Purification of Lignin Peroxidase from Cocos Nucifera Leaflets

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Abstract: The lignin peroxidases (LiP) are heme containing glycoproteins which catalyze the oxidation of a wide range of compounds with high redox potential. LiP has received considerable attention in the last decade because of its applications in various fields such as paper - pulp industry, food industry, textiles, cosmetics and bioremediation. The present study investigated a new source of LiP that is from Cocos nucifera leaflets. The enzyme was purified to homogeneity using gel filtration chromatography and ion exchange chromatography using Sephadex G50 and DEAE cellulose columns respectively. The molecular weight of the purified enzyme was determined to be 118 kDa using SDS-PAGE. The peroxidatic nature of LiP was confirmed through zymogram and ICP AES analysis. This is the first report of LiP from Cocos nucifera leaflets and its purification.

Keywords: Lignin Peroxidase; Cocos nucifera; Gel filtration chromatography; Ion exchange chromatography; Zymogram.

I. INTRODUCTION

Peroxidases (POD) are ubiquitous heme-containing proteins that utilize H_2O_2 for the oxidation of various organic and inorganic substances. They are widely distributed in bacteria, fungi, plants and animals. PODs are involved in a wide range of biological processes such as defense, immune response, pathogenicity, detoxification, and biomass degradation [1]. In response to external factors, they occur as distinctive isoenzymes which can be constitutive or induced [2]. The PODs that utilize guaiacol as the electron donor participate in lignification, cross-linking of cell wall polymers, degradation of indole acetic acid (IAA), ethylene biosynthesis, pathogen defense, and wound healing [3]. Peroxidases are grouped into three major classes based on the aminoacid sequence similarities as well as structural and functional properties. Class I enzymes are intracellular PODs of bacteria, archaeans, ascorbate peroxidases, and mitochondrial cytochrome c peroxidases; class II enzymes include the secretory fungal peroxidases; and class III include the plant peroxidases [4]. The two recently reported novel families of secreted fungal peroxidases are hemethiolate peroxidases (HTP) and dye-decolorizing peroxidases (DyPs) ([5], [6], [7], [8]). DyPs are the classical non-animal peroxidases including lignin peroxidase, manganese peroxidase, and versatile peroxidase which are phylogenetically unrelated to the catalase-peroxidase superfamily [9]. LiP is able to catalyze the oxidation of a variety of compounds with reduction potentials exceeding 1.4V [10]. Veratryl alcohol is often regarded as a model substrate for the enzymatic action of LiP [11]. LiP has been employed in different applications such as decolourization of kraft pulp mill effluents, production of vanillin and other aromatics, textile dye degradation and bleaching, mineralization of environmental contaminants, and xenobiotics degradation [12]. Recent reports suggest its potential as a novel skin lightening agent [13].

Cocos nucifera is a widespread tropical plant popularly known as coconut tree which belongs to the Arecaceae family. Nearly all parts of the plant are useful. The plant has been reported to show various biological properties such as analgesic, antiarthritic, antipyretic, antibacterial, antidiarrheal, antihelminthic, and hypoglycemic activities, while having antihypertensive, anti-inflammatory, antioxidant, cardioprotective, cytotoxic, hepatoprotective, vasodilatory, antiseizure, nephroprotective, and anti-osteoporosis effects [14].

Although plant PODs have been extensively studied in various species, lignin peroxidase activity has been reported only in soybean [15], tobacco [16] and *Musa paradisiaca* stem juice [17]. LiP activity has not been reported in *Cocos nucifera* and its investigation may offer many industrial applications. In this study we report the peroxidase as well as lignin peroxidase activity and describe the purification of LiP from *Cocos nucifera* leaflets.

II. MATERIALS AND METHODS

Extraction of lignin peroxidase

About 2g of fresh matured leaflets of *Cocos nucifera* was cut into 0.1cm long pieces and transferred to 250mL Erlenmeyer flask containing 50 ml of 0.1 M citrate buffer pH 5. The flask was kept in a rotary shaker at 15 C for 30 min at 150 rpm. The enzyme extract was centrifuged at 10000 rpm for 10 min at 4˚C and the supernatant was collected.

Enzyme activity

POD activity was assayed spectrophotometrically using guaiacol/H_2O_2 as substrate [18]. The increase in absorption as a result of the formation of the oxidized product (tetraguaiacol) was measured at 470 nm. The reaction mixture contained 0.5 mL citrate buffer pH 5.0 (0.1M), 0.25 mL H_2O_2 (0.2mM), and 0.1mL guaiacol (25 mM) and was incubated with 0.25 mL enzyme solution at 28° C.

LiP activity was determined by H_2O_2 dependent veratraldehyde formation from veratryl alcohol detected spectrophotometrically at 310 nm [19]. The reaction mixture contained 0.25 mL of enzyme solution, 0.25 mL veratryl alcohol (1mM), 0.25 mL H_2O_2 (0.2 mM) and 0.5 mL citrate buffer pH 5.0 (0.1M) at 28°C [20]. Protein concentration was determined by lowry method [21].

The POD and LiP activity were expressed in IU/mL. One International Unit (IU) is defined as the amount of enzyme activity which will catalyze the transformation of 1 micromole of the substrate per minute under standard conditions.

Purification of lignin peroxidase

a) Acetone precipitation

The crude enzyme was precipitated with 66% cold acetone and centrifuged at 18,000 rpm for 15 min [22]. The enzyme pellet was dissolved in a known volume of citrate buffer pH 5.0 (0.1M), and LiP activity was determined. Protein content in the enzyme solution was determined by Lowry's method [21].

b) Gel filtration chromatography

Gel filtration was carried out at 4 C. The sample was applied on to preequilibrated (using 0.1M citrate buffer, pH 5.0) sephadex G50 column (1.6x 47cm) and the enzyme fractions were eluted isocratically with 0.1M citrate buffer, pH 5.0. The flow rate was adjusted to 0.3mL/min. One mL fractions were collected and analyzed for enzyme activity as described earlier. The fractions showing LiP activity were pooled, and concentrated using 10kDa amicon filter for further purification by ion exchange chromatography.

c) Ion exchange chromatography

The sample after gel filtration was applied on to a preequilibrated (0.1M citrate buffer, pH 5.0) DEAE cellulose column (1.3x15cm). The elution was carried out using gradient of NaCl (0.0~1.0M) in citrate buffer pH 5.0 (0.1mM) at the flow rate of 1mL/min. Five mL fractions were collected and checked for LiP activity. The LiP fractions were pooled and concentrated as described earlier.

d) SDS PAGE

The homogeneity of the purified LiP was analyzed by sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS PAGE). The separating gel was 12% acrylamide in 0.375M Tris-HCl buffer pH 8.8 and stacking gel was 5.0 % acrylamide in 0.063M Tris-HCl buffer pH 6.8. Gel was run at a constant voltage of 80 volts. Proteins were visualized by silver staining.

e) Zymogram analysis

Zymogram analysis was performed by using L-dopa and 4-aminoantipyrine in presence of H_2O_2 in 0.1 M citrate buffer pH 5.0 [23].

f) Inductively Coupled Plasma – Atomic Emission Spectrometry

The peroxidatic nature of the enzyme was confirmed by subjecting the pooled ion exchange sample to Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES).

III. RESULTS AND DISCUSSION

Activity of crude enzyme extract

In the crude enzyme extracts from *C. nucifera* leaflets, the POD activity was found to be 16.65±0.49 IU/mL and the LiP activity was found to be 127.63±11.38 IU/mL. The assay result showed the dominance of LiP activity over peroxidase activity. POD activity has been reported in various vegetables and found to vary with respect to the source from which they have been extracted [24]. It has been demonstrated that all PODs are not considered similar as some show direct peroxidatic oxidation of veratryl alcohol [15]. The present study confirmed the LiP activity of *C. nucifera* leafets crude enzyme as it catalyzed direct peroxidatic oxidation of veratryl alcohol.

Purification of lignin peroxidase

The crude enzyme was then subjected to the purification process. The fractions 29 to 37 from the Sephadex G50 column showed highest enzyme activity (peak 4 in Fig 1a). These fractions were pooled, concentrated and subjected to ion exchange chromatography. On ion exchange chromatography the LiP activity was obtained in the 0.1 M NaCl gradient fraction. All active fractions represented in Fig 1b were pooled and concentrated. The SDS-PAGE revealed a single band (lane 3 of Fig 2) indicating that the purified enzyme was homogeneous. The calculated molecular weight of the purified lignin peroxidase is

approximately 118 kDa. Moreover, the purification of *C. nucifera* LiP gave only single peak with positive activity, indicating that, under the conditions used for extraction and purification, only one form of the enzyme is observed. Multiple isozymes have been noticed in the case of LiP's from other sources ([25], [26]). The LiP was purified from *C. nucifera* with a yield of 0.6% (Table 1).

TABLE 1: PURIFICATION OF LIGNIN PEROXIDASE

Fraction	Total Volume (mL)	LiP activity (IU/mL)	Protein content (mg)	Total LiP activity (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification fold	% Yield
Crude	60	97.6±1.0	0.7	5856.00 ± 60.0	42	139.42	-	-
Acetone precipitation	5	279.60±3.2	0.904	1398.00 ±16.1	4.52	309.29	2.21	24
Sephadex G50	5	139.00±1.8	0.074	695.00±9.0	0.37	1878.37	13.47	11.86
DEAE Cellulose	1	34.50±2.6	0.0054	34.50±2.6	0.0054	6388.89	45.82	0.6

Purification scheme for lignin peroxidase

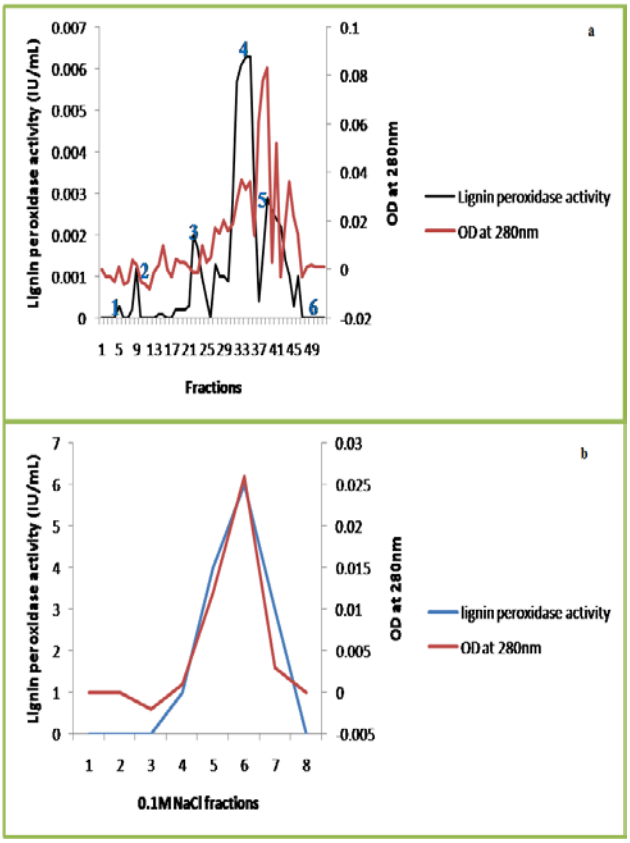

Fig 1: The elution profile of LiP on a) Sephadex G50 column and b) DEAE Cellulose column

TABLE 2: ICP -AES

Sl.No	Sample	Fe2599	Unit
1	LiP	40.16	ppm
	Detection limit	0.01	ppm

Confirmation of peroxidatic nature by ICP AES

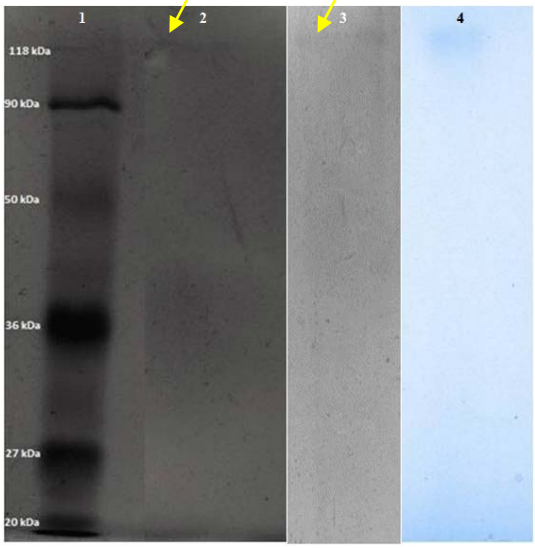

Fig 2: Purification of LiP: Lane 1- Marker, Lane 2- LiP after gel filtration chromatography; Lane 3- LiP after ion exchange chromatography; Lane 4- zymogram analysis

LiPs are oligomannose type glycoprotein with a molecular weight range of 38 kDa to 43 kDa [27]. The presence of a single band in lane 2 of Fig. 2 indicates that the purified enzyme from *C. nucifera* has a molecular weight of 118 kDa. Zymogram analysis (Fig2, Lane 4) performed using L-dopa and 4-aminoantipyrine in presence of H_2O_2 in 0.1M citrate buffer showed a red band in the respective region confirming the peroxidase nature. Inductively Coupled Plasma – Atomic Emission Spectrometry detected the presence of Fe^{2+} (cofactor of heme present in lignin peroxidase) and confirmed that the fraction obtained in ion exchange chromatography was purely lignin peroxidase. The Fe^{2+} concentration in the sample was determined to be

40.16 ppm (Table 2) which confirms the peroxidatic nature. The lowest molecular weight of 17.8 kDa for LiP has been reported in the *Streptomyces viridosporus* [28] and highest molecular weight of 110-130 kDa has been reported in *Acinetobacter calcoaceticus* NCIM 2890 [29]. Plant peroxidases have also been shown to possess the enzyme with varying molecular weights in different range ([15], [17]). Thus, the molecular mass of the LiP purified from *C. nucifera* was found to be in the same range as that of LiPs purified from other bacterial, fungal, and plants.

IV. CONCLUSION

The lignin peroxidase activity was studied in *Cocos nucifera* and the respective enzyme was purified to apparent electrophoretic homogeneity. The particular enzyme catalyzes the efficient oxidation of veratryl alcohol in the presence of H_2O_2 than the oxidation of guaiacol thus establishing the LiP activity rather than peroxidase activity. The molecular weight of the purified enzyme was determined to be 118kDa by SDS PAGE and the peroxidatic nature was affirmed by zymogram analysis and ICP AES.

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