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Biochemical characterization of a peroxidase isolated from Caribbean plant: *Euphorbia cotinifolia*

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ABSTRACT

A Caribbean copper plant peroxidase (CCPP) is purified from the latex of *Euphorbia cotinifolia*, using anion exchange chromatography. The molecular mass and isoelectic point of the enzyme is 43.11 kDa and pH 8.1 respectively. The peroxidase is found to be sensitive towards general phenolic substrates like guaiacol, pyrogallol, α-aminopterin, phloroglucinol, o-phenelenediamine and dianisidine dihydrochloride. The substrate specificity of CCPP was distinct from that of other peroxidases, and the best substrate for CCPP was guaiacol at pH 6.0 and 50 °C. Sucrose and Ca^{2+} enhance the activity whereas the activity is significantly inhibited by NaN_3 and Na_2SO_3. The strong absorption at 650 nm reveals the presence of Cu ions as a prosthetic group. Spectroscopic studies reveal that CCPP has high α-helicity. The enzyme was found to be very stable at room temperature and retained more than 80% activity even after a period of 2 months and was stable for more than 6 months at 4 °C without any additive or preservative. Adequate amount of latex, easy purification method, broad substrate specificity, and high stability against pH, temperature, chaotrophs and organic solvents makes this enzyme a potential candidate in biotechnological and industrial applications.

1. Introduction

Peroxidases (E.C. 1.11.1.7), a ubiquitous enzymes [1] is widely distributed in plants, microbes, fungi and vertebrates. They are present in multiple ionic forms, and in vivo they are one of the key enzymes controlling the plant growth and differentiation. The multiple isoperoxidase form found within the same plant source can differ significantly with respect to molecular mass, pH and temperature optima, isoelectric point, substrate specificity, amino acid and sugar composition and heat stability. Functionally, these enzymes act as an oxidoreductase that catalyzes a reaction, in which H_2O_2 act as the acceptor and another compound act as the donor of hydrogen atoms [2].

Three dimensional structure analysis and on the basis of amino acid sequence homology, it can be broadly classified into three major classes. Plant ascorbate peroxidase, cytochrome c peroxidase and bacterial peroxidase belong to same evolutionary branch and are designated as class I, whereas secretary fungal enzymes, ligninolytic peroxidase [3], manganese (Mn) peroxidase from fungi [4] are classified as class II peroxidase and plant secretory peroxidases, vacuolar peroxidases such as horseradish peroxidases, that usually contain a ferriprotoporphyrin IX prosthetic group linked to His residue are designated as class III peroxidases [5]. Peroxidases have further been classified into anionic and cationic group according to their electrophoretic mobility. Usually class III peroxidase are assigned to have physiological roles in the primary and secondary metabolic processes like, catabolized phenolic compounds for biosynthetic and catabolic functions [6], cross linking of cell wall polysaccharide, cell elongation regulation, wound healing, abiotic stress, ethylene biosynthesis, scavenging of peroxides, oxidation of toxic compounds, defense mechanism towards pathogens, biodegradation reactions, metabolism of plant hormone, indole acetic acid (IAA) oxidation, lignification of cell wall [7], anthocyanin degradation [8], leaf senescence and for use in pulp and paper industries [9]. In food industry they are associated with development of flavor, color, texture and nutrition quality of food [10]. Commercially, peroxidases are involved in production of alkaloid, biosensor construction, air pollution damage control [11], food processing, food storage, biotransformation of organic compounds, treatment of industrial waste water containing phenols and aromatic amines [12], production of oxidants, lignin degradation in fuels, bio-bleaching process, immunoassay (ELISA kit), site directed mutagenesis [13], production of secondary antibodies for research

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; GuHCl, guanidine hydrochloride; H_2O_2, hydrogen peroxide; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine; EDTA, ethylenediaminetetraacetic acid.

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and medical diagnosis, production of dimeric alkaloids, preventing deterioration of quality of many frozen fruits, biotransformation of organic compounds, preventing deterioration of prepared foods e.g. beer, porridge, conscous etc. [14] and recently the combination of peroxidase and IAA has been introduced as a novel cancer therapy [15].

Euphorbia cotinifolia is a deciduous tropical shrub (family Euphorbiaceae) and has many medicinal applications: primarily, its leaves, which have molluscidal [16] and antiviral properties. Among antiviral properties it exhibited high antiherpetic action in its leaf/stem water methanol extracts [17]. In the course of screening of latex for various activities, the latex of plant showed peroxidase activity and encouraged us to look further in detail. In this regards, in the present study, a peroxidase has been purified from the plant is being introduced and characterized. Its wide application and stability at temperatures, pH, hydrogen peroxide, urea and GuHCl was therefore important to explore the biochemical components of the latex of this plant in view of its applications in biotechnology and food industries.

2. Materials and methods

5 mL of latex was collected from incisions on the leaves and stems of *Euphorbia cotinifolia* in 45 mL of 10 mM Tris buffer, pH 8.4 and stored at 20 °C for 72 h. Major chemicals used in this study and their sources are given here under: sepharose, BSA, GuHCl, urea, DMSO, glycerol, β-mercaptoethanol, Coomassie brilliant blue R-250, phloroglucinol, o-phenelenediamine, avidin and horseradish (Sigma Chemical Co., USA), guaiacol (Sisco Research Lab, India), pyrogallol, α-aminopterin (Spectrochem, India), dianisidine dihydrochloride (Himedia), H_2O_2 (Loba Chemie Limited), Coomassie brilliant blue G-250 (Eastman Kodak), ampholine carrier ampholites (LKB), Molecular weight marker (Banglore Genei). All the other chemicals obtained were of highest purity commercially available.

2.1. Purification

2.1.1. Step 1. Removal of gum

The latex, collected in 10 mM Tris buffer of pH 8.4 is stored at −20 °C for 72 h. The latex was thawed at room temperature and centrifuged at 10,000 × *g* for 20 min to remove any insoluble material. Clear supernatant obtained in this process is considered as crude latex for further applications.

2.1.2. Step 2. Anion exchange chromatography on DEAE sepharose

Crude latex was applied to anion exchange chromatography on a DEAE-sepharose fast flow column (5.0 cm × 5.0 cm) pre-equilibrated with Tris pH 8.4 and eluted isocratically with 0.75 M NaCl to remove loosely bound protein with no peroxidase activity. The tightly bound proteins were eluted with a linear gradient of 0.75–1.25 M and the fractions of 3 mL volume were collected at a flow rate 3 mL/min. The fractions were assayed for protein content, extent of homogeneity and peroxidase activity. The homogenous as well as active fractions were pooled, dialyzed and stored in closed vials at 4 °C for further biochemical characterization.

2.2. Peroxidase assay and protein content

Peroxidase activity was determined spectrophotometrically using H_2O_2 as substrate I and one of the hydrogen donors such as guaiacol, pyrogallol, α-aminopterin, phloroglucinol, o-phenelenediamine and dianisidine dihydrochloride as subsequent reducing substrates. The absorbance was measured at 470 nm, 420 nm, 420 nm, 420 nm, 445 nm and 420 nm respectively and substrate specificity was studied under optimal condition of pH, buffer condition, etc., as determined earlier for other plant peroxidases [18]. One unit of activity is defined as the amount of peroxidase that oxidizes 1 μmol of substrate per minute under standard conditions and its specific activity was noted as units of activity per milligram of protein. Concentration of protein was also determined spectrophotometrically at 280 nm as well as Bradford assay [19] using BSA as a standard.

2.3. Electrophoresis

SDS–PAGE was used to assess the homogeneity of enzymes preparation as well as estimation of molecular mass. Purified enzyme (25 μg) from the homogenous pool fractions (30–45) were mixed with sample loading buffer and loaded on 12.5% SDS–PAGE under both non-reducing and reducing conditions [20]. The gels were stained with 0.2% Coomassie brilliant blue R-250. Molecular mass standard used were phosphorylase b (93.7 kDa), BSA (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa), and chicken egg white lysozyme (14.3 kDa).

2.4. Zymogram

25 μg of the purified enzyme was mixed with sample loading buffer and analyzed on 12.5% SDS–PAGE without prior boiling [21]. Gel was run at 200 V for 1 h and soaked in 2.5% Triton X-100 for displacement of SDS. Gel was washed with water to remove Triton X-100 and incubated for 5 min in 10 mM Tris buffer, pH 8.4 containing 20% H_2O_2 (v/v). For color development 1 mL of 0.2 mM pyrogallol was added to the above mixture followed by termination of the reaction with 1 mL of 1% EDTA.

2.5. Isoelectric focusing

The isoelectric point (p*I*) of purified peroxidase was determined by isoelectric focusing on polyacrylamide disk as described [22]. An electrophoretic run was carried out with ampholine carrier ampholytes pH 7.0–9.0.

2.6. Mass spectrometry

The mass spectra were recorded on a MICRO-MASS QUATTRO II mass spectrometer (Micromass, Altricem, UK) equipped with electrospray ionization ion [23]. Protein sample (20 μM) was precipitated with 5% TCA, and the resulting precipitate was washed with chilled acetone. The pellet obtained was dissolved in ultra pure water and passed through ZIP-TIP C-18 resin before the measurement. The resulting sample was injected into the ion source using Harvard Apparatus model 11 Syringe pump. The source and sample was operated at 80 °C and at 30 °C respectively. The electrospray capillary was set at 3.5 kV and the cone voltage at 20 V. The intensity and signal stability for ESI-MS were significantly increased by adding a small amount of methanol (3%) to the aqueous solution of peroxidase. The scanned range of mass spectrometer was from *m*/*z* 500 to 2200 in 6 s. The charge states and the deconvolution of the spectra was carried out using MassLynx and MaxEnt software respectively [24].

2.7. Antigenic properties

40 μg of the purified CCPP in 0.05 M Tris buffer, pH 8.4 was emulsified with Freund's complete adjuvant and injected subcutaneously at multiple sites as described [25]. Preimmune serum was collected prior to the immunization of the rabbit and used as a control for immunoassays. The presence of antibodies was confirmed by Ouchterlony's double immunodiffusion method, described by Ouchterlony and Nilsson [26].

2.8. pH and temperature optima

The reaction mixture, composed of 20 μg of peroxidase in an appropriate buffer of desired pH and 500 μl of substrate (0.2 mM guaiacol) solution at the same pH, was equilibrated for 1 min at room temperature. The enzyme was assayed using 20% H_2O_2 (v/v) as substrate I and one of the hydrogen donors such as guaiacol as reducing substrate II. The assay was carried out at 37 °C using buffers such as KCl–HCl (pH 0.5–1.5), glycine-HCl (pH 2.0–3.5), sodium acetate (pH 4.0–5.5), sodium phosphate (pH 6.0–7.5), Tris (pH 8.0–10.0) and sodium carbonate (pH 10.5–11.0), all at molarities of 50 mM. Assay was performed at every pH without addition of enzyme and used as control.

For temperature optimum measurement, 20 μg of the purified peroxidase was incubated at 30 min in the temperature range of 20–80 °C and assayed with 20% H_2O_2 (v/v) and 0.2 mM guaiacol for 1 min. Activity was measured as described above.

2.9. Peroxidase activity as a function of hydrogen peroxide concentration

The effect of initial hydrogen peroxide concentration on the peroxidase activity of purified peroxidase was investigated at pH 8.4 and 25 °C with different concentration of hydrogen peroxide. The absorbance was measured at 470 nm with 0.2 mM guaiacol used as reducing substrate.

2.10. Effect of additives on peroxidase

20 μg of the purified peroxidase was incubated with different additives such as EDTA (1 mM), sucrose (10%, w/v), sodium azide (1 mM) and DMSO (2%, v/v) for 30 min and assayed as described above.

2.11. Effect of salts on peroxidase

To study the effect of salts, the enzyme was incubated with increasing concentration of salts in the range of 10–100 mM for 30 min and assayed as mentioned above. The salts used for the present study were NaCl, $CaCl_2$, $MgCl_2$, and Na_2SO_3.

2.12. Spectroscopic studies: absorbance, fluorescence, and circular dichroism

Absorbance measurements were carried out on a Beckman DU-640B spectrophotometer. Absorbance spectra were recorded between 300 and 900 nm. Protein concentration for all absorbance measurements was 0.05 mg/mL. To assess the presence of copper ions, the enzyme was reduced with ascorbic acid followed