Latent Polyphenol Oxidases from Sago Log (*Metroxylon sagu*): Partial Purification, Activation, and Some Properties

Galila Hassan Onsa, Nazamid bin Saari,* Jinap Selamat, and Jamilah Bakar

Department of Food Science, Faculty of Food Science and Biotechnology, University Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Latent polyphenol oxidase (LPPO), an enzyme responsible for the browning reaction of sago starches during processing and storage, was investigated. The enzyme was effectively extracted and partially purified from the pith using combinations of nonionic detergents. With Triton X-114 and a temperature-induced phase partitioning method, the enzyme showed a recovery of 70% and purification of 4.1-fold. Native PAGE analysis of the partially purified LPPO revealed three activity bands when stained with catechol and two bands with pyrogallol. The molecular masses of the enzymes were estimated by SDS−PAGE to be 37, 45, and 53 kDa. The enzyme showed optimum pH values of 4.5 with 4-methylcatechol as a substrate and 7.5 with pyrogallol. The LPPO was highly reactive toward diphenols and triphenols. The activity of the enzyme was greatly enhanced in the presence of trypsin, SDS, ethanol, and linoleic acid.

Keywords: *Metroxylon sagu; pith; latent polyphenol oxidase; extraction; partial purification; characterization*

INTRODUCTION

Sago palm (*Metroxylon sagu*) is considered to be a main source of starch in Southeast Asia. In Malaysia, sago palm plantations and sago starch industries are located in Peninsular Malaysia and Sarawak (East Malaysi), covering a total area of 20300 ha with a productivity of 10−12 tons/ha of sago starch annually (Department of Statistics, Malaysia, 1997). Sago starch contains almost 88% carbohydrate (Encyclopædia Britannica, 1997). The starch has been used as a major ingredient in various foods: soups, cakes, puddings, and sauce thickeners. It is also used as a raw material in the production of monosodium glutamate, high fructose syrup, and caramel. It was found that the degree of whiteness of sago starch deteriorates markedly upon storage. Therefore, browning of the starch has been one of the most critical problems facing the industry.

Several studies have reported the involvement of polyphenol oxidase in the oxidation of the polyphenols from plants (Mayer and Harel, 1978). The resulting quinones formed are further polymerized to produce a brown pigment. In some plants these enzymes can either exist in latent (LPPO) or active soluble form (SPPO). LPPO of higher plants is generally considered to be a plastid enzyme and in the thylakoid membrane (Mayer and Harel, 1978). Sanchez-Ferrer et al. (1989a,b) and Marques et al. (1994) described LPPO as a hydrophilic protein with a short hydrophobic tail that anchors to the membrane. Large amounts of enzyme activity up to 50−120 times can be produced when the enzyme is exposed to different treatments such as ionic detergents (SDS), proteolytic enzymes, acid or base treatments, fatty acids, and alcohols. These activation processes have been reported for different plants: LPPO of table beet (Escribano et al., 1997); LPPO of broad bean leaf (Sanchez-Ferrer et al., 1990); LPPO of lettuce (Chazarra et al., 1996); and LPPO of William pear (Gauillard and Richard-Forget, 1997). These facts could be the reasons for the rapid browning reaction that happened during storage and processing of sago starch.

The existence of LPPO in sago log has not been previously reported. To develop a method of preventing the browning reaction in sago logs, this study was initiated with the aim of purifying and characterizing LPPO from sago pith.

MATERIALS AND METHODS

Plant Material. Sago (*M. sagu*) pith was collected from Batu Pahat, Johor, Malaysia. The tree was between 2 and 4 years old with a trunk diameter of 52 cm and a height of 280 cm.

Reagents Used. Triton X-100, Triton X-114, Tween 80, and all substrates used in this study were purchased from Sigma Chemical Co. Tris-base and sodium dodecyl sulfate (SDS) were purchased from Wako Pure Chemicals Industry, Osaka, Japan. Mono- and disodium phosphates were obtained from Fluka. All chemicals were used without further treatment.

Extraction of LPPO. Chloroplast was isolated from fresh sago pith by osmotic shock as described by Sanchez-Ferrer et al. (1989a,b) with some modifications. A 600 g of sago pith was blended and homogenized with 0.1 M phosphate buffer, pH 6.8, containing 0.4 M sorbitol and 1.0 mM ascorbic acid. The slurry was filtered through two layers of cotton sheath and centrifuged at 150*g* for 10 min. The resulting pellet was discarded, and the supernatant was centrifuged at 20000*g* for 30 min. The pellet of the chloroplast membrane was digested with different detergents (nonionic detergents Triton X-114, Triton X-100, and Tween 80 and ionic detergents deoxycholate sodium salt and SDS) independently at 1.5% (v/v) in 0.1 M Tris buffer, pH 6.8, followed by stirring for 20 min and standing overnight in a cold room at 4 °C. The treated pellets were centrifuged at 60000*g* for 20 min, and the supernatant was considered to be the LPPO extract.

Partial Purification of LPPO. The supernatant resulting from the successful detergent extraction was subjected to

* Author to whom correspondence should be addressed (fax + 603-89423552; e-mail nazamid@fsb.upm.edu.my).

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Table 1. Extraction of LPPO from Sago Pith

detergent	total vol (mL)	protein (mg/mL)	act.[a] (units × 10^3/mL)	sp act. (units × 10^3/mg of protein)	total act. (10^3 units)	extraction rate
no detergent						
buffer only	9	4.2	0.35	0.08	3.1	
nonionic						
Triton X-100	18	5.3	3.31	0.62	59.6	19.2
Triton X-114	15	3.2	4.43	1.37	66.5	21.4
Tween 80	10	6.5	3.37	0.52	33.7	10.8
ionic						
SDS	18	4.4	0.77	0.18	13.8	4.4
sodium deoxycholate	15	6.5	1.12	0.18	17.3	5.6

[a] Activity was assayed spectrophotometerically at 420 nm with 0.1 M pyrogallol in 0.1 M phosphate buffer, pH 7.0.

temperature-induced phase partitioning using the method of Sanchez-Ferrer et al. (1989a,b). The concentration of the detergent selected was increased from 1.5 to 8%. The extract was kept at 4 °C for 30 min and then warmed to 35 °C for 15 min. The treated extract was centrifuged at 5000*g* for 10 min at room temperature. The clear supernatant obtained was described as a partially purified LPPO.

LPPO Activity Assay. LPPO activity was determined spectrophotometerically at 420 nm by monitoring the reaction at 25 °C for 6 min. The reaction mixture contained 2.9 mL of 0.1 M buffer (acetate buffer, pH 3.0−7.0, and Tris buffer, pH 7.0−10.0), 1.0 mL of 0.05 M substrate, and 0.1 mL of enzyme solution. One unit of enzyme activity was defined as the amount of enzyme causing a 0.001 change in the absorbency per minute.

Protein Concentration. Protein concentration was determined according to the Hatree (1972) method using bovine serum albumin as the standard protein.

Electrophoresis Study. Polyacrylamide gel electrophoresis (PAGE) for native protein was carried out using a discontinuous gel system as described by Laemmli (1970) using a Hoefer Mini Cell (model SE 260). The separating gel containing 10% polyacrylamide was run under nonreducing and nondenaturing condition. Sample was mixed with glycerol and bromophenol blue. Ten microliters of the mixture was applied. Electrophoresis was carried out for 50 min at 4 °C with a current of 15 mA on a 1.5 mm thick gel. The gels were stained for LPPO activity by 0.1 M catechol in 0.1 M acetate buffer, pH 4.5, and 0.1 M pyrogallol in phosphate buffer, pH 7.5. For molecular mass determination, SDS−PAGE was carried out using the same system; sample was treated with glycerol, SDS, mercaptoethanol, and bromophenol blue according to the Laemmli (1970) treatment method. Electrophoresis was carried out for 50 min at room temperature with a current of 30 mA on a 1.5 mm thick gel. The gel was later stained with Coomassie brilliant blue R-250 overnight.

Substrate Specificity. A Rank Brothers oxygen electrode (Rottisham product) was used to determine the activity of sago LPPO toward different substrates. The activity was assayed at 30 °C. The reaction mixture contained 1.0 mL of 0.05 M substrate, 2.9 mL of phosphate buffer, pH 7.0, and 0.1 mL of enzyme solution. The activity was calculated from the tangent of the trace of oxygen consumption.

Activation of LPPO. LPPO was incubated with SDS (0.63 mM), trypsin (1000 units/mL), ethanol, and linoleic acid independently for 0, 5, and 10 min and 24 h. The activity was initiated by the addition of 50 μL of the treated enzyme into the reaction mixture and measured spectrophotometrically at 420 nm.

RESULT AND DISCUSSION

Enzyme Extraction. The experiment was carried out to extract LPPO from a thylakoid pellet of sago pith using either 0.1 M Tris buffer, pH 6.8, or detergents. The effect of various detergents on the extractability of LPPO is given in Table 1. Detergents used enhanced the extractability by 4.4−21.4-fold compared to when the enzyme was dissociated with buffer alone. LPPO was better extracted from sago pith with nonionic detergents as compared to anionic detergent. Among the nonionic detergents, Triton X-114 was the most effective because it enhanced the enzyme extraction by 21.4-fold, whereas with Triton X-100 and Tween 80, the extractabilities were only 19.2- and 10.8-fold, respectively. With ionic detergent, the extractabilities were 5.6- and 4.4-fold for SDS and deoxycholate, respectively. The parameters that describe detergent behavior with regard to solubilization of membrane proteins as reported by Judith (1990) are the critical micelle concentration (cmc) (the detergent concentration at which micelles begin to form) and the hydrophile−lipophile balance (HLB) (an index of detergent hydrophobicity). We observed that the larger the HLB value, the more hydrophilic the detergent and the less efficient the extractability of the enzyme. We could suggest that the electrical charge and the HLB of the detergents are the main determining factors for dissociation of LPPO from sago pith. The HLB for nonionic detergents Triton X-114, Triton X-100, and Tween 80 were 12.4, 13.5, and 15, respectively (Judith, 1990).

Partial Purification of LPPO. The supernatant of Triton X-114 extract, which had the highest specific activity (1371.5 units/mg protein) and the best extractability (21.4-fold), was subjected to a further purification step by increasing the concentration of TX-114 to 8% and warmed to 35 °C for 15 min. Aggregation of micelles formed was separated by centrifugation into two phases, a detergent-rich phase with some phospholipid and remaining phenols and a clear detergent-poor phase with LPPO activity. This could be justified by the fact that nonpolar residues predominate in the membrane-bound protein, forming a hydrophobic exterior that makes a micelle-like structure (Sanchez-Ferrer, 1989a,b). In this purification procedure the purification rate was increased from 1.7 to 4.1 and the yield was 70%. The degree of purification and the recovery rate are in ranges similar to those found when Triton X-114 was used to purify LPPO from lettuce (Chazarra et al., 1996) and potato leaf (Sanchez-Ferrer et al., 1990), which were found to be 4.3−4.5 and 70%, respectively. Although the specific activity was low, the procedure developed was able to remove all phenolic compounds present to give a clear extract, and the enzyme activity remained stable for more than a month when stored between 4 and −20 °C.

Electrophoresis Study. PAGE revealed the different forms of the enzyme present in the thylakoid membrane of sago pith. When the gel was run under nondenaturing, nonreducing condition and stained with 0.1 M catechol, the partially purified enzyme showed three active bands (Figure 1A). However, the gel stained with 0.1 M pyrogallol at pH 7.5 showed two active bands

Table 2. Purification of LPPO from Sago Pith

step	total vol (mL)	protein (mg/mL)	act. (units × 10^3 mL)	sp act. (units × 10^3/mg of protein)	total act. (units × 10^3)	purifn (fold)	recovery (%)
Triton X-114 extract	18	4.73	2.61	0.551	46.98		100.0
supernatant of 1.5% TX-114 digest	11	3.03	3.97	1.309	43.64	2.4	92.9
supernatant of 8% TX-114 digest	8	1.80	4.10	2.277	32.80	4.1	69.8

[a] Activity was assayed with 0.05 M 4-methylcatechol in 0.1 M acetate buffer, pH 4.5.

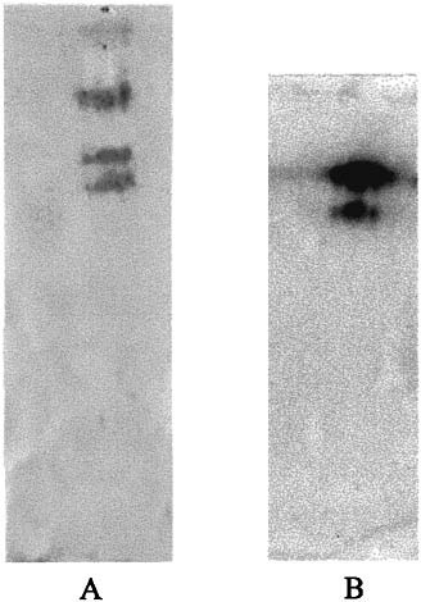

Figure 1. Native PAGE of the partially purified LPPO: (A) activity staining by 0.1 M catechol; (B) activity staining by 0.1 M pyrogallol. Electrophoresis was done on 10% polyacrylamide. Thirty micrograms of the partially purified LPPO was applied.

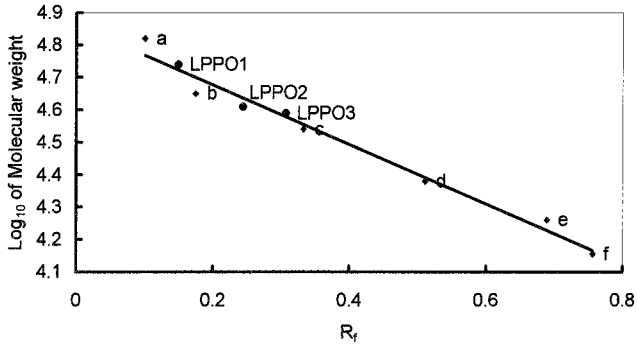

Figure 2. Molecular masses of the three isozymes of LPPO: (◆) protein markers used [(a) bovine albumin (66.0 kDa); (b) egg albumin (45.0 kDa), (c) pepsin (34.7 kDa); (d) trypsinogen (24.0 kDa); (e) lactoglubulin (18.4 kDa); (f) lysozyme (14.3 kDa)]; (●) LPPO isozyme molecular masses [LPPO1, 53 kDa; LPPO2, 45 kDa; LPPO3, 37 kDa]. The molecular mass was determined by SDS−PAGE on 10% gel electrophoresis.

(Figure 1B). These results suggest the probable existence of 3 units of diphenolase and 2 units of triphenolase. When SDS−PAGE was carried out, three bands appeared with Coomassie brilliant blue R-250. The molecular masses of the three isozymes were determined as 37, 45, and 53 kDa (Figure 2).

Activation Process. To characterize the partially purified enzyme, a detailed study of the enzyme activation was carried out. Treatment of LPPO with detergent (SDS), alcohol (ethanol), fatty acid (linoleic acid), or protease (trypsin) enhanced the activity differently (Figure 3). Trypsin as proteolytic enzyme cleaves the polypeptide chains on the carboxyl side of arginine and

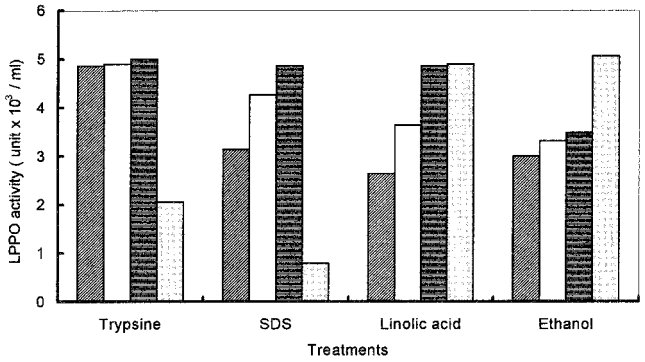

Figure 3. Activation of sago pith LPPO by different treatments. The partially purified LPPO was treated with SDS, ethanol, linoleic acid, and trypsin at time intervals of 0 min (diagonally slashed bar), 5 min (white bar), 10 min (horizontally striped bar) and 24 h (dotted bar).

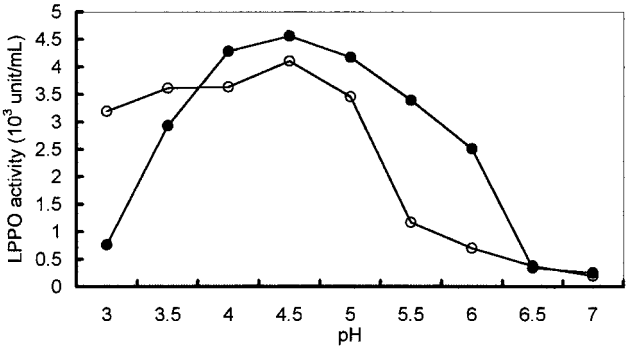

Figure 4. Effect of pH on activity of sago pith LPPO treated with SDS (●) and without SDS (○). The reaction mixture contained 0.1 mL of LPPO, 1 mL of 0.05 M 4-methylcatechol, and 2.9 mL of 0.1 M buffer (sodium acetate buffer, pH 3.5−7.0, and Tris-HCl buffer, pH 7.0−8.0).

lysine residues to produce several tryptic peptides. In this work, trypsin was found to be the most effective and fastest activator. Compared to other treatments, it gave almost full activation (5 × 10^3 units/mL) at 0 min. However, the enzyme lost its activity after prolonged incubation. With SDS treatment, however, full activation was achieved after 10 min of incubation. As with trypsin, prolonged incubation with SDS decreased the enzyme activity. This was also reported by Sanchez et al. (1989a,b) for spinach leaves LPPO. Another characteristic behavior of sago LPPO activated with SDS is illustrated in Figure 4. It was found that at low pH (pH <4) SDS inactivated the enzyme. The highest value of activation process by SDS was obtained at pH 5.5−6. This behavior was similar to that reported for lettuce (Chazara et al., 1996) and table beet (Escribano et al., 1997). The effect of SDS in the activation process of LPPO was a result of conformational change due to the binding of small amount of SDS to the active site as suggested by Moor and Flurky (1990). With linoleic acid and ethanol treatment, the enzyme was fully activated by incubation for 24 h. This suggests that both could

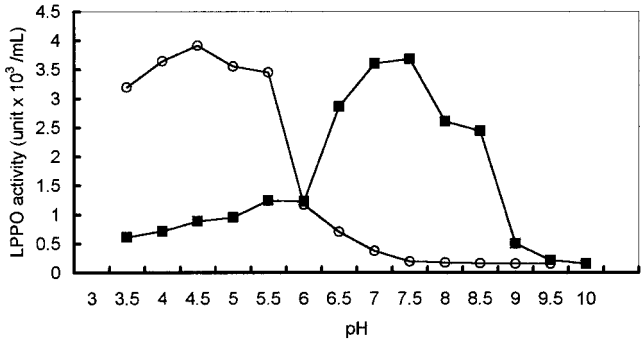

Figure 5. Effect of pH on sago pith latent diphenolase (○) and triphenolase (■). The reaction mixture contained 2.9 mL of 0.1 M buffer (sodium acetate buffer, pH 3.0−7.0, and Tris-HCl buffer, pH 7.0−10.0), 0.1 mL of LPPO, and 1 mL of 0.05 M substrate (diphenol; 4-methylcatechol, triphenol; pyrogallol).

Table 3. Substrate Specificity of LPPO from Sago Pith

substrate (0.05 M)	act. (μg of oxygen/min)	relative act.
monophenols		
benzoic acid	0.00	0.00
p-cresol	0.00	0.00
diphenols		
4-methylcatechol	4.94	100
chlorogenic acid	4.10	83.1
epicatechin	4.60	93.1
DL-DOPA	3.28	66.4
p-coumaric acid	1.09	22.1
catechol	4.57	92.5
triphenol		
pyrogallol	3.86	78.1

[a] Activity was assayed by oxygen electrode.

cause some conformational changes in the structure of sago LPPO upon prolonged incubation.

Effect of pH. pH is a determining factor in the expression of enzymatic activity; it alters the ionization states of amino acid side chains or the ionization of the substrate (Voet and Voet, 1990). Sago LPPO activity with 4-methylcatechol as a substrate showed an optimum pH at 4.5 (Figure 5). This low pH optimum could be a result of greater enzyme activity being induced by acid shock. A similar result was obtained by Escribano et al. (1997) for table beet, by Fraignier et al. (1995) for cherry, and by Sanchez-Ferrer et al. (1993) for potato leaf. The same pH optimum was obtained when the enzyme was treated with SDS. It was also observed that the activity of SDS-treated enzyme was decreased at pH <4 and that maximum activation was achieved at pH 5.5−6.0. Very few studies have been done on the pH profile of LPPO with pyrogallol as a substrate. However, sago LPPO has the ability to catalyze the oxidation of triphenols well at pH 7.5, forming a bell shape curve between pH 3.0−10.0 (Figure 5). This result seems to be related to the number of carboxyl groups of the substrates and to the binding ability of the substrates under acidic or alkaline condition.

Substrate Specificity. The oxidizing ability of the partially purified LPPO of sago pith was determined using monophenols, diphenols, and triphenols as substrates. The relative activity was calculated by considering 4-methylcatechol as 100% activity. From Table 3 it is seen that the enzyme showed no activity with monophenols, which agreed with the finding of Gauillard and Forget (1997) for William pear. However, the enzyme was active toward diphenols at the same pH. Among diphenols tested, 4-methylcatechol gave the highest activity followed by epicatechin, catechol, and chlorogenic acid, which are considered to be the major in vivo substrates in sago log (Ozawa et al., 1991; Anthonysamy et al., 1998). The enzyme also showed high activity (78%) with pyrogallol.

This work presented the purification and some properties of LPPO from sago pith. The paper showed the efficiency of Triton X-114 in the extraction of sago LPPO from the thylakoid membrane. This procedure was able to remove all of the phenols present to give a clear and stable enzyme solution. The partially purified enzyme revealed the presence of three isozymes, 3 units of diphenolase with optimum pH 4.5, and 2 units of triphenolase with optimum pH of 7.5. The enzyme was highly activated by trypsin followed by SDS. Further purification and characterization of sago LPPO are necessary to understand its behavior toward the browning reaction on sago starch during storage and processing.

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