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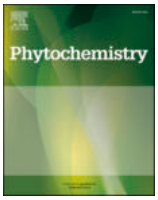

L-DOPA synthesis in Mucuna pruriens (L.) DC. is regulated by polyphenol oxidase and not CYP 450/tyrosine hydroxylase: An analysis of metabolic pathway using biochemical and molecular markers

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A B S T R A C T

Mucuna pruriens L., commonly known as velvetbean or cow-itch, is a self-pollinated tropical legume of the family Fabaceae, known for its medicinal properties. The active principle L-DOPA extracted from the plant is a potent drug used in the treatment of Parkinson's disease. Although, it is hypothesized that a single step reaction can produce L-DOPA, the presence of optional routes makes the pathway more intricate. For instance, the catecholamine biosynthetic pathway, which leads to L-DOPA production, could occur by hydroxylation of tyrosine to L-DOPA either by polyphenol oxidase (PPO) or tyrosine hydroxylase (TH). Furthermore, Cytochrome P450 (CYP) enzymes can also cause hydroxylation of tyrosine, resulting in L-DOPA synthesis. Therefore, the present investigation was focused on validating the step, which catalyzes the synthesis of L-DOPA, at the biochemical and molecular levels. Enzyme inhibitor studies showed significant inhibition of PPO enzyme with corresponding decrease in L-DOPA synthesis while TH and CYP inhibition had no effect on L-DOPA synthesis. Activity staining of non-denaturing PAGE gel for PPO and TH showed activity only to PPO enzyme. Following in-gel assay and tryptic digestion of the excised stained gel portion, peptide recovery and LC-MS/MS analysis were performed. Degenerate primers based on peptide sequence resulted in an 800bp amplicon. The subsequent sub-cloning, RACE analysis and BLAST search resulted in the isolation of full-length PPO coding sequence of 1800 bp. Structure prediction and phylogenetic analysis of the obtained sequence revealed strong similarity to other plant PPO's like *Glycine max*, *Vigna radiata* and *Vicia faba* of the same family.

1. Introduction

Mucuna pruriens (L.) DC. (Fabaceae) is an important medicinal plant reported to be indigenous to eastern India. The therapeutic value is attributed to L-DOPA, an effective drug against Parkinson's disease, a neurodegenerative disease caused by dopamine depletion in the brain. L-DOPA is considered to be the standard drug for the treatment, as administration of dopamine directly is not useful due to the blood-brain barrier. However, L-DOPA being the precursor of dopamine can cross the barrier and be converted to dopamine. Though L-DOPA can be chemically synthesized, the resultant racemic DL-mixture is inactive and further separation of enantiomerically active L-DOPA from this mixture is difficult. Therefore, conventional production of L-DOPA involves the extraction of it from *M. pruriens* seeds and marketed as tablets under various brand names like Sinemet, Atamet, Parcopa and Stalevo by pharmaceutical companies. The global demand for L-DOPA is high as it is estimated to be 250 tons per year. Consequently, the demand-supply gap is also high as the yield from field grown plants of *M. pruriens* is estimated to be low. Though alternative approaches like biotransformation of tyrosine using plant/fungal extracts and *in vitro* production of L-DOPA using cell suspension cultures of *M. pruriens* were reported, the yield and production cost are not always viable and satisfactory. Interestingly, L-DOPA is one of the major precursor molecule for several secondary metabolites like catecholamines, alkaloids, melanin in plants and hence execute various metabolic actions in plants.

The seeds of *M. pruriens* contain an average L-DOPA concentration of 48.9 g/kg (Chikagwa-Malunga et al., 2009). Further, additional medicinal properties like anti-diabetic, aphrodisiac, anti-neoplastic, anti--epileptic, anti-microbial (Sathyanarayana et al., 2011), anti-venom (Guerranti et al., 2001) and anti-helminthic activities have been reported (Jalalpure et al., 2008). The plant has also been shown to be neuroprotective (Misra and Wagner, 2007), and has demonstrated analgesic and anti-inflammatory activities as well (Hishika et al., 1981).

Although it is reported that a single step reaction can produce L-DOPA in mammals, the presence of optional routes in plants makes the pathway more intricate. Similarly, the recent revelation of alternate pathways catalyzed by CYP 450 class of enzymes for L-DOPA production and regulation in other plants (Polturak et al., 2016), animals and

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insects (Kim and Muturi, 2012) is indicative that there exist at least three different optional routes for L-DOPA synthesis. Therefore, the hypothetical pathway is suggested as in Fig. 1.

Tyrosine hydroxylases are iron-containing monooxygenases dependent on cofactor, tetrahydrobiopterin, purported to catalyze the rate-limiting regulatory step of conversion of L-tyrosine to L-DOPA. The *in vivo* enzyme activity of TH along with PPO is reported in plants like *Portulaca grandiflora* (Yamamoto et al., 2001) and *M. pruriens* (Luthra and Singh, 2010). In contrast to considerable amount of knowledge available on the biochemical and molecular aspects of TH in animal species like insects (Gorman et al., 2007) and mammals (Jin et al., 2006), very little is known about TH genes in plants, as full-length TH gene is not yet reported from any plant species.

Cytochrome P450 dependent monooxygenases are enzymes containing a heme group, which catalyzes hydroxylation reactions. In *Beta vulgaris,* the initial step in betalain synthesis is the hydroxylation of L-tyrosine to L-DOPA followed by downstream steps resulting in the formation of colored pigments like betacyanin or betaxanthin, collectively known as betalains (Sunnadeniya et al., 2016; Polturak et al., 2016.) Similarly, recent *in vitro* and *in vivo* studies performed in rodents indicate that dopamine and serotonin may be formed in the brain *via* CYP450 mediated pathway (Haduch et al., 2013).

Polyphenol oxidases (PPOs) are copper metalloproteins that play a critical role in producing various derivatives of polyphenolic compounds. Primarily, PPO is an enzyme that catalyzes the hydroxylation of monophenols into ortho-diphenols (cresolase activity) and the oxidation of o-diphenols into quinones (catecholase activity) in the presence of molecular oxygen. These quinones spontaneously polymerize to form dark-colored phytomelanins, seen as brown colored patches on damaged plant tissue. Interestingly, enzyme nomenclature differentiates the monophenol oxidase as laccase (EC 1.10.3.2) and the o-diphenol: oxygen oxidoreductase as catechol oxidase (EC 1.10.3.1). However, depending on the source of enzyme it may act on two categories of substrates like monophenols and diphenols and hence commonly known as polyphenol oxidase (Tyrosinase; EC 1.14.18.1). For example, tyrosinase from mushroom shows both monophenol oxidase and o-diphenol oxidase activity. Similarly, PPO's in plants like apple (Goodenough et al., 1983), avocado (Kahn and Pomerantz, 1980), eggplant (Perez-Gilabert and Carmona, 2000), grape (Valero et al., 1989, 1998), and potato (Sanchez-Ferrer et al., 1993), possess both types of activities. However, PPO's in some plants like lettuce (Heimdal et al., 1994), longan (Jiang, 1999), pineapple (Das et al., 1997), field bean (Paul and Gowda, 2000) and sunflower (Raymond et al., 1993) lack the hydroxylation properties and act only on o-diphenols *i.e.,* having only diphenolase activity. Biochemically, diphenolase is the most prevalent form of PPO (Yoruk and Marshall, 2003). However, when both monophenolase and diphenolase activity existed in the same PPO, the ratio of respective activities was found to be 1:10 to 1:40 (Nicolas et al., 1994; Marshall et al., 2000).

Therefore, given the optional routes available for L-DOPA synthesis

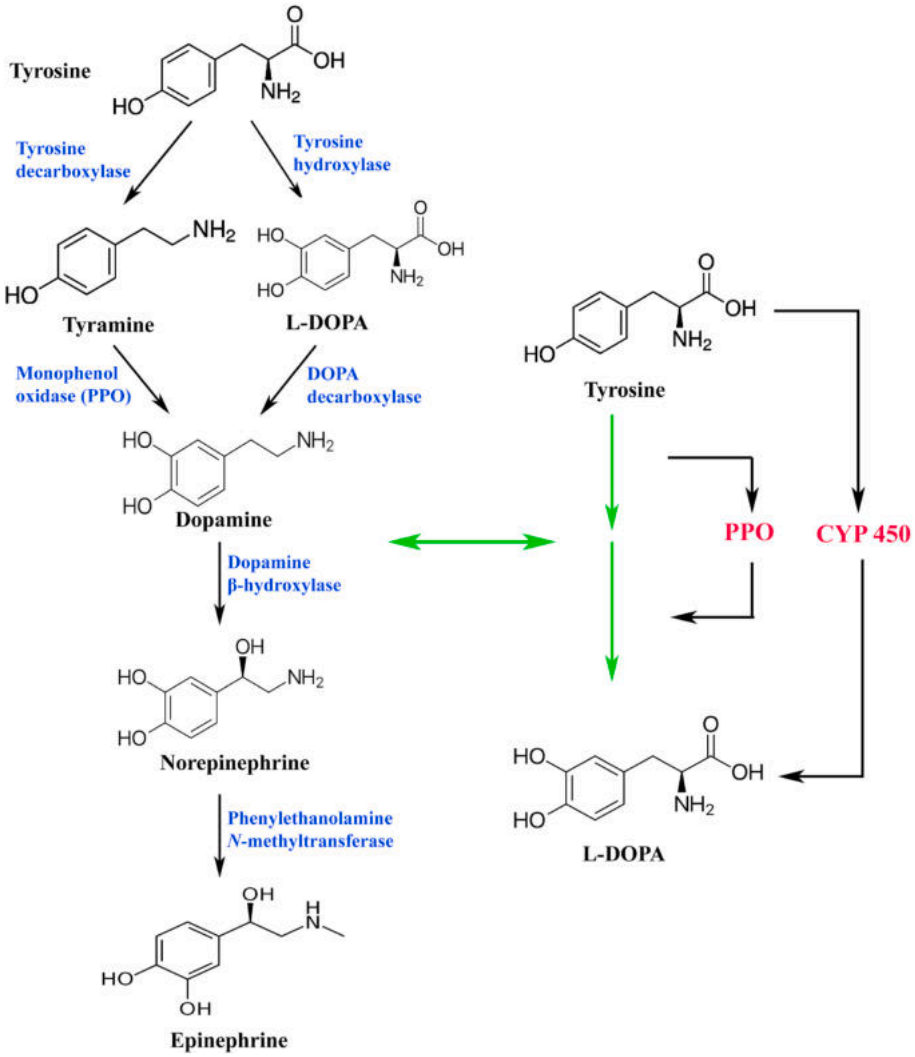

Fig. 1. The proposed hypothetical pathway for catecholamine biosynthesis in *Mucuna pruriens*.

in various plant species, the objective of the present investigation was to experimentally validate the enzymatic step which hydroxylates L-tyrosine to L-DOPA in *M. pruriens*, at the biochemical and molecular levels.

2. Results

2.1. Partial purification of the enzyme

The crude enzyme extract from *M. pruriens* leaves was precipitated by ammonium sulfate. The precipitate was dialyzed by the phosphate buffer. The dialyzed enzyme was purified double the fold and gel filtrated using sephadex G100 column and eluted with phosphate buffer. The eluted fractions 5–8 were collected and found to have higher enzyme activity as in Fig.S1a. These fractions were pooled together and purification fold was found to be 8 fold when compared with the crude enzyme extract (Table S1).

From the pooled enzyme fractions, 75% (v/v) was passed through DEAE – Cellulose column and eluted with Tris-HCl buffer, while the remaining 25% (v/v) was used for protein estimation. Different fractions were collected and the fractions with enzyme activity were pooled again. It was found that the purification fold was higher and the enzyme activity was significantly lower in comparison with crude enzyme extract as well as sephadex G100 column passed fractions as shown in Table S1. More than 30% of PPO got eluted from the column as unbound enzyme. However, these fractions were not considered for further studies, as similar to the flow through, they did not show any appreciable TH/PPO/CYP450 activity. The bound proteins were eluted using 50 mM and 250 mM sodium chloride in 10 mM Tris-HCl buffer (pH 8.0) (Fig S1b). Elution with 50 mM NaCl generated a protein peak showing PPO enzyme activity. However, elution with 250 mM NaCl showed significantly lower enzyme activity. The enzyme obtained was used for further studies.

Similarly, TH enzyme activity was studied through purification steps. However, the pooled enzyme fractions after DEAE-cellulose column purification showed only marginal TH enzyme activity (Fig. S1c). Therefore, pooled fractions showing PPO activity alone was taken for further electrophoretic and peptide recovery analysis. Interestingly, the pooled fractions after Sephadex chromatography retained the TH activity and hence it was used for preliminary characterization of the enzyme.

2.2. Characterization of enzyme

2.2.1. Effect of pH and temperature on enzyme activity

A wide pH range of 3–10 was taken for estimation of optimum pH of *M. pruriens* PPO, as changes in pH may affect the shape as well as charge properties of the substrate, making it impossible to undergo catalysis or binding to the active site. *M. pruriens* PPO has an optimum pH of 6.0 using catechol as the substrate (Fig. 2). However, the substrate specificity seems to change with the shift in pH (Ma et al., 2016). When L-DOPA was used as substrate, the optimal pH was found to be 7.0 whereas pH was 5.0 with L-tyrosine as substrate. The highest enzyme activity was obtained at an optimal pH of 6.0 using the substrate catechol. Generally, optimal pH was found to differ among plant PPO's. In the case of TH enzyme activity, optimal pH was 7.0 when L-tyrosine was used as substrate (Fig. 2).

The effect of temperature was investigated in a wide range from 5°C to 40°C. The maximum enzyme activity of PPO was obtained at 30°C (Fig.S2). Optimal temperature for maximal TH activity was seen at 25 °C across the temperature regime of 5°C-40°C (Fig. S2).

2.2.2. Substrate specificity and concentration

Substrate specificity was determined using different substrates such as L-DOPA, L-tyrosine and catechol. Out of which, catechol was found to be the best substrate for PPO followed by L-DOPA (Table 1). The relative enzyme activity of PPO with L-tyrosine as substrate was only 11.07% compared to 73.57% with catechol. However, still L-tyrosine proved to be a substrate for PPO enzyme activity. Catechol was prepared in different concentrations like 5 mM, 15 mM, 30 mM, 50 mM, 100 mM and 300 mM. Enzyme activity was shown at maximum rate when 50 mM catechol was used as substrate. Optimal substrate concentration was hence found to be 50 mM for *Mucuna* PPO (Fig. 3). When substrate specificity studies were performed for TH fraction using the same set of substrates mentioned above, the highest enzyme activity was shown with L-tyrosine (Table 1) at the concentration of 30 mM (Fig. 3).

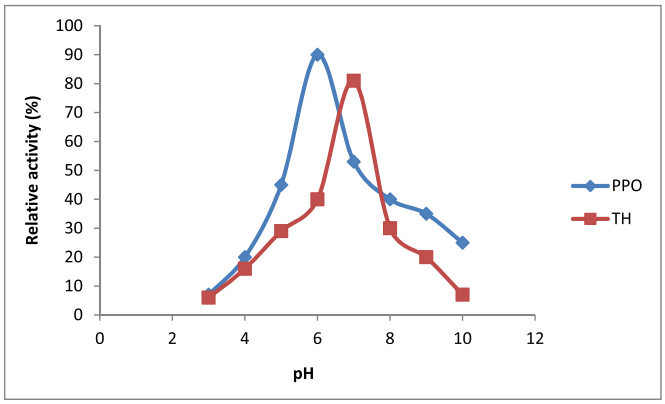

Fig. 2. Effect of pH on the activity of partially purified enzymes from *Mucuna pruriens*. The assay was performed using 50 mM catechol and 30 mM L-tyrosine as substrates for the PPO and TH enzyme activity, respectively. Four different buffers with their optimal buffering capacity in the pH range of 3–10 were used in separate assays.

2.2.3. Effect of inhibitors

Six inhibitors with different concentrations were used to determine their potential for inhibition of PPO activity. Among the inhibitors used in the study, sodium metabisulfite showed maximum enzyme inhibition (98%) followed by citric acid (95%) and sodium chlorite (93%) (Table 2). Kojic acid was the least effective (1%) inhibitor for PPO enzyme activity. However, there was no evident inhibition for TH enzyme activity with the set of TH inhibitors used in this experiment (Table 2). The percentage of inhibition was negligible (1–3%) with metyrosine and 3-iodotyrosine. Enzyme inhibition studies were carried out for CYP450 activity using inhibitors specific for these enzymes. The percentage of enzyme inhibition using specific inhibitors was monitored and compared with the untreated control sample. L-DOPA quantification carried out using HPTLC showed that L-DOPA synthesis was decreased to 0.04% by PPO inhibitors like L-ascorbic acid and kojic acid when compared to the control (0.13% dry weight) (Fig. 4). The effect of CYP450 inhibitors cimetidine and quinidine was negligible as the percentage L-DOPA decreased only to 0.12% and 0.11% respectively, compared to the control.

Table 1
Substrate specificity of partially purified enzymes from *M. pruriens*.

Substrate	Crude extract		Dialysed sample		Column fractionated sample	
	Enzyme activity (U/min/mg)		Enzyme activity (U/min/mg)		Enzyme activity (U/min/mg)	
	PPO	TH	PPO	TH	PPO	TH
L-tyrosine	3.1	9.2	1.8	10.8	2.4	12.1
L-DOPA	7.9	-	5.6	-	6.5	-
Catechol	20.6	-	11.5	-	12.4	-

The enzyme activity determined after each level of purification like ammonium sulfate precipitation, sephadex G-100 column chromatography and DEAE-column purification is given in the table with '-' indicating that no value was detected.

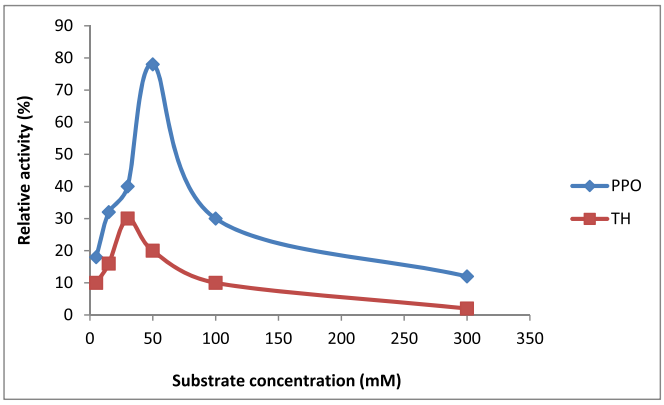

Fig. 3. Effect of substrate concentration on partially purified enzymes. The assay was performed for PPO activity with 50 mM catechol as substrate at pH 6.0 while keeping the temperature for reaction at 30 °C. For TH activity, 30 mM L-tyrosine was the substrate and assay done at pH 7.0 and 25 °C.

Table 2
Effect of enzyme inhibitors on partially purified enzymes from *M. pruriens*.

Compounds	Concentration (mM)	Inhibition (%)
PPO Inhibitors		
L-ascorbic acid	0.05	6 ± 0.3
	10	95 ± 0.23
Citric acid	0.05	10 ± 0.74
	10	50 ± 0.71
Sodium chlorite	0.05	4 ± 0.26
	20	93 ± 0.15
Kojic acid	0.05	1 ± 0.58
	20	1 ± 0.25
L-Cysteine	0.02	1 ± 0.84
	5	90 ± 0.13
Sodium metabisulfite	0.01	9 ± 0.41
	10	98 ± 0.32
TH Inhibitors		
	0.02	1 ± 0.25
Metyrosine	10	2 ± 0.79
3-iodotyrosine	0.02	3 ± 0.93
	10	2 ± 0.46

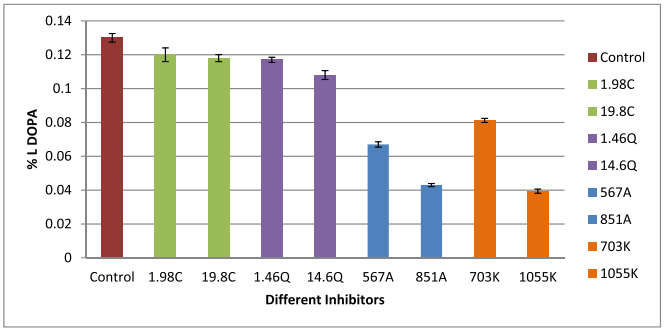

Fig. 4. Effect of enzyme inhibitors on L-DOPA production in callus cultures of *M. pruriens* was estimated using HPTLC. The culture without inhibitor was treated as negative control and cultures with different concentration of the inhibitor were the test samples. (Control-untreated, C = Cimetidine at 1.98 μM and 19.8 μM; Q = Quinidine at 1.46 μM and 14.6 μM; A = L-ascorbic acid at 567 μM and 851 μM; K = Kojic acid at 703 μM and 1055 μM).

2.3. Electrophoresis

Activity staining of PPO/TH was performed by carrying out a non-denaturing (native) PAGE. Clear and distinct bands appeared on the gel when catechol (Fig.S3a) and L-DOPA (Fig.S3b) were provided as the substrate for PPO. Activity staining for TH enzyme fraction resulted in a blank gel (Fig.S3c). The enzyme purity was determined by using SDS-PAGE. The molecular weight of the purified enzyme was found to be 59 kDa on average (Fig S4) as determined by SDS-PAGE under reducing conditions.

2.4. Amino acid sequence and LC-MS analysis

The data acquired from LC-MS/MS were analyzed by the Mascot server in MS/MS ion search and thereafter compared with National Center for Biotechnology Information (NCBI) non-redundant protein blast database (plants). The server identified presence of a putative conserved oxidase domain. Based on the transit peptide sequence (Table 3) obtained through analysis, degenerate primers were designed for PCR amplification of the desired fragment.

2.5. PCR amplification of cDNA using PPO and TH specific primers

An amplicon of 250bp was obtained (Fig. 5a) using MTH F and MTH R primers designed for tyrosine hydroxylase and there was no amplification using the remaining fifteen primers (Table S2). Sequencing result of the amplicon obtained was not showing any relevant similarity upon BLAST search. Therefore, degenerate primers were designed based on the peptide sequence derived through LC-MS/MS. The 800bp amplicon (Fig. 5b) obtained through PCR reaction was cloned and sequenced. The nucleotide sequence showed similarity to polyphenol oxidase by 85%. The 5′ and 3’ coding sequences obtained through RACE analysis resulted in the construction of full-length cDNA with an open reading frame of 1800 bp (Fig. 6). It was further compared with the already reported plant PPOs using sequence similarity search and found >80% similarity to *Glycine max* PPO. Additionally, the sequence also had two characteristic copper binding (tyrosinase) signature domains (Fig.S5) which are seen in other reported plant PPOs. The sequence was submitted in the GenBank database with the accession number MK140603.

2.6. Structure prediction and phylogenetic analysis

From the deduced amino acid sequence of the 1800 bp ORF, the structure prediction of PPO protein was carried out by using the software’s Phyre^2 (Kelly et al., 2015) and SWISS Model (Arnold et al., 2006). The residues (100%) were modeled at >90% confidence and the PPO protein was modeled based on heuristics to maximize alignment coverage and percentage identity by Phyre^2 software. Secondary structure of the protein showed 17% α helix and 13% of β strands in total. The predicted secondary structure (Fig. 7a) of PPO with specific model dimensions (Fig. 7b) was shown. The model of PPO enzyme obtained with its active sites, protein-ligand interaction and metal linkages are depicted in Fig. 7c, wherein the conserved histidine residues directly interacting with the two Cu^{2+} ions are also seen. Phylogenetic relation of *M. pruriens* PPO with other reported plant PPOs of same family as well as of other plant types was obtained by sequence analysis using MEGA software (Fig.S6). Phylogenetic analysis of *M. pruriens* PPO (MpPPO) showed significant extent of similarity relationship with other reported plant PPOs of *Vigna radiata, Vicia faba, Glycine max* and *Juglans regia*.

3. Discussion

It is widely accepted that the non-protein amino acid L-3,4- dihydroxyphenylalanine (L-DOPA), which is present in plants, animals and fungi, is synthesized by conversion of L-tyrosine by a hydroxylation reaction catalyzed by the enzyme TH, *via* a single step process (Soares et al., 2014). Largely, animals followed this step through the catecholamine pathway. However, L-DOPA synthesis in fungi is catalyzed by novel tyrosinase having both cresolase and catecholase activity (Taranto et al., 2017). Interestingly, L-DOPA synthesis in plants shows much complicated scenario, wherein it is presumed that either TH, CYP450 or

Table 3
The total amino acid sequence obtained through LC-MS/MS of the recovered peptide after in-gel activity.

STLPEVKDQH	SEEKKNEVNG	TSNAVVTSYW	GITRPKVRRE	DGTEWPWNCF
MPWDSYHSDV	SIDVTKHHTP	KSLTDKVAFR	AVKFLRVLSD	IYFKERYGCH
AMMLETIAAV	PGMVGGMLLH	LKSLRKFQHS	GGWIKALLEE	AENERMHLMT
MVELVKPSWH	ERLLIFTAQG	VFFNAFFVFY	LLSPKAAHRF	VGYLEEEAVI
SYTQHLNAIE	SGKVENVPAP	AIAIDYWRLP	KDATLKDVVT	VIRADEAHHR
DVNHFASDIH	HQGKELKEAP	APIGYH		

The highlighted portion (in red) corresponds to the conserved oxidase domain of PPO.

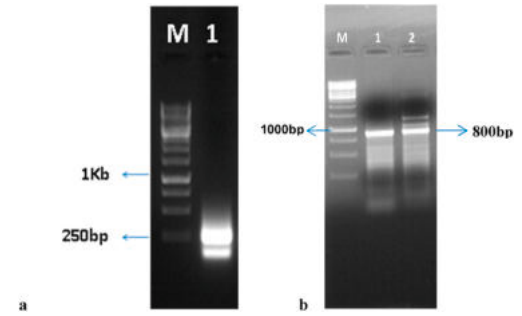

Fig. 5. The amplicons generated using degenerate primer approach. (a) Lane M-DNA ladder, Lane 1–250 bp amplicon generated using MTH –F and MTH-R primer pairs of TH gene (b) Lane M-DNA ladder, Lane 1 and 2–800 bp amplicon using primers deduced from the peptide sequence derived through LC-MS/MS.

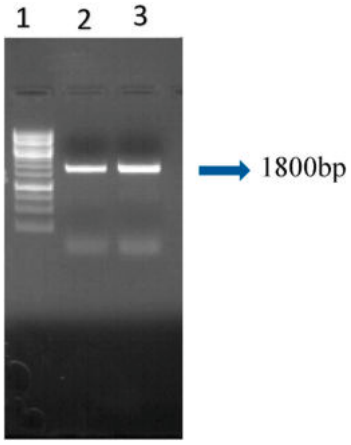

Fig. 6. The 1800 bp amplicon of full-length PPO cDNA obtained after deducing the 5′and 3′ ends through RACE analysis. Lane 1- 1 Kb DNA marker, Lane 2 and 3 the amplicon in duplicate after amplification using gene specific primers.

PPO could catalyze the conversion of L-tyrosine to L-DOPA in different species. Therefore, present investigation was carried out to elucidate the enzyme and its corresponding gene involved in the catalytic conversion of L-tyrosine to L-DOPA in *M. pruriens*. The presence of PPO and TH enzyme activity during the procedure of crude enzyme purification is consistent with earlier reports of both activities observed in *Portulaca grandiflora* (Yamamoto et al., 2001) and TH activity in *M. pruriens* (Luthra and Singh, 2010). The optimal pH for PPO activity was reported to range from 4.0 to 8.0 with pH 5.0 in most of the cases. In our study, the optimum pH shifted from 5.0 to 7.0 depending upon the kind of substrate catalyzed like tyrosine (pH 5.0) catechol (pH 6.0) and L-DOPA (pH 7.0). It is not unusual, as binding of the substrate and further catalysis is pH dependent in PPO. The PPO with two copper-binding domains showing both the monophenolase and diphenolase activity, also known as tyrosinase, has an active site architecture which is heavily influenced by the ionic state of the two copper ions and the ability of enzyme to bind molecular oxygen. In brief, the enzyme can exist in four possible oxidative states, depending on the valence of the two Cu^{2+} ions present in the active site. The interaction of which can lead to unusual kinetic behavior of the enzyme including its preference for diverse substrates (Ramsden and Riley, 2014). The normal state of the enzyme is known as met-tyrosinase (Cu^{2+}-OH-Cu^{2+}) followed by deoxy-tyrosinase (Cu^{2+}- Cu^{+}), oxy-tyrosinase (Cu^{2+}- O2– Cu^{2+}) and deact-tyrosinase (C^{2+}- C^{0}). The pH of the environment is an important factor that influences the ionic state of the Cu^{2+} ions and hence the binding of different substrates. The TH enzyme activity showed optimal catalysis at pH 7.0, which corroborates the earlier study in *M. pruriens* wherein the high TH activity was measured at pH range from 6.0 to 7.5, with minimal activity at pH 4.0 and 8.0.

During partial purification of the enzymes, as the purification fold increased, significant reduction in yield was observed. Also, the elution of unbound proteins was also detected in the purification procedure. Excessive loading of the protein (enzyme) may not be the reason for unbound protein as only 8.0 mg of the protein was loaded onto the column. The possible explanation is that the plant extract contains many phenolic compounds which could interact with the purification procedure adversely affecting the yield. During cell disruption in extraction procedure, the spatially separated PPO enzyme and the phenolics interact and irreversible binding occurs, which changes the hydrophobic and ionic properties of the enzyme. This drastically changes the elution behavior of the enzyme and its purity, in successive purification steps (Mishra and Gautam, 2016). Furthermore, variation in purity may also affect other parameters like temperature, pH, cofactors etc., required for optimal and maximum activity. Additionally, the elution of unbound proteins also indicates the presence of various isoforms (Saby et al., 2011). All the three enzymes, PPO, TH and CYP have ample isoforms which show spatial and temporal expression in various tissues. For instance, there are four TH isoforms in humans differentially expressed in diverse neuron populations in brain (Lewis et al., 1993). Similarly, the number of PPO isoforms is so diverse that it varies from nil in *Arabidopsis thaliana* (Araji et al., 2014) to 19 in *Salvia miltiorrhiza* (Li et al., 2017). The CYP gene family is one of the largest in the plant kingdom with 1059 CYP sequences identified. Many plants contain numerous CYP proteins with *Oryza sativa* cvs *japonica* and *indica* having the highest number of 458 predicted CYP sequences (Nielsen and Moller, 2005).

The enzyme inhibition studies of partially purified enzymes, using specific inhibitors, showed significantly higher percentage of inhibition of PPO activity (98%) than TH (1–3%), thereby strongly dismissing the role of TH. The *in vivo* initiated callus cultures were monitored for the effect of CYP450 and PPO inhibitors, wherein no significant inhibition was found in comparison with that of untreated callus control. The percentage inhibition well corresponds with decrease in L-DOPA production as in the case of PPO but with negligible or no effect in the case

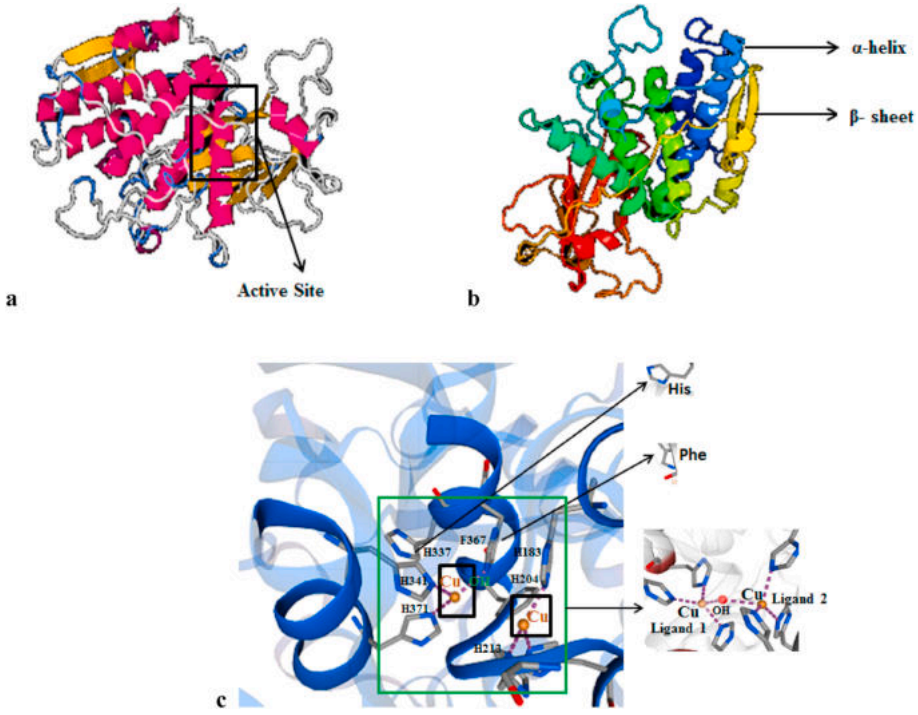

Fig. 7. Homology modelling and secondary structure prediction of PPO enzyme from *Mucuna pruriens* (a) Predicted secondary structure of PPO (b) Phyre^2 protein model for PPO with 3D model dimensions (in Å) (X:49.941 Y:64.463 Z:57.979). Image colored by rainbow N → C terminus (c) Three dimensional SWISS protein model for PPO enzyme with two active copper binding ligands (copper ions bridging oxygen moiety is illustrated as small yellow spheres highlighted in the box), conserved histidine residues and metal complex interactions (in dotted lines) . Chain A for Ligand 1: H.183, H.204, H.213, F.367, H.371; metal interactions: A:H.183, A:H.204, A:H.213. Chain A for Ligand 2: H.337, H.341, F.367, H.370, H.371; metal interactions: A:H.337, A:H.341, A:H.371). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of TH and CYP, apparently indicating the active involvement of PPO in L-DOPA synthesis.

Though TH enzyme activity was observed, isolation of TH gene was quite challenging albeit different degenerate primer pairs from various sources were used. Out of the 16 different degenerate primers used, only PCR amplification of a 250 bp fragment was obtained with the primers MTH F and R of *Musa acuminata*. However, sequence analysis of the PCR amplicon showed an insignificant similarity upon BLAST search. Further gene mining efforts revealed that not a single full-length TH gene of plant source has been deposited in the Genbank database except for certain partial cds of *M. acuminata* with accession number EU880276.1. Therefore, it is presumed that the synthesis of L-DOPA in *M. pruriens* could be the step catalyzed either by a CYP450 class of enzyme or a Polyphenol oxidase. The compelling reasons to assume the role of CYP enzymes were derived from recent studies carried out in *Beta vulgaris* (Sunnadeniya et al., 2016; Polturak et al., 2016). The first step in betalain synthesis is the conversion of L-tyrosine to L-DOPA, as in the case of catecholamine synthesis in *M. pruriens*. This step was earlier reported to be catalyzed by PPO or TH (Gandia-Herrero and Garcia-Carmona, 2013) but later found to be catalyzed by the CYP450 class of enzymes like CYP76AD1 along with CYP76AD5 and CYP76AD6, as all three are having the tyrosine hydroxylase activity. Similarly, the synthesis of caffeic acid from p-coumaric acid, presumed to be mediated by PPO (Vaughan and Butt, 1969), was later proved to be by CYP450 enzymes (Schoch et al., 2001; Franke et al., 2002). However, as enzyme inhibitor studies have strongly ruled out the involvement of CYP enzyme and TH, it was pertinent to focus now on PPO for further intensive studies. Furthermore, reports on PPO's mediating the synthesis of specific metabolites in many plant species had warranted further studies on *M. pruriens* PPO. For instance, PPO with monophenolase activity specifically catalyzing the conversion of L-tyrosine to L-DOPA in *Portulaca grandiflora* (Yamamoto et al., 2001), *Juglans regia* (Araji et al., 2014) and synthesis of 8-8 linked lignans in *Larrea tridentata* are few examples that highlight their active involvement in plant secondary metabolism (Sullivan, 2015). Therefore, it is interesting to note that the observed tyrosine hydroxylase activity in the present investigation could be the gene product of the PPO gene and not TH/CYP gene.

As degenerate primer based isolation of TH gene was found to be futile, reverse translation approach was used for the isolation of the candidate gene. The DEAE-column purified pooled enzyme fractions loaded in SDS-PAGE under denaturing conditions identified an enzyme monomer of size 59 kDa. Further in-gel activity of the enzyme in native PAGE gel, with catechol as substrate, showed only PPO activity and not TH activity, indicative that the protein monomer could be PPO. In plants, PPO is generally expressed in the size range 30–70 kDa (Fujita et al., 1995) with an N-terminal transit peptide of 8–12 kDa which can be removed proteolytically. As PPO activity is dependent on its molecular weight (Taranto et al., 2017), diverse PPO isoforms of size range have been reported. For example, *Beta vulgaris* PPO (41 kDa), *Solanum tuberosum* PPO (57–60 kDa), *Lycopersicon esculentum* PPO (57–62 kDa), *Brassica oleracea* PPO (53.1 kDa) (Gawlik-Dziki et al., 2007) and *Hevea brasiliensis* PPO (32 kDa and 34 kDa; Wititsuwannakul et al., 2002) are few representatives. As such, the 59 kDa protein is within the size limit and subsequent peptide analysis yielded the amino acid sequence. The reverse translation approach eventually resulted in the full-length cDNA of 1800bp with significant sequence similarity to other PPO's, particularly from the family Fabaceae. The nucleotide sequence on further analysis showed its active tyrosinase enzymatic region at two sites with copper-binding signature, a hallmark of the PPO enzyme. The recent report on biochemical characterization of the catecholamine pathway in different tissues of *M. pruriens* showing a spike in PPO expression level compared to TH (Singh et al., 2018), also strongly supports our findings.

The prediction of secondary structures and similarity analysis using phylogenetic tree construction for polyphenol oxidases has been widely studied in plants and fungal systems (Marusek et al., 2006). According to Marusek, secondary structure predictions provided a detailed indication that the C-terminal domains of PPO share similar tertiary domains to the heme group. The secondary structure prediction of PPO, in the present study, presented specific model dimensions of α helix (17%) and β strands (13%) which indicates the propensity of amino acid L-tyrosine at the helix strand interface. It is reported that accuracy of secondary structure prediction is dependent on amino acid propensities in helix-strand and helix-helix packing (Hu and Koehl, 2010). The crystal structure of PPO from other plant species like *Ipomoea batatus* and *Vitis vinifera*, which were deduced and published in the protein bank, has provided better insight about the conserved amino acids and the pockets

where the two Cu^{2+} ions are packed (Mishra and Gautam, 2016). The active site structure of these and other plant species shows few conserved amino acid residues linked to the metal ions. The presence of histidine residues in the predicted secondary structure of *M. pruriens* PPO, linked three each to the metal ions, is in agreement with the earlier reports. Moreover, the deduced nucleotide sequence of *M. pruriens* PPO showing >80% similarity to other reported plant PPOs of the same and related family indicates the common ancestry shared among the species.

4. Conclusion

In summary, the study provides ample validation that the observed tyrosine hydroxylase enzyme activity in *M. pruriens*, in the present and earlier investigations, is the translated product of the PPO gene and not CYP450 or TH gene. We hypothesized that any of these genes could be the candidate gene, as all of them are known to translate protein products with hydroxylase activity. However, enzyme inhibitor and in-gel activity studies strongly indicated the active role of PPO and ruled out the possibility of TH and CYP450. The 1800bp coding sequence isolated is the first report of full-length PPO cDNA from this species. The study not only proves the earlier assumption that there is no TH gene in plants but also substantiates the occurrence of alternate pathways for L-DOPA synthesis in different plants. As isoforms of PPO's do exist, it warrants further research on the number of isoforms in *M. pruriens*, their differential expression and functional roles in plant physiology, metabolism and defense.

5. Experimental

5.1. Plant material

The seeds *of Mucuna pruriens* L. DC. (Fabaceae) were obtained from Indian Institute of Horticultural Research, Bangalore, with the Indigenous Collection (IC) No. IT-1 IIHR MP125. The seeds were grown in pots and maintained in the Indian Institute of Science (IISc) campus, Bangalore. Leaves were harvested from a two-month old plant in the winter season (20 ± 5°C), washed thoroughly and proceeded for enzyme and RNA extraction.

5.2. Enzyme extraction, purification and characterization

5.2.1. Enzyme extraction

The *M. pruriens* leaves (300 g fresh weight) were homogenized in three volumes of 0.1 M phosphate buffer (pH 5.7) supplemented with 2% (w/v) PVPP as a phenolic scavenger, 0.5 M NaCl, 10 mM ascorbate, 1.0 mM phenyl methyl sulfonyl fluoride (PMSF). The homogenate was filtered through a double layer of cheesecloth and centrifuged at 10,000 (xg)for 15 min at 4°C (Beckman Coulter, California,USA). The supernatant was treated with ammonium sulfate to obtain 80% saturation at 4°C and then centrifuged at 10,000 (xg) for 15 min at 4°C.

5.2.2. Partial purification of the enzyme

The crude enzyme extract was extensively dialyzed against the same buffer at 4°C for 24 h with three changes of the buffer during dialysis. The dialyzed enzyme solution was fractionated by a sephadex G-100 column (1.2 cm × 60 cm). The column was equilibrated with 0.1 M phosphate buffer (pH 5.7). The 1.0 mL dialyzed enzyme solution was passed through the column and was eluted by the same phosphate buffer. The elution rate was adjusted to 1.0 mL per 5 min. An elution of 3.0 mL was collected each time. The elution process was continued until obtaining two bed volumes of the column and zero absorbance at 280 nm. Protein was qualitatively detected by nanodrop (NanoDrop One, Thermo Fisher Scientific,USA) at 280 nm and quantitatively estimated by standard Bradford method. The eluted sample fractions were then determined for both PPO and TH activity. The values obtained were plotted against the sample numbers. Those fractions having enzyme activity for PPO and TH were pooled separately.

For further purification of the pooled fractions, samples were passed through DEAE –cellulose column (1.2 cm × 60 cm) after equilibrating the column with 10 mM Tris-HCl buffer (pH 8.0). Elution was done with 50 mM NaCl and 250 mM NaCl in 5.0 mL fractions. Different fractions having enzyme activity for PPO and TH were pooled separately and respective enzyme activity assay was carried out. The purified enzyme extract was stored at 4°C for further analysis.

5.2.3. Enzyme assay and characterization

The partially purified enzyme fractions after Sephadex gel filtration chromatography step was used for PPO and TH enzyme assays.

PPO enzyme activity was determined by measuring the initial rate of quinone formation as indicated by an increase in absorbance at 420 nm spectrophotometrically (Hitachi U2900) at 15 s intervals at 30 °C by using 50 mM catechol as substrate. The PPO enzyme activity was determined by reaction mixture which contained of 0.1mLpurified extract, 2.9 mL of 100 mM phosphate buffer (pH 7.0) and 1.0 mL of 50 mM catechol. The PPO activity was assayed in triplicate measurements. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001 per min. Based on enzyme activity, relative activity was defined as the ratio between the enzyme activity of the sample of interest and activity of the control sample (reaction mixture without enzyme) and therefore could be expressed in percentage. The protein content was determined using the Bradford protein dye-binding method with bovine serum albumin as the standard.

Standard assay reaction for TH enzyme activity was carried out in a total volume of 100 μL containing 100 mM potassium phosphate buffer (pH 5.7), 30 mM L-tyrosine, 100 mM ascorbic acid (pH 7.0) and tetrahydrobiopterin as cofactor. The reaction was terminated by adding 25 μL of orthophosphoric acid (Steiner et al., 1996). Colorimetric reaction was performed by adding equal volumes of 0.1 N HCl, nitrite molybdate reagent and 0.1 N NaOH. The absorbance of reaction mixture was read at 530 nm.

5.2.4. pH and temperature profile of PPO and TH

The optimum pH of partially purified PPO and TH enzymes was investigated by measuring its activity at wide pH range. Different buffers like 0.1 M KH_2P0_4/K_2HP0_4, 0.1 M sodium acetate, 0.1 M glycine-NaOH and 0.1 M Tris-HCl in the pH range 3–10 was used. The effect of temperature on PPO and TH enzyme activity was carried out by assaying the reaction mixture at various temperatures ranging from 5°C to 40°C. Results were presented as the mean (±) standard error of the values in triplicates.

5.2.5. Effect of substrate concentration and specificity on the enzyme

The substrate specificity of the enzymes was determined by measuring its activity against L-DOPA, catechol and L-tyrosine. Varying concentrations of the substrate (5–300 mM) was used for the reaction assay.

5.2.6. Effect of inhibitors on PPO, TH and CYP450 enzyme activity

The specific inhibitors for PPO enzyme activity like L-cysteine, sodium metabisulphite, L-ascorbic acid, citric acid, sodium chlorite, and kojic acid, at concentrations ranging from 0.01 mM–20 mM, were used in the assay. The assay comprised of 1.0 mL of catechol (50 mM), 0.1 mL enzyme extract, 0.5 mL inhibitor and 2.9 mL phosphate buffer (0.1 M, pH 7.0). Reaction mixture without inhibitor served as the negative control.

The specific inhibitors for TH enzyme activity (metyrosine and 3-iodotyrosine) were used in the concentration range of 0.02–10 mM, to measure the percentage of inhibition of TH activity. Standard reaction mixture contained 1.0 mL of L-tyrosine (30 mM),0.1 mL enzyme extract, 0.5 mL inhibitor and 2.9 mL potassium phosphate buffer (0.1 M, pH 5.7).

Quinidine and cimetidine along with L-ascorbic acid and kojic acid are the specific inhibitors used for CYP450 and PPO respectively.

Different concentrations of these inhibitors (1.46 and 14.6 μM of quinidine, 1.98 μM and 19.8 μM of cimetidine, 567 μM and 851 μM of ascorbic acid, 703 μM and 1055 μM of kojic acid) were supplemented into callus cultures of *M. pruriens* to measure percentage inhibition of CYP450 activity. Untreated callus cultures in basal media with 0.9 μM of 2,4-D served as the negative control. The percentage L-DOPA content was quantified using HPTLC.

The percentage of inhibition was calculated using the equation,

$$\%\text{Inhibition} = [(A0 - A1) / A0] \times 100$$

A0- Initial PPO/TH activity (without inhibitor).
A1- PPO/TH activity with inhibitor.

5.3. L-DOPA extraction and quantification

Callus cultures supplemented with CYP450 inhibitors were harvested and the fresh weight as well as the dry weight of the samples determined. The dried callus (200 mg) was ground using pestle and mortar (in the presence of liquid nitrogen) and the powdered samples were extracted with 5 mL of 1:1 formic acid: ethanol (Pulikalpura et al., 2015) for 2 h and filtered. The filtrate was extracted again with formic acid: ethanol for three to four times and the final extract was made to 25 mL. The extracts were pooled and then concentrated using a rotary evaporator (Rotavapour R-100, Buchi, Switzerland) and then quantified using HPTLC (CAMAG, Switzerland). The solvent system used was n-butanol: acetic acid: water (282 nm).

5.4. Electrophoretic procedures and estimation of molecular weight

The DEAE-column purified pooled samples were used for electrophoretic and subsequent assays. According to the method of Laemmli (1970), SDS–PAGE was performed in 12% acrylamide gels using 10% SDS to determine the homogeneity and molecular weight under denaturation conditions by using vertical electrophoresis. Electrophoresis was run at 50–100 V for 2.5 h at room temperature. Gels were stained with Coomassie Brilliant Blue R-250 (protocol 1658100, Bio-Rad).

5.4.1. Native PAGE and in-gel-activity staining

Non-denaturing PAGE was carried out in 12% acrylamide gel (10% resolving: 5% stacking), without using SDS and β-mercaptoethanol. Electrolyte for the electrode was Tris-glycine (pH 8.3) without SDS. Bromophenol blue (0.02%) was used as tracking dye. For visualizing the band, 30 μg of the protein sample was loaded on the native gel. The electrophoresis was carried out at 100mA/gel for 5 h at 4 °C. After electrophoresis, the gel was stained for PPO enzyme activity by incubating in 0.1 M sodium-phosphate buffer (pH 7.2) containing 50 mM catechol and L-DOPA separately as substrates for 1 h for color development. Another set of the gel was stained for TH enzyme activity by incubating in a specific TH assay reaction mix containing 30 mM L-tyrosine as substrate for 1 h for development of color.

5.4.2. In-gel assay of PPO and TH by SDS-PAGE

After electrophoresis, the gel was stained with 0.2% Coomassie Brilliant Blue-250, and further destained with a destaining solution (methanol/water/glacial acetic acid 40/50/10,v/v/v) until protein band became visible. The protein which was separated by electrophoresis through SDS-PAGE was renatured by washing it in washing buffer (0.1 M $NaPO_4$ buffer, pH 7.2) for 1 h to remove SDS. Subsequently, the gel was washed in 0.1 M potassium phosphate buffer (pH 5.7) and incubated in reaction mixture and other colorimetric reagents specific for PPO and TH, respectively.

5.4.3. In-gel protein digestion and peptide recovery

The stained protein bands were in-gel digested by destaining the gel on equilibration with 50 mM ammonium bicarbonate (Rosenfield et al., 1992). Following the modified protocol, the stained gel regions were excised and destained by adding 50 mM ammonium bicarbonate made in 50% acetonitrile (ACN) (v/v) and incubated at 37°C for about 20 min. When the gel appeared to be completely dehydrated, it was partially rehydrated with 100% ACN. Further, 10 mM dithiothreitol (DTT) was added to the gel and incubated at 56°C for 45 min followed by iodoacetamide (10 mg mL^{-1}) and 20 μL of porcine trypsin solution (10 ng μL^{-1} in 50 mM ammonium bicarbonate). After absorption of the protease solution, 5.0 μL of ammonium bicarbonate was added until the gel slices were completely immersed. The digestion was carried out for 16 h at 37 °C and then peptide extraction was done by the addition of extraction solution (5% formic acid, 60% ACN, 35% water). The resulting peptides were recovered by shaking in a vortexer for 20 min. The extracted solution was concentrated in a speedvac (CentriVap, Labconco, USA) evaporator. The dried tryptic peptide was subjected to mass spectrometry (LC-MS/MS) to obtain its amino acid sequence.

5.5. RNA isolation and cDNA preparation

Total RNA was isolated from *M. pruriens* leaves (100 mg) based on the modified procedures as described by Zeng and Yang (2002).

5.5.1. Synthesis of cDNA and PCR amplification of PPO and TH genes

Reverse transcription was carried out using the Thermo Scientific™ Revert Aid™ first strand cDNA synthesis Kit (Thermo Fisher Scientific, USA) by an oligo (dT) priming method. For the isolation of TH gene, 16 primer pairs (Table S2) were designed based on the conserved regions of the tyrosine hydroxylase gene of known organisms. Similarly, degenerate primers were also designed based on the amino acid sequence data obtained from the LC-MS/MS analysis.

5.5.2. PCR amplification, cloning and sequence analysis

The Complementary DNA (cDNA) was used as a template for PCR amplification of the gene/oxidase domain with primers FP1, FP2, RP1 and RP2 (Table S3). The reaction mix for PCR contained 2.0 μL of 5X Phusion buffer, 1.0 μL dNTP (10 mM),1.0 μL each of forward and reverse primers (10 pmol), 100 ng cDNA as template, 0.25 μL (0.5U) Phusion® high-fidelity DNA polymerase (New England Biolabs, USA). Reactions were optimized by adding DMSO (0.75 μL) and made up to 25 μL total reaction volume with Milli-Q water. The standardized PCR (BioRad C1000 Thermal cycler) was performed for 35 cycles with initial denaturation at 94°C for 3min (step 1), cyclic denaturation at 94°C for 30s (step 2), annealing at 58 °C for 40s (step 3), extension at 72°C for 30s (step 4) followed by a final extension at 72°C for 10min. Similarly, the PCR products obtained in both the cases were purified by agarose gel electrophoresis using DNA Gel Extraction Kit (Thermo Fisher scientific, USA) and ligated into a pJET (Thermo Fisher Scientific, USA) cloning vector. The plasmid was transformed into *E. coli* DH5α and positive colonies were screened on Luria-Bertani (LB) agar plates containing ampicillin with a final concentration of 100 μg mL^{-1}, as selection marker. After confirmation by PCR amplification and restriction enzyme digestion of plasmids, one positive plasmid was sequenced at Medauxin Sequencing facility (Bangalore). Nucleotide sequences and the deduced amino acid sequences of the gene were analyzed using the BLAST program at the database of the National Center for Biotechnology Information.

5.6. Rapid amplification of cDNA ends(RACE)of the partial sequence

Separate 3′ and 5′RACE analysis was performed using RLM-RACE kit (Thermo Fisher Scientific, USA) to obtain the extreme 3′ and 5′ ends of the gene. The PCR amplification was performed using primers provided in the kit and the gene specific primers FP20, RP20, FP21 and RP21 (Table S3). The amplified PCR product was cloned and sequenced.

5.7. BLAST search and construction of a similarity tree

Basic Local Alignment Search Tool (BLAST) analysis of the full-length sequence obtained from RACE analysis was carried out to calculate the statistical significance in terms of sequence and query coverage, percentage identity and blast score. The phylogenetic tree was constructed based on similarity index to investigate the hierarchial relationship among different biological entities using MEGA software Version 7 (2016).

Contribution of authors

S.G conducted all experiments concerning tyrosine hydroxylase and polyphenol oxidase. J.M.V contributed in cloning experiments. J.K.S performed works related to cytochrome P450 enzymes. C.J.B and P.P provided the fundamental ideas and conceptualized the research problem. S.G prepared the draft manuscript and valuable suggestions and corrections were made by P.P

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112467.

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