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Isolation and partial characterization of phenol oxidases from *Mangifera indica* L. sap (latex)

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ABSTRACT

Mango sap (latex), a by-product in mango industry, was separated into upper non-aqueous phase and lower aqueous phase. Aqueous phase contains very low protein (4.3 mg/ml) but contains high specific activities for peroxidase and polyphenol oxidase. The aqueous phase of sap was subjected to ion-exchange chromatography on DEAE-Sephacel. The bound protein was separated into three enzyme peaks: peak I showed peroxidase activity, peak II showed polyphenol oxidase activity and peak III showed activities against substrates of peroxidase as well as polyphenol oxidase. On native PAGE and SDS-PAGE, each peak showed a single band. Based on the substrate specificity and inhibitor studies peak III was identified as laccase. Although they showed variations in their mobility on native PAGE, these enzymes showed similar molecular weight of 100,000 ± 5000. These enzymes exhibited maximum activity at pH 6 however, polyphenol oxidase showed good activity even in basic pH. Peroxidase and polyphenol oxidase showed stability up to 70 °C while laccase was found to be stable up to 60 °C. Syringaldazine was the best substrate for laccase while catechol was the best for polyphenol oxidase. Thus, mango sap a by-product in mango industry is a good source of these phenol oxidases.

1. Introduction

Polyphenol oxidase is a generic term used for the group of enzymes that catalyses the oxidation of different phenolic compounds. Based on substrate specificity these polyphenol oxidases have been named as tyrosinase or monophenol monooxidase or cresolase (EC 1.14.18.1), catechol oxidase or diphenol oxidase (EC 1.10.3.1), laccases or *p*-diphenol oxygen oxidoreductase (EC 1.10.3.2) and these enzymes are present in various fungi and plant tissues [1,2]. The presence of laccase in the exudates of Japanese lacquer tree, *Rhus vernicifera*, was discovered as early as 1883 [2,3]. Peroxidase (EC 1.11.1.7; donor: hydrogen-peroxide oxidoreductase) is an another oxidative enzyme present in different tissues of plants and animals. Peroxidase oxidises a variety of phenolic and amines in the presence of hydrogen peroxide while polyphenol oxidases oxidizes phenolic compounds in the presence of molecular oxygen. There is a considerable overlap in their substrate specificities of these three phenol oxidases. However, these enzymes may be differentiated by their specificities to certain substrates and inhibitors [4,5]. Some of the attributed functions of these three groups of enzymes are similar. These oxidases have been implicated in plant senescence, fruit ripening and defensive role against insects [6–8] and are also involved in protein cross-linking [9]. Due to wide substrate specificity, laccase and peroxidase are used for a wide variety of applications in food processing and biological applications, analytical chemistry, immunochemical studies, water purification, biotransformation of various valuable chemicals and biosensors [2,10–12].

Mango is one of the major tropical fruits and India produces about 40% of the world production. Mango fruit has duct system, which continues into the stalk but ends before the abscission zone. These fruit ducts contain a viscous liquid referred to as mango sap (latex) [13,14]. During harvesting of mango fruits, sap initially spurts and then oozes out onto the surface of the fruits and causes sap-injury. Sap-injury is characterised as darkening or browning of the peel due to contact with the sap. Sap-injury not only reduces consumer acceptance of the fruit, but also lowers shelf-life of the fruit as the injured regions of the peel tend to be more susceptible to fungal or bacterial infections. De-sapping of the mangoes is one of the methods practiced to control sap-injury and the sap thus obtained is currently being wasted.

Sap can be separated into two phases, aqueous and non-aqueous. Earlier, we have reported that non-aqueous phase has high specific activities of polyphenol oxidase (catechol oxidase) and peroxidase [15]. In the present study we report the purification of sap enzymes and their properties. The presence of PPO/laccase has been reported by few workers [15–17] while presence of peroxidase in sap has been reported only by Saby John et al. [15]. In the

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present study we have purified these enzymes in a single step by ion-exchange chromatography on DEAE-Sephacel and studied their biochemical properties.

2. Materials and methods

2.1. Plant materials and chemicals

Raspuri mango variety grown in CFTRI campus, Mysore, Karnataka, India, was used in the present study. Sephadex G-200 was obtained from Pharmacia Biochemicals, Uppsala, Sweden. DEAE-Sephacel, SDS, β-mercaptoethanol, acrylamide, *N*,*N*′ bisacrylamide, Tris, protein markers, *o*-dianisidine, syringaldazine, *p*-phenylenediamine, guaiacol, catechol were obtained from Sigma, USA. All other chemicals used in this study were of analytical reagent grade chemicals. Glass double distilled water was used throughout this research.

2.2. Separation of aqueous phase from mango sap (latex)

As described earlier [18], mango fruits were harvested with pedicel intact and subsequently, the pedicels were detached from the fruit at the abscission zone and the sap was collected in to the glass tube for about 1 min. The sap thus collected was separated into aqueous and non-aqueous phases by centrifugation at 3000 × *g* at room temperature. Aqueous phase thus collected was stored at 4 °C until further use.

2.3. Protein estimation

Protein was estimated by the dye binding method described by Bradford [19] using bovine serum albumin as a standard.

2.4. DEAE-Sephacel ion-exchange chromatography

Mango sap proteins were diluted with equal volume of 50 mM Tris–HCl buffer, pH 8.0 and loaded onto DEAE-Sephacel column (3.0 cm × 16.5 cm) which was pre-equilibrated with the same buffer. The bound protein was eluted using 0.025 M, 0.05 M, 0.075 M and 0.1 M sodium chloride in 0.05 M Tris–HCl buffer, pH 8.0, in a step-wise manner. The flow rate was maintained at 10 ml/h and 2 ml fractions were collected, monitored for protein by determining the absorbance at 280 nm and assayed for PPO and POD in the individual peaks. The enzyme fractions, 85–100 (peak I); 120–130 (peak II); and 150–165 (peak III) were pooled separately and used for further studies.

2.5. Electrophoresis

2.5.1. Polyacrylamide gel electrophoresis of purified fractions (PAGE)

The fractions obtained from the chromatography were subjected to electrophoresis on 7.5% native gel and the protein loaded in each lane was 10 μg. The gel composition, running buffer composition, staining and destaining procedures were followed according to the method described by Laemmli [20] without SDS and β-mercaptoethanol. The protein and enzyme staining was done as per the procedures described by Saby John et al. [15].

2.5.2. SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed on 10% gel in the presence of 0.1% sodium dodecyl sulphate according to the method of Laemmli [20]. A mixture of molecular weight markers containing carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (66,000), rabbit muscle phosphorylase b (97,400), *Escherichia coli* β-galactosidase (116,000) and rabbit muscle myosin (205,000), as well as the enzyme samples prepared in 1% SDS and 5% mercaptoethanol, were boiled for 5 min. The pH of the running buffer was 8.5 and a constant voltage of 50 V was employed. An amount of 10 μg of protein was loaded in each lane. Following the run, the proteins were stained with 0.25% Coomassie brilliant blue for 5 h and destained with 7% acetic acid, 10% methanol and 83% water. The molecular weights of the enzymes were calculated from the standard graph obtained using R_f values of standards vs. their molecular weight.

2.6. Determination of molecular weight of the purified enzymes using gel filtration chromatography on Sephadex G-200

The molecular weights of the purified enzymes were determined using Sephadex G-200 gel filtration column which was calibrated using protein standards. The void volume was determined using blue dextran.

2.7. Peroxidase assays

The peroxidase assay was carried out using 0.25% *o*-dianisidine as substrate as described by Saby John et al. [15]. One unit of activity was defined as that amount of enzyme which produced an increase in absorbance of 1/min at 460 nm. For substrate specificity studies of peak I enzyme increase in absorbance was monitored at 460 nm for diaminobenzidine and *p*-phenylenedaimine; 470 nm for guaiacol, 650 nm for tetramethylbenzidine. All these substrate were solubilized in water except guaiacol which was solubilized in ethanol.

2.8. Effect of inhibitors on peak I (POD)

The effect of varying concentrations of inhibitors on the activity of enzyme in peak I was determined. The compounds tested were sodium azide, potassium cyanide, hydrazine, dithiothrietol, ethylenediaminetetracetic acid and cetyltrimethylammonium bromide. With the exception of hydrazine which is alcohol-soluble, all the compounds were solubilized in water. Appropriately diluted enzyme (100 μl; 50–60 U) was incubated with 100 μl of varying concentrations of inhibitor in 600 μl of 50 mM sodium acetate buffer, pH 6.0 at room temperature for 5 min. Hydrogen peroxide (1%; 100 μl) and *o*-dianisidine (0.25%; 100 μl) were then added to the reaction mixture and the absorbance was recorded at 460 nm for 3 min. The enzyme unit was defined as mentioned earlier.

2.9. Polyphenol oxidase assays

The polyphenol oxidase assays were carried out using 0.5 M catechol substrate as described by Saby John et al. [15]. One unit of activity was defined as that amount of enzyme which produced an increase in absorbance of 1/min at 420 nm. For substrate studies of peaks II and III enzymes, increase in absorbance was monitored at 420 nm for catechol, 4-methyl catechol, *p*-quinol, and *o*-dianisidine; 460 nm for β-napthol; 525 nm for syringaldazine; 410 nm for *p*-phenylene diamine; 280 nm for tyrosine. All these substrate were solubilized in water except syringalazine and β-napthol which were solubilized in ethanol.

2.10. Effect of inhibitors on peaks II and III (polyphenol oxidases)

The effect of varying concentrations of inhibitors on the activities of enzymes in peaks II and III was determined. The compounds tested were ascorbic acid, cinnamic acid, citric acid, sodium chloride, sodium thiosulphate, cysteine, cetyltrimethylammonium bromide and polyvinylpyrrolidone. With the exception of cinnamic