis (n = 3). Different letters (a-f) in the same column are significantly different at $p < 0.05$.

Table 5. Effects of activators on lotus root PPO.

Activators	Concentration (mM)	Relative activity (%)			
		Pyrogallol		4-methylcatechol	
		0.05 M	0.1 M	0.05 M	0.1 M
SDS	10	228.86 ± 2.00^{b}	163.67 ± 1.10^{c}	185.72 ± 0.90^{a}	108.70 ± 0.30^{c}
	30	245.80 ± 0.90^{a}	228.80 ± 1.80^{a}	95.00 ± 2.10^{d}	117.28 ± 0.80^{b}
$MnCl_2$	10	117.30 ± 0.80^{d}	139.49 ± 0.70^{d}	127.78 ± 1.30^{c}	108.70 ± 0.50^{c}
	30	124.22 ± 1.20^{c}	194.32 ± 1.00^{b}	150.00 ± 0.80^{b}	120.77 ± 0.50^{a}

Values are means ± standard deviations of triplicate analysis (n = 3). Different letters (a-d) in the same column are significantly different at $p < 0.05$.

Effect of activators

The maximum activation effect were found when 30 mM SDS reacted with 0.05 M pyrogallol which resulted to nearly of 2.5-fold increase in the relative activity, while 30 mM $MnCl_2$ only contributed to the highest 1.9-fold increase of PPO activity by using 0.10 M pyrogallol (Table 5). The effect of SDS in the activation process was due to the binding of a small amount of SDS to enzyme‘s active site which leads to the alteration of enzymatic and physical characteristic, as well as limited conformation change of the enzyme (Concellón *et al*., 2004).

The degree of activation mostly increased with the increased in concentration of activators as seen from Table 5 for both pyrogallol and 4-methylcatechol. The only exception was a 5% decreased in relative activity was found when SDS was reacted with 0.05 M 4-methylcatechol. This result is in agreement with Sojo *et al*. (1998) who reported that a lower concentration of SDS was able to activate banana PPO, but PPO activity was inhibited at higher concentration. Most enzymes lose their biological activity upon treatment with SDS but not in the case of PPO. Some authors suggested that resistance of PPO to SDS might be due to the presence of disulphide bonds which hold and strengthen the PPO structure (Marri *et al*., 2003).

The divalent cation, Mn^{2+} ion, activated the oxidation of 4-methlycatehcol and increased 50% of the relative activity (Table 5). PPO activation by Mn^{2+} ion was also found in water caltrop PPO (Zhu and Zhan, 2010) and green bean PPO (Guo *et al*., 2009). However, $MnCl_2$ weakly inhibited the activity of banana PPO and meddler fruit PPO as reported by Yang *et al*. (2001) and Ayaz *et al*. (2008), respectively. It can be concluded that the degree of activation strongly depends on the plant materials as well as the extraction method used.

Conclusion

In the present work, PPO from lotus root was successfully purified using an aqueous two-phase partitioning approach with Triton X-114 up to 11.51× with a 43.6% yield. The optimum pH obtained was 8.0. PPO showed better affinity towards pyrogallol. High temperatures (> 90°C) for longer time (> 40 min) process were more effective to inactivate the PPO activity. Ascorbic acid was a better inhibitor, but Mn2+ had a positive effect on PPO activity. Further investigation on the usage of natural inhibitors such as onion, honey or garlic in preventing enzymatic browning during processing and storage can be carried out in order to improve the nutritional value and exterior quality of lotus root.

Acknowledgement

The authors would like to thank the Ministry of Higher Education (MOHE) for the financial support received for the completion of the present work under the Fundamental Research Grant Scheme (FRGS/1/2018/TK10/UCSI/02/1).

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