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Purification and some properties of polyphenol oxidase of longan fruit

Yue-Ming Jiang*

South China Institute of Botany, The Chinese Academy of Sciences, Guangzhou 510650, People's Republic of China

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Abstract

Polyphenol oxidase (PPO) was isolated from longan (*Dimocarpus longan* Lour.) fruit peel, with a 46-fold purification of PPO by ammonium sulfate, Sephadex G-200 and Phenyl Sepharose being achieved. Pyrogallol, 4-methylcatechol, and catechol were good substrates for the enzyme, and activity with chlorogenic acid, *p*-cresol, resorcinol, or tyrosine was not observed. The optimal pH for PPO activity was 6.5 with 4-methylcatechol. The enzyme had a remarkably temperature optimum (35°C) and was relatively stable, requiring a little more than 20 min at 50°C for 50% loss of activity. Reduced glutathione, L-cysteine, thiourea, $FeSO_4$ and $SnCl_2$ markedly inhibited PPO activity, whereas $MnSO_4$ and $CaCl_2$ enhanced PPO activity. Data obtained in this study might help to better understand longan fruit peel browning. © 1999 Elsevier Science Ltd. All rights reserved.

1. Introduction

The longan (*Dimocarpus longan* Lour.) is a highly attractive subtropical fruit. The fruit is a non-climacteric and is harvested when eating quality and visual appearance are optimal (Paull & Chen, 1987). Lu et al. (1992) reported that longan fruit was not susceptible to chilling injury and can be stored for about 30 days at a range of 1–5°C depending various cultivars. However, it has a very short shelf-life under normal ambient conditions due to skin colour loss (browning) and deterioration during storage and transportation (Prapaipong & Rakariyatham, 1990; Tongdee, 1994). The colour loss reduces its commercial value and has been considered a main postharvest problem (Reed, 1986; Lu et al., 1992; Saranant, 1992). Browning has been attributed to oxidation of phenolics by polyphenol oxidase, producing brown-coloured byproducts (Ferrar & Walker, 1996; Mayer & Harel, 1979; Walker & Ferrar, 1998; Zawistoski, Biliadeis, & Eskin, 1991).

PPO has been widely studied in various fruits such as apple (Harel, Mayer, & Sham, 1964; Janovitz-Klapp, Richard, & Nicolas, 1990), banana (Galeazzi & Sgarbieri, 1981), grape (Harel & Mayer, 1971), litchi (Tan & Li, 1984), peach (Wong, Lun, & Whitaker, 1971), pear (Rivas & Whitaker, 1973), and plum (Liu, Jiang, Li, Zhang, & Chen, 1994), but little has been known about longan fruit PPO. The objective of this study was to achieve purification and a better understanding of the properties of the longan fruit PPO that catalyses the browning reaction during fruit storage and transportation.

2. Materials and methods

2.1. Materials

Mature fruit of a major cultivar, Shixia, from a commercial orchard in Guangzhou, P.R. China was obtained on the day of harvest and stored at −20°C until peel extraction.

2.2. Extraction and purification of PPO

All steps were carried out at 4°C. The longan peel was homogenised with 0.1 M sodium phosphate buffer (pH 6.8) with Poly-Clar AT (insoluble high molecular weight grade of polyvinylpyrrolidone; 1% of fruit peel in weight) using a Ultra-AII (Guangdong, China). After filtration of the homogenate through muslin, the filtrate

* Fax: +86-20-87701031; e-mail: gzscib@public.guangzhou.gd.cn

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was centrifuged at 15,000 **g** for 20 min, and the supernatant was collected. The enzyme solution was fractionated with solid ammonium sulfate (50–80% saturation) and the precipitate was collected by centrifugation at 15 000 **g** for 20 min, redissolved in 0.01 M sodium phosphate buffer (pH 6.8) and dialyzed against the same buffer. The dialyzed solution was lyophilised, dissolved again in small volume of 0.01 M sodium phosphate buffer (pH 6.8), and applied to a Sephadex G-200 column (4.0 × 110 cm), preequilibrated with 0.01 M sodium phosphate buffer (pH 6.8). The enzyme solution was eluted with the same buffer and the fractions with highest enzymatic activity was pooled and lyophilised. Dissolved in small volume of 0.01 M sodium phosphate buffer (pH 6.8) with a final concentration of 1 M ammonium sulfate added, the fraction was loaded onto a column (1.5 × 38 cm) of Phenyl Sepharose (Fast Flow) equilibrated with buffer A (0.01 M sodium phosphate, 1 M ammonium sulfate, 1 M KCl, pH 6.8). The PPO was eluted with a gradient of 100, 80, 60, 40, 20, 10 to 0% buffer A in 0.01 M sodium phosphate buffer (pH 6.8). The active fractions from the Phenyl Sepharose column were pooled, lyophilised, and dissolved in a small volume of 0.01 M sodium phosphate buffer (pH 6.8). After overnight dialysis against the same buffer, the dialyzed solution was collected as PPO.

2.3. Enzyme assay and protein determination

PPO activity was assayed with 4-methylcatechol as a substrate by a spectrophotometric procedure (Zauberman et al., 1991). The assay was performed using 0.5 ml of 100 mM 4-methylcatechol, 1.0 ml of 0.1 M sodium phosphate buffer (pH 6.8) and 0.5 ml of the crude enzyme or 1.49 ml of 0.1 M sodium phosphate buffer (pH 6.8) and 0.01 ml of the enzyme solution purified by different steps (see Table 1). The increase in absorbance at 410 nm at 25°C was recorded automatically for 5 min (Beckman, DU-7). One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.001 in absorbance per minute. Protein content was determined according to the dye-binding method of Bradford (1976) with bovine serum protein as the standard.

2.4. Effect of pH on PPO activity and stability

Two kinds of buffer solutions were used for this study: 0.1 M sodium citrate for the pH range of 3.0 to 5.5 and 0.1 M sodium phosphate buffer for pH 6.0–8.0 (Fujita, Tono, & Kawahare, 1991). To determine the effect of pH on PPO activity, 0.5 ml of 100 mM 4-methylcatechol solution was added to 1.49 ml of various buffer solutions followed by the addition of 0.01 ml of enzyme solution. To determine the effect of pH on PPO stability, 0.01 ml of enzyme solution was incubated in 1.49 ml of various buffer solutions ranging from pH 3.0 to 8.0 for 24 h at 4°C. Residual PPO activity was assayed by mixing 0.5 ml of 100 mM 4-methylcatechol with the incubated PPO solution. The enzyme activity was performed according to the method described above and expressed a percentage of the maximum activity.

2.5. Effect of temperature on PPO activity and stability

The optimum temperature for PPO activity was determined by adding 0.01 ml of enzyme solution to 0.5 ml of 100 mM 4-methylcatechol and 1.49 ml of 0.1 M sodium phosphate buffer, pH 6.5 (Zhou, Smith, & Lee, 1993). The substrate and buffer were incubated for 5 min at various temperatures from 20°C to 60°C, prior to the addition of the enzyme solution. Spectrophotometric measurement for 5 min was carried out at 25°C. For thermal stability studies, 0.01 ml of enzyme solution with 1.49 ml of 0.1 M sodium phosphate buffer (pH 6.5) were incubated at various temperatures for 10, 20 and 30 min, rapidly cooled in an ice bath for 5 min, and then removed to 25°C. When the mixture reached room temperature (25°C), 0.5 ml of 100 mM 4-methylcatechol was added and the residual enzyme activity assayed and expressed relative to the maximum activity.

2.6. Substrate specificity

The reaction system consisted of 0.01 ml of enzyme solution, 1.49 ml of 0.1 M sodium phosphate buffer (pH 6.5) and 0.5 ml of various substrate solutions (see Table 2). The increase in absorbance at the optimum wavelength for each substrate was measured.

Table 1
Purification of polyphenol oxidase of longan fruit[a]

Step	Volume (ml)	Protein (μg/ml)	Activity (units/ml)	Specific activity (units/mg protein)	Total activity (units)	Purification (fold)	Yield (%)
Crude extract	12,000	11.5	9.0	7.8×10^2	108,000	1.0	100
$(NH_4)_2SO_4$	210	84.3	160	1.9×10^3	33,642	2.4	31.2
Sephadex G-200	80	8.8	96.8	1.1×10^4	7744	14.1	7.2
Phenyl Sepharose	15	7.4	266	3.6×10^4	3996	46.2	3.7

[a] Enzyme activity was assayed by using 0.5 ml of 100 mM 4-methylcatechol and 1.0 ml of 0.1 M sodium phosphate buffer (pH 6.8) and 0.5 ml of the crude enzyme or 1.49 ml of 0.1 M sodium phosphate buffer (pH 6.8) and 0.01 ml of the enzyme solution purified by different steps.

Table 2
Substrate specificity of polyphenol oxidase of longan fruit[a]

Substrate	Wavelength (nm)	Relative activity (%)
4-Methylcatechol	410	100
Catechol	400	91
Chlorogenic acid	400	0
p-Cresol	400	0
Pyrogallol	334	281
Resorcinol	400	0
Tyrosine	472	0

[a] All substrate was present at a final concentration of 25 mM except for chlorogenic acid and tyrosine, which were 2.5 mM. Activity was expressed relative to that with 4-methylcatechol (3.9×10^4 units/mg protein) = 100.

To further examine the oxidation of tyrosine, *p*-cresol, resorcinol and chlorogenic acid catalysed by PPO, the enzyme activity was also assayed polarographically using a Clark O_2 electrode (Model SSI-11A, Shanghai Scientfic Instruments, China), calibrated daily with sodium dithionite. PPO activity was measured using a 0.01 ml aliquot of enzyme solution in 1.4 ml of 0.01 M sodium phosphate buffer (pH 6.5). The solution was allowed to equilibrate at 25°C for 5 min. The reaction was initiated by adding 0.5 ml of various substrate solutions into the solution. Activity was determined based on the initial linear phase of oxygen consumption during the reaction.

2.7. Effects of inhibitors on PPO activity

To 0.5 ml of 100 mM 4-methylcatechol and 1.39 ml of 0.1 M sodium phosphate buffer (pH 6.5), 0.1 ml of inhibitor solutions at 0.1 mM or 1.0 mM concentrations were mixed immediately before the addition of 0.01 ml of enzyme solution. Relative enzymatic activity was calculated as a percentage of the activity without any inhibitor.

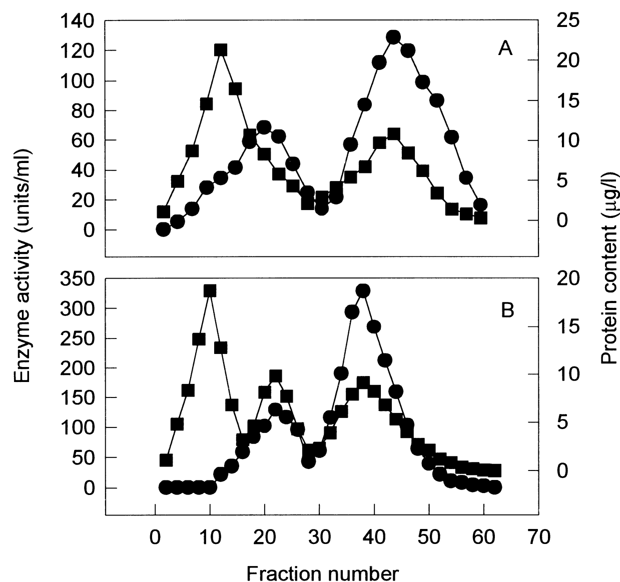

Fig. 1. Elution profiles of enzyme activity (●) and protein content (■) of longan polyphenol oxidase purified by (A) Sephadex G-200 and (B) Phenyl Sepharose.

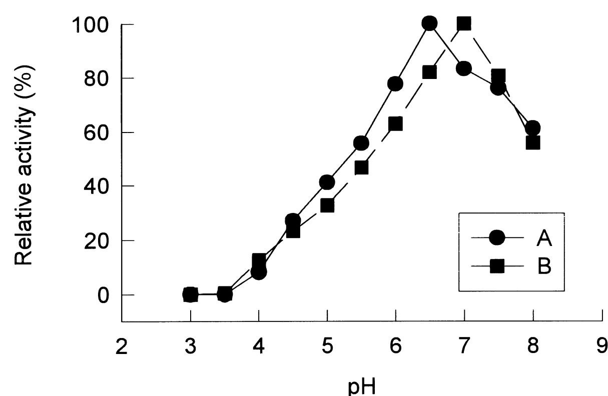

Fig. 2. Determination of the (A) optimum pH and (B) pH stability of longan polyphenol oxidase.

3. Results and discussion

3.1. Extraction and purification of PPO

A 46-fold purification of PPO relative to protein with a yield of 3.7% was achieved (Table 1), and the elution profile of the PPO on Sephadex G-200 and Phenyl Sepharose is shown in Fig. 1.

3.2. pH optimum and stability

The optimal pH for PPO activity was 6.5 with 4-methylcatechol as a substrate (Fig. 2). Differences in PPO pH optima with various substrates were reported (Aylward & Haisman, 1969) varying from 4.0 to 7.0, depending on the origin of the material, extraction method, and substrate.

The longan PPO was most stable at pH 7.0. The pH stability of PPO increased from pH 4.0 to 7.0, and then decreased from 7.0 to 8.0 (Fig. 2).

3.3. Optimum temperature and stability

The enzyme had a temperature optimum of 35°C (Fig. 3), and was relatively stable, requiring a little more than 20 min at 50°C for 50% loss of activity (Fig. 4).

3.4. Substrate specificity

The longan PPO had no activity towards monophenols (tyrosine and *p*-cresol), *m*-phenol (resorcinol), or chlorogenic acid (Table 2), which was consistent with the result obtained by the Clark O_2 electrode. The greatest activity was detected towards pyrogallol, followed by 4-methylcatechol and catechol. The inability of the PPO

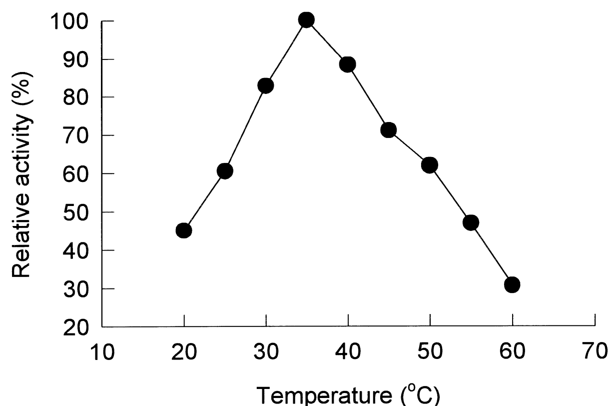

Fig. 3. The optimum temperature of longan polyphenol oxidase.

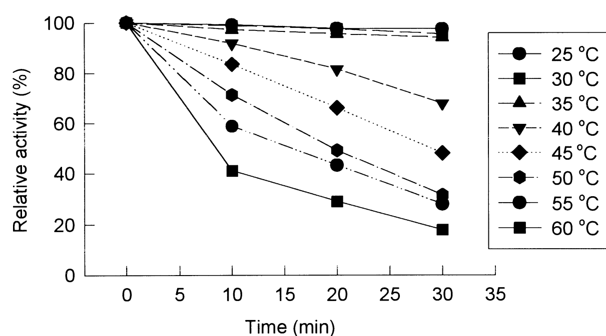

Fig. 4. Thermal stability of longan polyphenol oxidase.

used for this study to oxidase those phenolic compounds, suggests that the enzyme lacked cresolase and laccase activities and was a diphenol oxidase.

3.5. Inhibitors

The effects of various inhibitors on the partially purified longan PPO are shown in Table 3. A lag period was observed when D-glutathione, L-cysteine, ascorbic acid or thiourea was used as an inhibitor. However, it is noted that benzoic acid, *p*-coumaric acid, and ferulic acid did not completely inhibit the PPO activity; they are commercially used to prevent the browning (Walker, 1995; Walker & Ferrar, 1998). Of all the inhibitors tested in this study, D-glutathione (reduced form) was the most effective, followed by L-cysteine. In addition, the PPO activity was markedly inhibited by $FeSO_4$ and $SnCl_2$, but promoted by $MnSO_4$ and $CaCl_2$. Jimenez and Garcia-Carmona (1993) also reported that a latent activity of grape PPO can be activated in the presence of divalent cation such as Ca^{2+}, Mg^{2+} and Mn^{2+}.

Data presented here suggest glutathione appears to be a potent inhibitor of longan peel PPO but the cost of its use would be prohibitive on a commercial scale. Cysteine can easily form complexes with *o*-quinones and PPO was inhibited by the formation of additional products(Sanada, Suzue, Nakashima, & Kawada, 1972;

Table 3
Effect of various compounds on activity of polyphenol oxidase of longan fruit[a]

Compound	Relative activity (%)	
	0.1 mM	1.0 mM
$CaCl_2$	118	142
$CuSO_4$	106	118
EDTA	81	75
NaCl	85	81
$MnSO_4$	124	186
$SnCl_2$	48	11
Ascorbic acid	86	47
Benzoic acid	56	37
p-Coumaric acid	61	43
L-Cysteine	43	0
Ferulic acid	69	46
Glutathione (reduced)	14	0
Thiourea	34	19

[a] Substrate was present at a final concentration of 25 mM, and activity was expressed relative to that with 4-methylcatechol (3.9×10^4 units/mg protein) =100.

Janovitz-Klapp et al., 1990). Cysteine has been suggested to prevent enzymatic browning in processed fruit products (Santerre, Cash, & Vannorman, 1988). However, the application of L-cysteine as an anti-browning agent for longan fruit should be further studied as results with intact fruit peel would not necessarily be the same as with enzyme extract.

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