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Purification and characterization of membrane-bound *Borassus flabellifer* L. thermostable peroxidase

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Generating wealth out of waste is a goal in solid waste management. *Borassus flabellifer* shells with stone part are discarded as waste after removing its nutritious fruit. An enzyme extracted from the discarded stone part has potential application in molecular diagnosis and it is a wealth. The waste stone part bound *Borassus flabellifer* peroxidase was purified by salting-out, salting-in and DEAE-Cellulose anion exchange chromatographic technique to apparent homogeneity. Relative molecular weight under denaturating condition was around 40 kDa. The preparation had single isoenzyme as evidenced under nondenaturating condition. It was a glyco- and hemoprotein. It retained 100% activity for 120 h at 70°C. pH optima with benzidine, o-dianisidine, guaiacol and tetramethylbenzidine was around 5.0. Kinetic studies showed it had a higher affinity towards tetramethyl benzidine than other three substrates. The thermodynamic parameter of the enzyme with benzidine, o-dianisidine, guaiacol and tetramethylbenzidine was 35, 1.3, 35×10^6 KJ/mole and 1007×10^6 KJ/mole respectively. It obeyed Ping-Pong kinetics. The fluorescence intensities of the enzyme increased linearly as hydrogen peroxide concentration increased due to exposure of its hydrophobic moiety to the environment. Peel staining with Guaiacol substantiated it as a membrane-bound protein. Peroxidase was inhibited reversibly by various aromatic alcohols and its IC_{50} values were determined.

Keywords: Affinity, *Borassus flabellifer* peroxidase, Hydrophobic, Fluorescence, Substrate specificity

Peroxidases play an important role in immunotechnology (quantification of antigens, and toxins)[1,2], biosensor[3,4] (quantifying glucose, cholesterol, hydrogen peroxide) and in nano biosensor[5]. Generally peroxidases (EC number 1.11.1.7) are classified as families[6] of Class I (prokaryotic origin: intracellular Cytochrome C peroxidase), Class II (Fungal origin: extracellular Lignin peroxidase, Mn-Peroxidase) and Class III (Plant origin: Plant peroxidases: Green coconut water[7], date palm leaves[8], red pepper[9], soybean seed coat[10], and tomato[11]). Plant peroxidase exists in coconut water and in its endosperm[7]. The plant peroxidases possess broad substrate specificities[12], is monomeric glycoprotein andhaemoprotein[13]. Mechanism of Peroxidase catalysis is hydrogen peroxide oxidizes peroxidise to a two-electron deficient oxidant (Cpd I). Cpd I is reduced to an enzyme intermediate (Cpd II, an electron deficient) by a Guaiacol substrate. Cpd II is further reduced to native state by second Guaiacol substrate[6]. This oxidized Guaiacol polymerizes[12]. It has four conserved disulfide bridges and two calcium ions[13] to prevent thermal inactivation. The plant peroxidases are involved in the removal of hydrogen peroxide from chloroplast and cytosol, oxidation of toxic compounds[14], biosynthesis of lignin[15], Indole acetic acid catabolism[16] and ethylene biosynthesis.

Aqueous extract of *Borassus flabellifer* (BF) fruit showed antiulcer activity[17], used against ulcer, cancer, edema, epilepsy, boil and used in soft drinks[18-19]. BF fruit flour contained cytotoxic flabelliferin. The stone part of BF fruit contained carbohydrate, saponin, tannins, flavonoids, terpenoids, and glycosides. These stone parts are discarded as waste and left out in the environment without further utility. This left out stone part can be utilized for extracting valuable products. Stone parts contained a valuable enzyme which has potential applications in molecular diagnosis. In this article, we report the purification of the stone part membrane-bound peroxidase, which is relatively thermostable, active in ten times concentrated hydrogen peroxide solution and inhibited by various aromatic alcohols.

Materials and Methods

Chemical Components and Instruments

Acetic acid, Benzidine.2HCl, DEAE-Cellulose, o-Dianisidine. 2HCl, Glycine, Guaiacol, Sodium

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dihydrogen orthophosphate, sodium hydroxide, tetramethylbenzidine. 2HCl and tris hydrochloride was analytical grade (Himedia, Mumbai, India) and used without further purification. Cholesterol, m-Cresol, p-Cresol, α-Naphthol, β-Napthol, and Phenol were procured from SD Fine Chemicals (Mumbai, India). Quartz double distilled water (Bhanu Scientific Company) was used in preparing all solutions. Double beam UV-VIS Spectrophotometer (Elico, Hyderabad, India) and Fluorescence Spectrophotometer (Molecular Devices, USA) were employed in carrying out the experiments. The concentration of hydrogen peroxide[20] was quantified spectrophotometrically at 240 nm using a molar extinction coefficient of 39.4 $M^{-1}cm^{-1}$.

Enzyme preparation

The fresh fruits were plucked from the tree. The outer layer of fleshy endosperm was referred as the stone part. This was separated manually under aseptic condition from the edible endosperm. The edible endosperm is nutritious. Normally, the stone parts were discarded in the environment without further use. These waste bioresources were washed thoroughly with double distilled water to remove the adhering endosperm for further process. These solid stone parts were mechanically ground to form colloidal solution by adding water (100 gm: 50 mL distilled water). This colloidal solution was centrifuged at 10000 rpm for thirty minutes at 4°C, discarded the sediments and collected supernatant solution. The supernatant solution was saturated with 40% ammonium sulfate[21] and stored in the fridge at 8°C overnight. Discarded the pellets and solution was raised to 90% gradually by adding powdered ammonium sulfate and left at 4°C overnight. The resultant was centrifuged as mentioned earlier and collected the pellets. These pellets containing most of the peroxidases were dissolved in minimal volume of double distilled water and dialyzed against 20 mM acetate buffer pH 4.5 for 24 h with three changes [1:1000 mL]. This solution was considered as dialysate.

Purification of enzyme

The dialysate was loaded onto pre-equilibrated DEAE-Cellulose (20 mM acetate buffer, pH 4.5, Sigma Column size 1.0 × 60 cm). The anion exchanger was washed thoroughly with the same buffer to get rid of unbound and loosely bound proteins. The ionically interacting protein was eluted by applying a linear gradient (0-0.6 M sodium chloride). Fractions of 5 mL were collected. Each fraction was tested for peroxidase activity with Guaiacol as organic substrate and protein was quantified according to Lowry *et al.*[22] using Bovine Serum Albumin (BSA) as standard. Peroxidase-containing fractions were pooled and used for further studies (Fig. 1).

Enzyme activity

Amount of enzyme required to produce one micromole of tetraguaiacol in one milliliter the reaction mixture containing 0.2 M acetate buffer pH 4.5, 8.8 mM Guaiacol and 3.9 mM hydrogen peroxide per minute at 30°C.

Molecular weight determination and isoenzymes

Its apparent homogeneity and relative molecular weight of pooled peroxidase was determined under a denaturating condition in 10% separating gel as reported[23] with broad range molecular weight standard markers (Genei, Bangaluru). Bands were stained with Coomassie brilliant blue (CBB) 250. Identification of the number of isoenzymes present in the pooled sample was analyzed under non-denaturating condition in 7% gel. Activity staining was done by soaking the gel in 100 mL enzyme assay solution containing Guaiacol and hydrogen peroxide in 0.2 M acetate buffer, pH 4.5. This experiment was performed at room temperature, as it is thermostable

LC/MS method

The purified peroxidase was subjected to in-gel (Native gel) digestion with additional alkylation and reduction as published[24]. Briefly, digested peptides were reconstituted in 15 µL of 0.1% formic acid and 1.0 µL of the same was injected into the column. Digested peptides were subjected to 70 min RPLC gradient, followed by the acquisition of the data on LTQ- Orbitrap-MS. Data was generated using peaks software.

Thermostability of enzyme

0.9 mL of reaction mixture containing 0.2 M acetate buffer pH 4.5, 0.24 µg of enzyme and saturating guaiacol concentration (16 mM) in a test tube was incubated at 70°C for seven days in a water bath (A). The control (B) containing all the above components were incubated at 30°C. Both (A) and (B) were brought down to room temperature. The total volumes were made one milliliter by adding the required volume of double distilled water and swirled for a while. The reaction mixture was transferred to a

cuvette containing 0.1 mL of 39 mM hydrogen peroxide, inverted thrice and kept in cell holder. The Absorbance change was measured at 470 nm for every ten seconds up to two minutes and calculated slope value. The absorbance change was expressed in minutes. By using molar extinction coefficient of 26600 $M^{-1}cm^{-1}$ the enzyme activity (μM/min) was determined for both A & B at different days. The relative activity (%) = (B−A)/ B × 100 was obtained.

pH optima

0.9 mL of reaction mixture containing 0.2 M buffer (Acetate buffer pH 4.0, 4.5, 5.0 & 5.5, Phosphate buffer pH 6.0, 6.5, 7.0 & 7.5, Glycine/Sodium hydroxide buffer pH 8.0, 8.5 & 9.0), 0.24 μg of the enzyme, saturating guaiacol concentration (16 mM) in the test tube was incubated at 30°C for fifteen minutes in water bath. Brought down to room temperature and the reaction was initiated by adding 0.1 mL of 39 mM hydrogen peroxide. The enzyme activity was measured as mentioned in the above section.

Determination of kinetic parameters

0.9 mL of reaction mixture containing 0.2 M acetate buffer pH 4.5, 0.24 μg of enzyme, different substrate concentration (0.79-19 mM of Guaiacol (a), 31-744 μM of o-Dianisidine. 2HCl (b), 39-936 μM of benzidine. 2HCl (c), 32-768 μM of tetramethylbenzidine. 2HCl (d)) was incubated at 30°C for fifteen minutes in water bath. Brought down to room temperature and the reaction was initiated by adding 0.1 mL of 39 mM hydrogen peroxide. Product formation was measured at 470 nm for (a), (b), (c) and at 652 nm for (d). Molar extinction coefficient of product (a) was 2.66×10^4 $M^{-1}cm^{-1}$, (b) and (c) products were 83 $M^{-1}cm^{-1}$ and (d) was 3.9×10^4 $M^{-1}cm^{-1}$. The LB plot[25] was used in determining the maximal velocity and Michaelis–Menten constant. The R^2 value lies from 0.968 to 0.987. The experiments were done in triplicates. The average value was tabulated. Ping Pong kinetics was done at fixed Guaiacol concentration (9 and 19 mM) but at varying hydrogen peroxide concentrations.

Carbohydrate content

The carbohydrate content of this peroxidase was estimated by using Anthrone reagent[26].

Thermodynamic parameters

The thermodynamic parameters like Gibb's free energy change and K_{eq} were calculated as published[27].

Hydrophobicity of peroxidase

The methodology was followed as reported[28]. Briefly, 250 μL of reaction mixture contained peroxidase [0.24 μg] in acetate buffer pH 4.5, ANS (8- anilino, 1-naphthalene sulphonic acid) reagent and varying concentration of hydrogen peroxide. Each test has a control. The control contained ANS reagent and varying concentration of hydrogen peroxide in the same buffer. Average value of triplicates is considered for both test and control. Difference in fluorescence intensities were obtained and plotted in the graph.

Inhibition studies

The inhibition studies of BF peroxidase were examined with various romatic alcohols like Cholesterol (2 mM), m-Cresols (1.0 mM), p-Cresols (16 mM), α-Naphthol (18 mM), β-Napthol (13 mM) and Phenol (2 mM). The indicated concentration of each aromatic alcohol was mixed with BFP in assay buffer (BF Peroxidase, 0.36 μg, test) and incubated at 30°C separately for thirty minutes and dialysed against 20 mM assay buffer with three changes (1:1000 mL) overnight at room temperature to find out whether it is a reversible or irreversible inhibitor. The control contained 0.36 μg of BFP and water was dialysed separately. The assay was done as mentioned in enzyme activity section. The enzyme activity of test containing inhibitor and control without inhibitor are almost same. This experiment substantiated that the aromatic alcohols are reversible inhibitors.

In calculating $IC_{50,}$ 0.9 mL of each experiment contained acetate buffer pH 4.5, 8.8 mM Guaiacol, BFP (0.36 μg), and increasing concentration of inhibitor. Incubated at 30°C for fifteen min and absorbance change was measured at 470 nm by adding 0.1 mL of 39 mM hydrogen peroxide. Enzyme activity was plotted against inhibitor concentration. The concentration of inhibitor required to reduce half of its original activity is referred to IC_{50}. The inhibitory constant (IC_{50}) was calculated from the graph. The graphs are not shown.

Results and Discussion

The soft outer coat of endosperm coincided with tender endosperm whereas thick wall endosperm coincided with hard endosperm without water. The soft outer coat of endosperm has more peroxidases which are involved in gradual hardness of endosperm. This enhanced the dryness of endosperm. The green coconut (*Cocus nuciferae*; water and endosperm) has both soluble and endosperm peroxidase[7]. BF does not have water and endosperm peroxidase. Ajithadevi *et al.*[28] partially purified 100 kDa BF fruit peroxidase but the results are unclear.

In addition to peroxidases, the stone parts contained a high concentration of polyphenols, which cause browning of the crude extract during homogenization and colloidal preparation. The brown colored product does not affect the purification process as polyphenolates interact strongly with an anion exchanger. The purification scheme resulted in 12 fold increase in specific activity when compared to crude extract with a recovery of 76%. The results are shown in (Fig. 1) and tabulated in (Table 1). The purity of the preparation was checked by SDS-PAGE. It showed single band and its relative molecular weight was around 40 kDa The molecular weight of horseradish, oil palm leaves[29], date palm leaves[8], tomato[11] ranges from 30 kDa to 60 kDa. The native staining of the enzyme showed a the single brown colored band corresponding to peroxidase activity. These results are shown in Figs. 2 & 3. This is

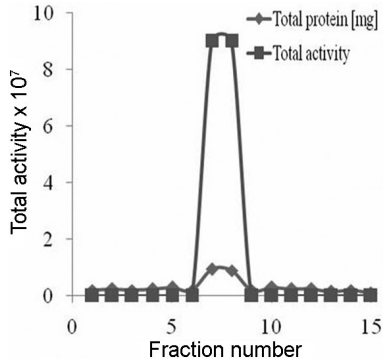

Fig. 1 — Elution profile of peroxidase from DEAE –Cellulose column. The protein was eluted at a flow rate of 0.3 mL/min.

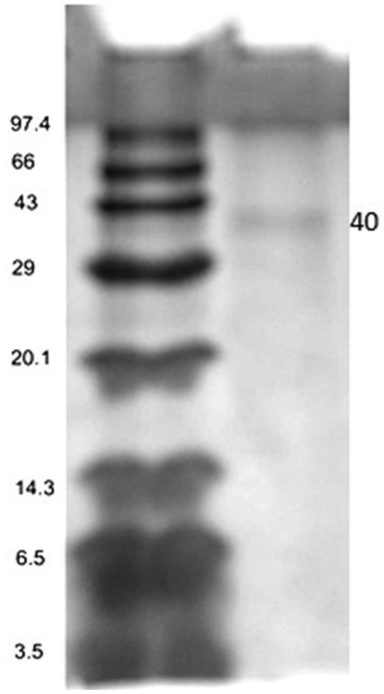

Fig. 2 — SDS-PAGE. Lane 1 contained predetermined high molecular weight markers. Lane 2 contained approximately 5 µg of the purified fraction.

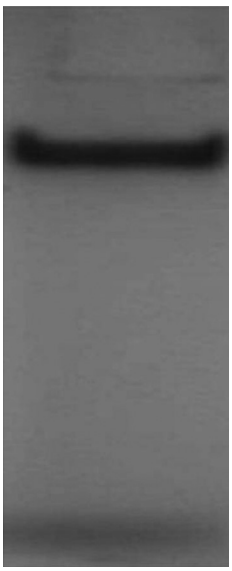

Fig. 3 — Native Gel. The lane contained 10 µg of purified fraction [brown band]. The blue band was Bromophenol blue dye.

Table 1 — Purification steps of membrane bound *Borassus flabellifer* peroxidase

Purification Steps	Total Activity × 106 [U]	Total Protein [mg]	Specific Activity × 106 [U/mg]	Purification fold	Yield%
Crude Extract	236	29.4	8.0	1	100
Ammonium sulfate precipitation	203	9.3	22	2.7	86
DEAE-Cellulose	180	1.8	100	12.5	76

uncommon for many reported plant peroxidase[30,31] which had many isoenzymes. The purified BFP exhibited higher activity around pH 5.0. It was similar to most of the reported peroxidase which showed maximum activity in the pH range between 4.5 to 6.5[7,8].

The enzyme lost its activity completely below pH 3 and above 9 due to its irreversible change in tertiary structure and detachment of haem from hemoprotein respectively. BF peroxidase was assayed at optimum pH and at different temperatures, ranging from 30 to 70°C. The enzyme exhibited maximum activity around 70°C. Buckwheat seed peroxidase showed optimum temperature around 25°C[33]. Turnip[34] and green coconut water peroxidases[7] showed temperature optima at 35 and 75°C, respectively. The BF enzyme remained active at 70°C for 120 h. The result is shown in (Fig. 4). This showed it is a heat-resistant enzyme[35]. The thermostability is due to the presence of disulphur bridges[36], carbohydrate[8]or calcium[37] in the enzyme. The LC/MS method proved the presence of calcium-binding peptides as in Calmodulin and peptides as in silk fibroin. This may be one of the possible reasons for its thermostability. Enzyme lost its complete activity when kept in boiling water bath for 10 min. The affinity of purified BFP towards Guaiacol, Benzidine, o-Dianisidine, and TMB was determined by LB plots[25] and tabulated. The typical graph is shown in (Fig. 5). The enzyme showed a higher affinity towards TMB compare to other three substrates. The K_m value of BF peroxidase with Guaiacol was 70 nM, which was very less than date palm leaves[8], green peas[37] and turnip roots[34] peroxidases. The relatively low K_m value suggested higher affinity towards Guaiacol but had the highest affinity towards TMB. The K_{eq} and free energy changes were obtained and tabulated along with kinetic parameters in (Table 2). The reaction was more feasible with TMB as its free energy change was −1007 KJ/mol. Further, the enzyme obeyed Ping-Pong kinetics that is common to plant peroxidase. The graph is shown in (Fig. 6).

The fluorescence intensity of the ANS-peroxidase intermediate complex increased as hydrogen peroxide concentration increased. The graph is shown in (Fig. 7). This showed clearly that there were conformational changes in the enzyme intermediate. These conformational changes exposed hydrophobic side chains of enzyme intermediate to the environment. These exposed hydrophobic side chains interact with ANS and enhanced fluorescence intensity.

The TMB stained stone part of the endosperm clearly substantiated that it possessed Membrane bound peroxidase. 22% of carbohydrate was present in BF peroxidase as in other plant peroxidases[38]. Jaiswal & Srivastava[39,40], Pravin *et al.* claimed that water logging stress on maize roots and drought tolerant cotton enhanced peroxidase production.

This may be correlated with drought tolerant *Borassus flabellifer L* with kinetic and thermodynamic behavior of cellobiase[4]

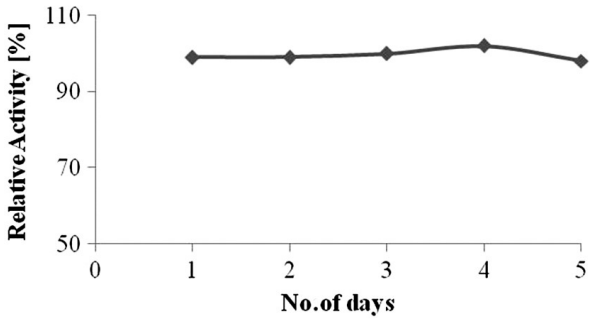

Fig. 4 — As in enzyme activity section except reaction mixture was incubated at 70°C for indicated days.

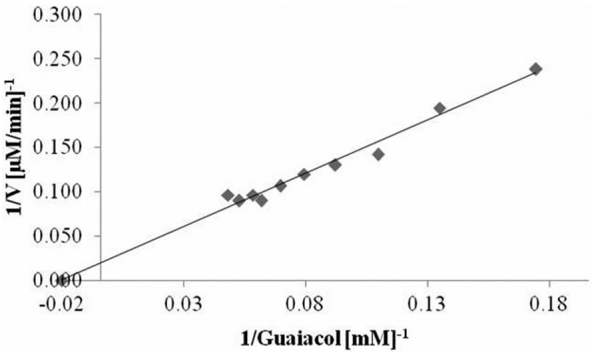

Fig. 5 — A representative graph. Double reciprocal plot with Guaiacol as substrate.

Table 2 — Kinetic and thermodynamic parameters of peroxidase with different organic substrates.

Substrate	V_{max} µM/min/mg	K_m	K_{eq} [M]	ΔG × 106 KJ/mole	K_{cat} $[min]^{-1}$	K_{cat}/K_m $[min^{-1}M^{-1}]$
Guaiacol	245833	70 nM	14 × 106	-35	1630	23 × 109
Benzidine.2Hcl	195833	70 µM	14 × 103	-35	199 × 103	28 × 108
O-Dianisidine 2Hcl	4166666	1.2mM	826	-1.3	215 × 103	18 × 107
TMB.2Hcl	95833	2.5 nM	4 × 108	-1007	182	73 × 109

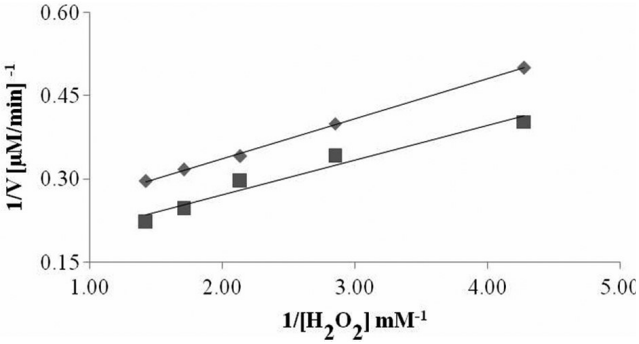

Fig. 6 — Ping-Pong kinetics at indicated hydrogen peroxide concentrations.

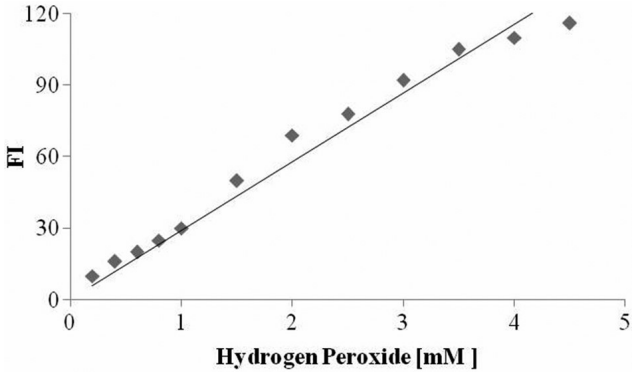

Fig. 7 — Increase in fluorescence intensity at a specified concentration of hydrogen peroxide

Table 3 — Inhibition studies with various aromatic alcohols and its IC_{50}

S. No	Inhibitor	Concentration of Guaiacol [mM]	IC_{50} [mM]
1	Cholesterol	8.8	0.25
2	o-Cresol	8.8	-
3	m-Cresol	8.8	0.26
4	p-Cresol	8.8	9
5	α- Naphthol	8.8	0.4
6	β- Naphthol	8.8	10
7	Phenol	8.8	1.0

Normally HRP acts upon pollutants like aromatic alcohols and its substituted alcohols to a polymer[12]. Since the dialysed enzyme restored its activity the aromatic alcohols are reversible inhibitor. All aromatic alcohols inhibited reversibly BFP activity except o-Cresol due to unknown reason. The results are tabulated in (Table 3). The reason for reversible inhibition may be the presence of electron donating methyl group in cresols which increased bond strength or electron density between hydrogen and oxygen in hydroxyl group and resist oxidation by BFP intermediate. The electron withdrawing methoxy group in Guaiacol reduced relatively the bond strength or electron density between hydrogen and oxygen in hydroxyl group and does not resist oxidation by BFP intermediate. Cresols are reversible inhibitors but Guaiacol is a substrate. Our future aim is to find out the type of reversible inhibition, sequencing BFP and to perform molecular biological studies which will further clarify structural and functional properties of this thermostable enzyme.

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