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Purification and Characterization of Ionically Unbound Polyphenol Oxidase from *Cinnamomum tamala*

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Abstract

Polyphenol oxidase (EC. 1.10.3.1 PPO), an ionically unbound and thermostable enzyme, was extracted from the leaves of *Cinnamomum tamala* having aroma flavor used to add in Indian food and vegetables as a part of spices to enhance the taste of recipes. The enzyme was purified 2.63-fold with a total yield of 9.5% by ammonium sulfate precipitation followed by Sephadex G-100 gel filtration chromatography. The purified enzyme exhibited a clear single band on sodium dodecyl sulfate (SDS) PAGE. It was found to be monomeric protein with molecular mass of about 25 kD. The zymographic study using crude extract as enzyme source showed a very clear band around 25 kD. The enzyme was optimally active at pH 7.0 and 50^{o}C. It was active in a wide range of pH (3-9) and temperature (30–90^{o}C). From the thermal inactivation studies in the range 60-80^{o}C, the half-life ($t_{1/2}$) values of the enzyme varied from 19 to 72 min. The inactivation energy (Ea) value of PPO was estimated to be 94.5 kJ mol^{-1}; it showed higher specificity with substrate catechol (K_m=6.8mM). Among the metal ions and reagents tested, Cu^{2+} (indicating its role as cofactors), Fe^{2+}, Hg^{2+}, protocatechuic acid, and ferrulic acid enhanced the enzyme activity, while K^{+}, Mg^{2+}, Co^{2+}, kojic acid, L-ascorbic acid, ethylenediamine tetraacetic acid (EDTA), urea, sodium azide, β-mercaptoethanol, and L-cysteine inhibited its activity.

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1. Introduction

Polyphenol oxidase (PPO; E.C. 1.10.3.1) enzyme is ubiquitous in nature. PPOs belong to a group of copper-containing metalloproteins, and are members of oxidoreductases that catalyze the oxidation of a wide range of phenolic compounds by utilizing molecular oxygen [1, 2]. PPO has several physiological and biochemical roles in plants and other organisms. It is a very important enzyme in the food industry due to its involvement in the enzymatic browning of edible plants. It catalyzes the oxidation of o-diphenol to o-quinone (diphenolase, catecholase activity) in the presence of oxygen, and the final polymerized product, is undesirable brown, red, or black pigments [3]. Enzymatic browning impairs the sensory properties and marketability of the product and also lowers its nutritional value [4]. Several industries like petroleum refining, coal conversion, steel and iron manufacture, resins and plastics, wood preservation, dyes and chemicals, etc. produce wastewater containing phenol and its derivatives.

Removal of phenols and its derivatives from wastewater by using enzymes is attracting much attention, as enzymes have many potential advantages over conventional biological treatment. Although peroxidase enzymes (PODs) have the potential to treat a large variety of phenolic compounds, they are being replaced by PPOs. In recent years, PPOs have garnered significant interest because of their high capacity for oxidizing aromaticcompounds. This feature makes the use of PPOs very suitable for some biotechnological applications in the food industry [2, 5], pulp and paper industry [5], textile industry [6], medicine [7, 8, 9, 10], and environmental technology [11]. The enzyme of interest can be used for the development of biosensors to detect phenolic compounds for various purposes; also the ability of PPOs to act on phenolic compounds can be used for the degradation of phenols in industrial waste waters [11].

The leaves of *Cinnamomum tamala*are used extensively in northern India as a spice-Tejpat, though hydro-distilled essential oils of *C. tamala,* screened for their antifungal activity against *Trichophyton mentagrophytes* and *Microsporum audounil* causing ring worm diseases in animals and humans, exhibited fungicidal or fungistatic toxicity and were more effective than the synthetic antifungal agents, clotrimazole, griseofulvin or nystatin. Plants of *C. tamala* are used in many ayurvedic preparations, e.g. sudarshan choorna and chanderprabhavati [41].

In view of the applications just described and their significance in the food & medicine industries, the present investigation deals with the purification and characterization of PPO from the leaves of *Cinnamomum tamala*.

2. Materials and Methods

2.1. Experimental plant materials

The mature leaves of *Cinnamomum tamala* were collected from a local cultivated area. The leaves were washed properly, cut into small pieces, crushed, and used for enzymes extraction.

2.2. Reagents

Sephadex G-100 was obtained from GE Healthcare, UK. Brilliant blue R was from Sigma Chemical Co. (St. Louis, MO). NaH_2PO_4, Na_2HPO_4, $FeSO_4.7H_2O$, $CuSO_4.H_2O$, $MgSO4.7H_2O$, $(NH_4)_2SO_4$, kojic acid, protocatechuic acid, ferulic acid, amino acids kit, urea, catechol, and all other chemicals used were of analytical grade, purchased from Merck, India.

2.3. Preparation of crude enzyme

Crude extract of PPO enzyme was obtained from 100 gm of *Cinnamomum tamala* leaves, and crushed into thin paste using a pre-chilled pestle and mortar. To this, 20mL of 100mM chilled phosphate buffer (pH 7) containing 0.1% polyvinylpyrrolidone (PVP) and 0.01% triton X-100 were added. Then the obtained mixture was pressed to separate the filtrate through cheesecloth. The filtrate was centrifuged at 11000 rpm and 4^{o}C for 15 min. The supernatant was collected and used as source of crude enzyme, and stored at 4^{o}C for further study.

2.4. Enzyme and protein assay

Polyphenol oxidase assay [12, 13] was carried out in an ultraviolet/visible (UV/VIS) spectrophotometer (Systronics, India) by measuring the change in the absorbance (at 410nm at 50^{o}C) due to oxidation of catechol (200mM) in the presence of molecular oxygen in 1 mL reaction mixture containing 20 µL of enzyme, 20 µL of 200 mM catechol, and 960 µL of phosphate buffer (pH 7).

One unit of enzyme activity is defined as the amount of enzyme producing a 0.001 absorbance change per minute under the standard assay conditions [14]. Protein concentration at each step was determined by the method of Lowry *et al*. [15] using bovine serum albumin as standard. Except for the chromatographic experiments, the enzyme activity values presented are the mean values of assays conducted in triplicate.

2.5. Purification of Polyphenol Oxidase

2.5.1. Ammonium sulfate precipitation

The crude extract of PPO was precipitated by slowly adding solid ammonium sulfate [13, 16, 17] for 20% saturation at 4^{o}C. During the addition of ammonium sulfate, the extract was constantly stirred to avoid the local precipitation of protein. The precipitated pellet was collected by centrifugation at 11,000 rpm for 20 min at 4^{o}C. The supernatant was used for further fractionation at 40, 60, and 80% ammonium sulfate saturations. Protein pellets obtained at each step were dissolved in 1.0mL phosphate buffer (100mM, pH 7), dialyzed against the same buffer, and analyzed for polyphenol oxidase activity. The fraction showing polyphenol oxidase activity was used as partially purified enzyme.

2.5.2. Sephadex G-100 gel filtration chromate-graphy

The partially purified enzyme (now used for further purification) was loaded onto a Sephadex G-100 column (50cm×1.5 cm) pre-equilibrated with 100 mM phosphate buffer, pH 7.0. The enzyme was eluted with the same buffer at a flow rate of 1.0 mL/3 min. The single activity peak fractions were collected. Furthermore, the enzyme was concentrated and dialyzed against the same buffer. Finally, the dialyzed enzyme solution was then used as almost purified enzyme preparation.

2.5.3. Polyacrylamide gel electrophoresis

With the help of 10% SDS-PAGE under denaturing conditions, the purity of the enzyme was checked according to the method of Laemmli [18].

The gel was stained with brilliant blue R. The RECO 004, BLUE medium-range protein marker mixture (Real Biotech Corporation, Taiwan) was used in SDS-PAGE, and ranged from 15 to 100 kD. For the zymographic pattern, PPO activity in the non-denaturing polyacrylamide gel was assayed by incubating the gel in the phosphate buffer (pH, 7.0) containing 200mM catechol at 50^{o}C for 15 min.

2.5.4. Determination of optimum pH

The effect of pH on PPO activity over the pH range of 3-9 was measured in 100mM buffer. Sodium acetate (pH 3-5), Sodium phosphate (pH 6-7) and Tris-HCl (pH 8-9) were used under standard assay conditions for determining the optimum pH. The relative PPO activity (%) was calculated after assaying the enzyme at different pH buffers. Three replicates have been performed for this assay.

2.5.5. Determination of optimum temperature and thermal stability

The effect of temperature on unbound PPO was measured in the range of 30-90^{o}C. The heat inactivation studies of PPO were carried out in the temperature range of 60–80^{o}C for various time periods (20, 40, 60, 80, 100 and 120 min). The enzyme was taken in test tubes and placed into a water bath, which was pre-set at the appropriate temperature. Aliquots of the enzyme solution were withdrawn at different time intervals, rapidly cooled down in an ice bath, and assayed for the remaining activity (A) under the assay conditions described above followed by three replicates, performed for this assay. The enzyme sample incubated at 50^{o}C was used as the blank (A_o). The residual activity of the enzyme was estimated under standard assay conditions in each case, and expressed as relative activity (%) with reference to the activity observed in case of the enzyme incubated at 50^{o}C. First-order inactivation constant (k) was calculated from the slope of natural logarithm (ln) of A/A_o versus absolute time. The half-life of the enzyme ($t_{1/2}$) was calculated by using the equation $t_{1/2}$=0.639/k. Decimal reduction time (D value) was estimated from the relationship between k (heat inactivation constant) and D value: D=ln (10/k). The activation energy (Ea) for denaturation of the enzyme was determined by an Arrhenius plot of log reaction rate constant (ln k) versus the reciprocal of the absolute temperature (T^{-1}).

2.5.6. Determination of the effect of various metal ions and reagents

The effect of several metal ions were tested like Co^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+}, Hg^{2+} Ca^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, and K^{+} (0.5mM), amino acids like L-cysteine, L-asparagine, L-ascorbic acid, L-proline, and L-glutamine (0.5mM) while some phenolic compounds were also tested like hydroxycinemic acid derivative ferulic acid (0.5 µM), hydroxybenzoic acid derivative like protocatechuic acid (0.5µM) and phytohormones like indole 3-acetic acid and gibberellic acid followed by α-naphthalene acetic acid and Vit. B were incubated with fixed enzyme concentration at room temperature. Some potent inhibitors were also tested like kojic acid, sodium azide, L-cysteine, β-mercaptoethanol, ethylenediamine tetraacetic acid (EDTA), urea, and NaCl (0.5mM) were incubated with fixed enzyme concentration at room temperature. Enzymatic activities were expressed as relative values (%) with reference to the activity of the enzyme without any reagent. All the experiment performed thrice at a time.

Thus the effects of various inhibitors on enzyme activity were examined by incubating a mixture consisting of 20 µL of the enzyme solution and 50 µL of the inhibitors (0.5mM) for 5 min at room temperature. Then enzyme activity was assayed under standard conditions. Enzymatic activities were expressed as relative values (percent) with reference to the activity of the enzyme without any of these respective reagents.

2.5.7. Determination of kinetic constants

The apparent K_m and V_{max} were determined from a Lineweaver-Burk [19] plot by following the optimum pH and temperature conditions. The substrate used was catechol with concentration 1.0-12mM followed by three replicates, performed for this assay.

3. Results and Discussion

3.1. Enzyme purification

The enzyme purification steps are summarized in Table 1. PPO was purified 2.63-fold with 9.7% yield from the crude enzyme extract. First, the proteins in the crude extract were precipitated with ammonium sulfate. The fractionation with ammonium sulfate yielded four fractions (20–40%, fraction A; 40–60%, fraction B; 60–80%, fraction C; and 80–100%, fraction D), and all the fractions were analyzed, consequently, for the PPO activity. Out of these four fractions, only fraction C had PPO activity, while the other fractions did not show any detectable PPO activity. After this step, the enzyme was purified 1.35-fold with 41.7% recovery. Then the fraction C containing partially purified enzyme was loaded onto Sephadex G-100 column. The enzyme was eluted with 100mM phosphate buffer, pH 7.0.

Table 1. Steps for purification of polyphenol oxidase from *Cinnamomum tamala* leaves

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery
Crude extract	31599	3590	8.8	1	100
$(NH_4)_2SO_4$ (60-80%)	13099	1095	11.9	1.35	41.7
Sephadex G-100	2997	129	23.23	2.63	9.7

The elution profile is shown in Figure 1. After analysis, the enzyme was found to be in the fraction numbers 11 to 23 (Figure 1). All the fractions having PPO activity were pooled together, concentrated, and loaded on 10% SDS-PAGE to check their purity. In 10% SDS-PAGE, only one protein band appeared at around 25 kD (Figure 2a). The purification steps basically employed here are relatively simple and used routinely in a general protein laboratory. The enzyme has been purified to homogeneity in just two steps: ammonium sulfate precipitation and gel filtration chromatography. In most of the reports, PPO has been purified in several steps [20, 21, 22] though there are a few reports also describing the purification of PPO in just two steps [13, 16, 17]. The single band of the enzyme around 25 kD in both SDS-PAGE and zymographic pattern shows monomeric protein nature. The procedure was the same as for SDS-PAGE except that SDS and β-mercaptoethanol were not added.

There are earlier reports about PPO from butter lettuce (*Lactuca sativa var. capitata* L.) [20], edible yam (*Dioscorea opposita*) [22], wheat bran [23], muscat bailey A [24], lychee fruit [25] and loquat fruit [26], having a molecular mass of around 37 kD, 44 kD, 40 kD, 60 kD, 75.6 kD, and 58 kD, respectively. Most of these PPOs have been reported to be a monomeric protein, which is similar to our report of PPO from *Cinnamomum tamala* leaves. When the zymographic study of PPO was performed using the crude extract of PPO as a source of enzyme, one activity band was visible, the band appeared almost at the same position as in SDS-PAGE (Figure 2b). The enzyme having a molecular mass of around 25 kD was attempted for its purification.

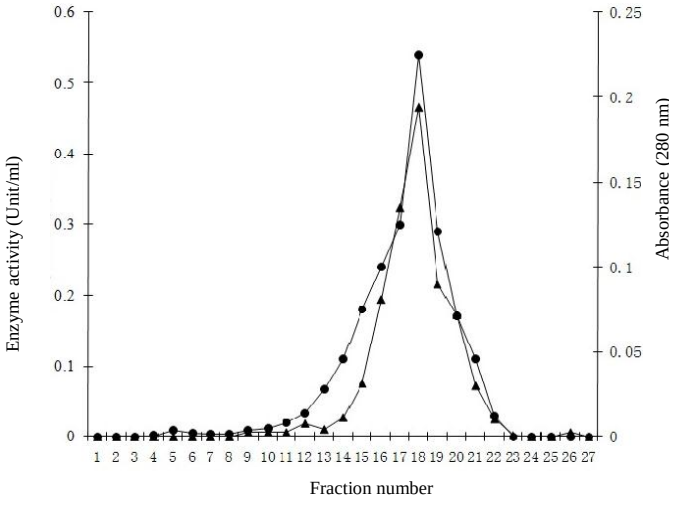

Figure 1. Elution profile of polyphenol oxidase activity (♦) and absorbance at 280nm (▲) in a Sephadex G-100 column equilibrated with 100 mM sodium phosphate buffer (pH 7.0); 60-80% ammonium sulfate-precipitated fraction containing partially purified enzyme was loaded onto the column. Samples of 1.0mL were collected at a flow rate of 20mL/hr.

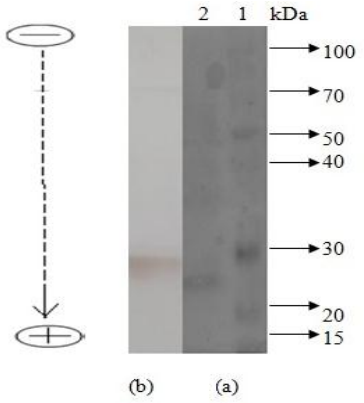

Figure 2. SDS-PAGE, native PAGE, and activity staining of *Cinnamomum tamala* leaves polyphenol oxidase. (a) 10% SDS-PAGE: lane 1, molecular mass standards; lane 2, purified enzyme (b) Zymographic pattern of crude PPO

3.2. Optimum pH

When assaying the enzymatic activity of PPO from *Cinnamomum tamala* leaves in the pH range of 3-9 using catechol as substrate, the maximum activity was observed at pH 7 (Figure 3). By enhancing the pH from acidic to neutral, the activity of enzyme increased and started to decline after pH 7. The same optimum pH for PPO using the same substrate (catechol) has also been reported by [4,27,28] in cucumber, pear (*Pyrus communis*), Anamur banana (*Musa cavendishii*), and Jerusalem artichoke (*Helianthus tuberosus*), respectively.

There is also a report from [17] showing the optimum pH of PPO as 8.2, 7.2, 5.6, 5.6, 5.0, and 4.8, for pyragallol, 4-methyl catechol, D-tyrosine, caffeic acid, p-cresol, and L-DOPA, respectively.The optimum pH for PPO ranges from acidic to alkaline region (generally from pH 4 to 8), which is dependent on the enzyme sources and substrates. Even in the case of fruits, the optimum pH of PPO may vary depending on the origin of the material, the extraction method, the maturity of the fruit, and the substrate [17]. The optimum activity of PPO from butter lettuce (*Lactuca sativa* var. capitata L.) [20], Barbados cherry (*Malpighia glabra*) [21], wheat bran [23], muscat baily A grape juice [24], loquat fruit [29], Henry chestnuts (*Castenea henryi*) [30], taro (*Colocasia antiquorum*) [12], artichoke (*Cynara scolymus*) [21], and bayberry (*Myrica rubra* Sieb. et Zucc.) with catechol as substrate [31] has been reported at pH 5.5, 6.3, 5.5–6, 4.5, 5, 6, 6.5, 5–7, and 7.2, respectively. The optimum pH for maximum PPO activity is also subject to changes when assayed in the presence of the modulator SDS [32]. PPO is, generally, more active in a pH range from 4 to 8 catalytically, and the enzyme activity drastically drops at a more acid environment. Modes of pH-induced inhibition are attributed to protonation of catalytic groups essential for catalysis,

conformational changes in the active site of the enzyme, irreversible denaturation of the protein, and/or reduction in the stability of the substrate as a function of pH [32].

3.3. Optimum temperature and thermal stability

The optimum temperature of PPO from *Cinnamomum tamala* leaves is 50°C (Figure 4). Above 50°C, the enzyme activity decreases gradually. Optimum temperature of PPO varies for different plant sources, as well as the substrate used in the assay. The optimum temperature of this enzyme is quite high and comparable with that (50°C) reported for the PPOs from cucumber [27] and strawberry [33] against the catechol and pyrocatechol substrates, respectively. Generally, PPOs from other sources have been reported to have optimum temperature below 50°C. Thermal inactivation of *Cinnamomum tamala* leaves PPO was studied at 60, 70, and 80°C for different time periods (10, 20, 40, 60, 80, 100 and120 min). From Figure 5, it is clear that the rate of heat inactivation (k) gets greater with increasing of both temperature and time, and follows first-order kinetics, shown in Table 2. The half-life ($t_{1/2}$) values can also be used to characterize the enzyme stability. The $t_{1/2}$ value for this enzyme decreases with increasing the temperature from 60 to 80°C (Table 2). At 70°C, the $t_{1/2}$ value of this enzyme is 27 min, which is higher than for any other PPO reported so far under the same experimental conditions. Generally, PPO is considered to be a less thermostable enzyme. Different thermo-stabilities have been reported in different cultivars and multiple forms of PPO from the same source, and also between fruit tissue homogenates and their respective juices [34].

D value (decimal reduction time) denotes the time in which, at a given temperature and pressure, 90% of the initial activity of enzyme is reduced. D values ranged from 192 min at 60°C to 50 min at 80°C (Table 2). D values for PPO from taro (*Colocasia antiquorum)* have been reported to be 124, 47.5, and 20.4 min at 60, 70, and 80°C, respectively [12], reported D values of 30.3-56.6 min at 73°C and 8.1–14.4 min at 78°C for various apple cultivars.In addition, the Arrhenius Ea value for thermal inactivation of *Cinnamomum tamala* leaves' PPO was found to be 94.5 kJ mol^{-1} from the slope of the ln (k-1)/T plot, which indicates a high resistance of this enzyme to increase in temperature. The Ea value of 94.5 kJ mol^{-1} compares well with the 91.3kJ mol-1 of banana leaf PPO [13].

3.4. Enzyme kinetics

The apparent K_m and V_{max} were determined from Lineweaver–Burk [19] plots by following the assay conditions at optimum pH and temperature. The K_m and V_{max} values of PPO for the substrate catechol were 6.8 mM, 81000 IU/min, respectively. The K_m value is a measure of the affinity of the enzyme for the substrate. A smaller K_m value means higher affinity of the enzyme with the substrate, and vice versa. The higher Km value of *Cinnamomum tamala* leaves PPO indicates that the affinity of this enzyme is low. Affinity of plant PPO for phenolic substrate is relatively low. The reported Km value for persimmon fruit PPO, marula fruit, banana PPO, banana leaf PPO, and quince is 12.4mM [35], 4.99mM [36], 18mM [37], 8mM [13], and 4.54mM [38], respectively. The K_m value obtained in this study is within the range of the reported values**.**

Table 2. Inactivation parameter of *Cinnamomum tamala* leaves polyphenol oxidase

Temperature (°C)	K (min^{-1})	$t_{1/2}$ (min)	D (min)	Determination Coefficient (R^2)
60	0.0124	57	192	0.9902
70	0.0267	27	80	0.9829
80	0.0350	19	50	0.9838

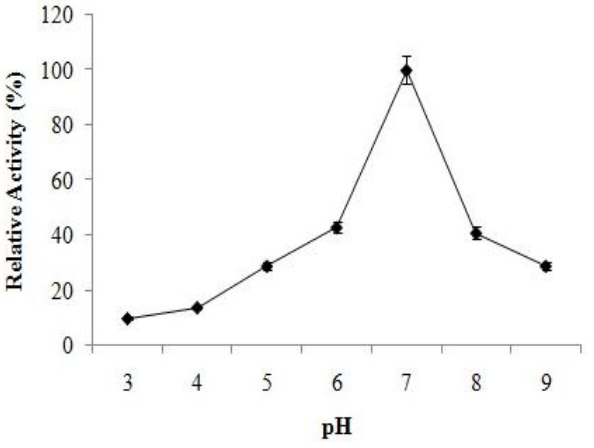

Figure 3. pH effect on *Cinnamomum tamala* leaves polyphenol oxidase

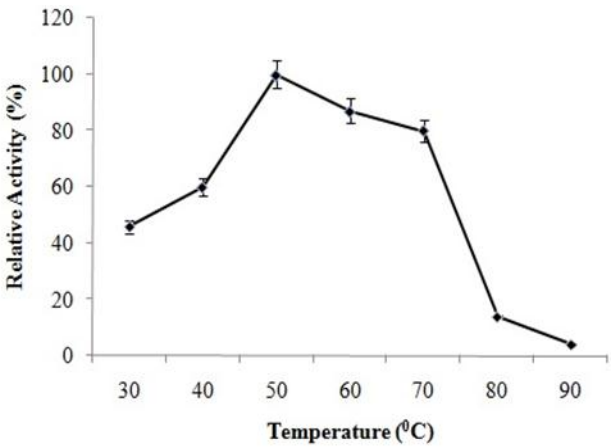

Figure 4. Temperature effect on *Cinnamomum tamala* leaves polyphenol oxidase

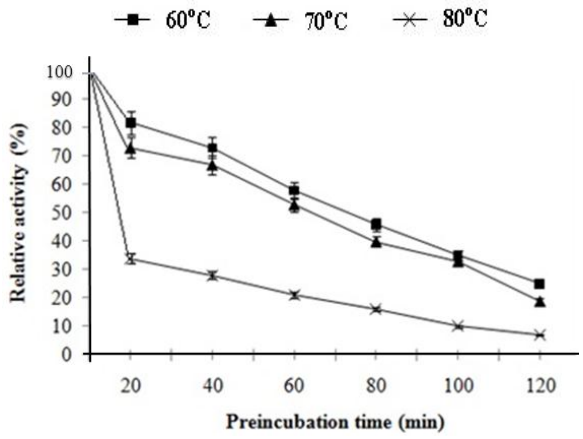

Figure 5. Stability of purified polyphenol oxidase from *Cinnamomum tamala* leaves polyphenol oxidase as a function of temperature. The thermal stability of the enzyme was determined by incubating enzyme at 60^0C (■), 70^0C (▲) and 80^0C (✱) for the indicated time, respectively, and the remaining activity was assayed under standard conditions

3.5. Effect of various metal ions and reagents

A wide variety of proteins and enzymes incorporate metal ions or metal complexes into their overall structure, and trigger enhancement of their activity. Effects of metal ions and other chemical reagents on the enzyme activity were studied at their final concentration (0.5mM) using catechol as the substrate. As shown in Figures 6A and 6B, addition of Fe^{2+}, Hg^{2+} (0.5mM) to the assay reaction mixture showed enhancement in PPO activity, and Cu^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, and Ni^{2+} (0.5mM) moderately enhanced the enzyme activity while Co^{2+} and KCl strongly inhibited the PPO activity, and moderate inhibition was reported with NaCl [13, 28]. It has also been reported that Cu^{2+} and Fe^{2+} are activators of PPO while Co^{2+} and Ca^{2+} inhibit the activity of enzyme. Phenolic compound (Figure 6C.) like ferulic acid showed good activator followed by protocatechuic acid and vitamin B. In case of phytohormones, α-NAA has shown to be a potent inhibitor followed by GA, and activity of the enzyme is marginally activated by IAA. Figure 6D clearly shows that effectors like kojic acid are strong inhibitors while marginally inhibited by EDTA, β-marcaptoethanol and sodium azide. Similar results have been reported for kojic acid, [35,13] sodium azide, [13,17, 28, 29] L-ascorbic acid, [4,3,17,28,38,39] β-mercaptoethanol [13,17,28,29,30], and EDTA [13,30]. However, Shi *et al.* [40] have reported that azide can act as either an inhibitor or an activator of the copper enzymes like PPO, and its action depends only on its concentration. While no measurable changes occurred in case of urea and sodium sulfide.

In addition to those given in Figure 6E, various amino acids were tested on PPO activity to prevent the enzymatic browning reaction. Ascorbic acid and L-Cystein could be preferred as potent inhibitors while L-Aspartate, L-Glutamine, L-Proline, L-Lysine, L-Tyrosine and L-Alanine almost showed negligible effects on PPO activity. Due to browning caused by PPO in the food industry and its great significance in melanogenesis, further research on potential inhibitors of PPO is important.

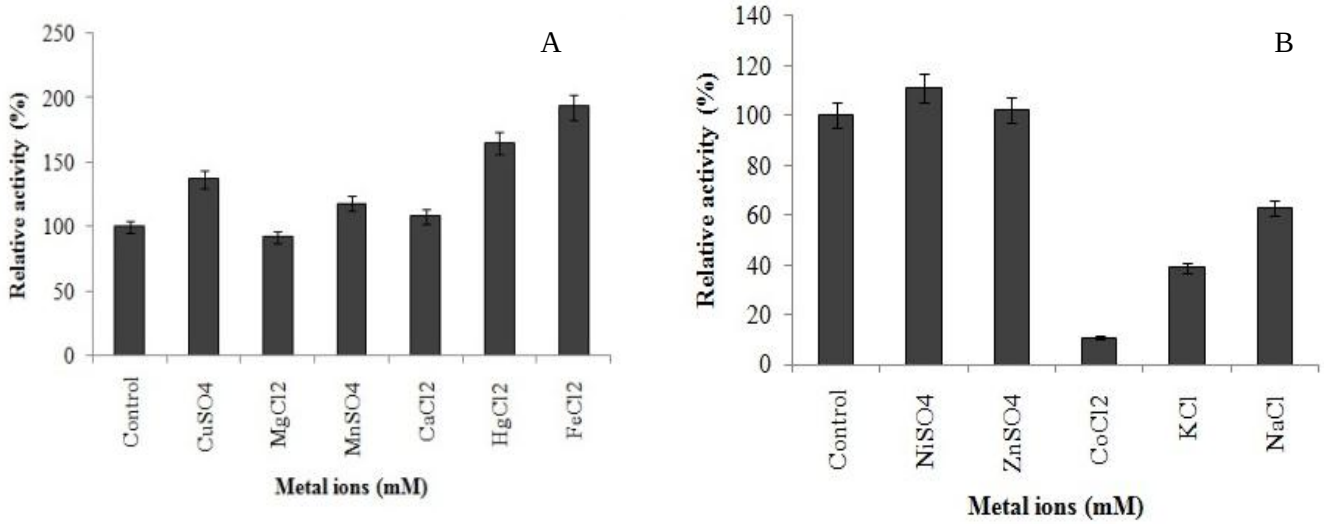

Figure 6 A & B. Effect of metal ions on the activity of purified polyphenol oxidase from *Cinnamomum tamala* leaves

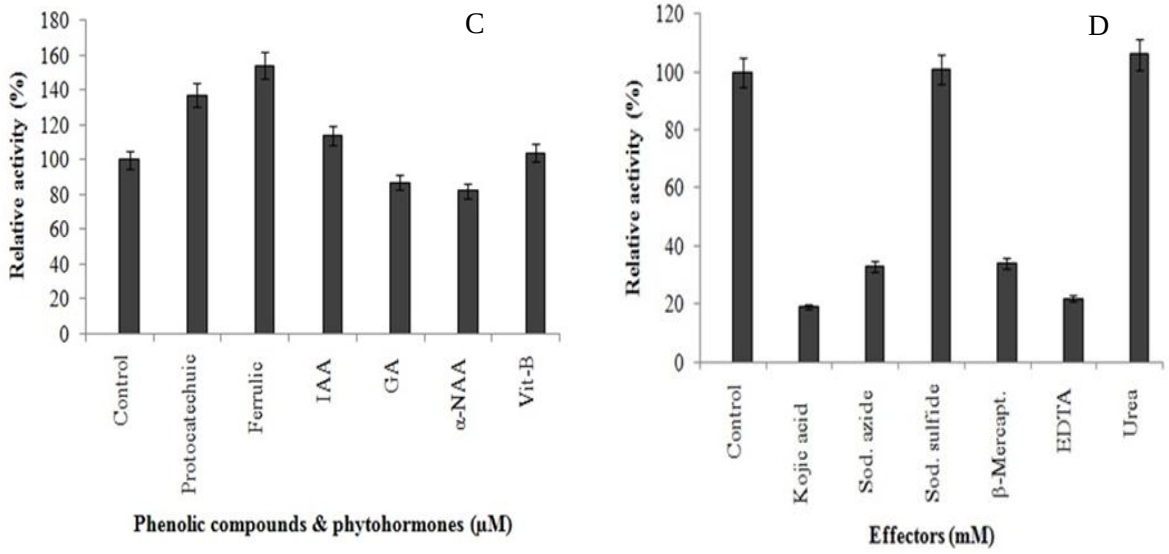

Figure 6. C) Effect of phenolic compounds and phytohormones, D) effectors on the activity of purified polyphenol oxidase from *Cinnamomum tamala* leaves

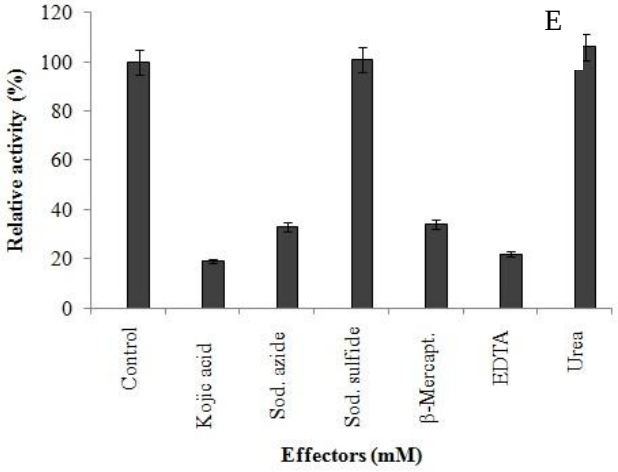

Figure 6E. Effect of various amino acids on the activity of purified polyphenol oxidase from *Cinnamomum tamala* leaves (with 5% error bar, n =5).

Ascorbic acid inhibits browning reaction by acting as a reducing agent, and has emerged as the best alternative to sulfite. It is commonly being used as an anti-browning agent in the manufacturing of fruit juices, frozen sliced fruit, and canned fruits and vegetables, due to safety concerns, as use of sulfites in fresh fruits and vegetables was banned by the Food and Drug Administration [32]. However, effectiveness of inhibitors against different PPOs could significantly vary, and therefore, specific control measures for individuals systems would also be needed.

Our report is in agreement with the earlier reports [13, 17, 29] for L-cysteine as a strong inhibitor of PPO. Among the hormones, only IAA was found to be slightly activating the activity of PPO enzyme, while GA and α-NAA marginally inhibited the enzyme activity.

4. Conclusion

According to this study, it can be concluded that *Cinnamomum tamala* leaves are able to release high levels of thermostable PPO enzyme of industrial importance under economic laboratory extraction condition along with 0.1% polyvinylpyrrolidone (PVP). The enzyme of interest can be used for the development of biosensors to detect phenolic compounds for various purposes; also the ability of PPO to act on phenolic compounds can be used for the degradation of phenols in industrial waste waters. Although peroxidase enzymes have the potential to treat a large variety of phenolic compounds, they are being replaced by PPO. This application requires PPOs active in a wide range of pH and temperature.

5. Acknowledgment

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6. Conflict of interest

The authors verified that there is no declaration of interest.

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