Turk J Biol
35 (2011) 575-583
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doi:10.3906/biy-0912-26

Characterization of poly phenol oxidase in two in vitro regenerated cultivars of *Mucuna: Mucuna pruriens* L. and *Mucuna prurita* H.

Raghavendra SATHYANARAYANA[1,*], Vadlapudi KUMAR[1], Chapeyil Kumaran RAMESH[2], Mahadevappa PARMESHA[2], Mahaboob Habeebulla Moinuddin KHAN[3]

[1]Department of Biochemistry, (Kuvempu University-P.G. Centre) Davangere University, Shivagangothri, Davangere - 577 002, Karnataka - INDIA

[2]Department of Biotechnology, Sahyadri Science College, Shivamogga, 577203, Karnataka - INDIA

[3]Department of Chemistry, Jawaharlal Nehru National College of Engineering, 577201 - SHIVAMOGGA

Received: 14.12.2009

Abstract: Polyphenol oxidases (PPOs EC 1.14.18.1) were isolated from *Mucuna pruriens* and *Mucuna prurita* and confirmed as tyrosinases involved in L-DOPA production. PPOs were extracted by using a 0.05 M phosphate buffer, pH 7.0. The purified enzyme was resolved into a single band by PAGE, the enzyme was confirmed as PPO by activity staining, and SDS-PAGE analysis revealed that the purified PPO of both the species is a tetramer. Substrate specificity experiments were carried out with catechol, L-DOPA, L-tyrosine, and p-cresol. Of these, catechol was evaluated as the most suitable substrate based on the determined K_m and V_{max} values. The optimum pH and temperature were determined to be 6.5 to 7.0 and 30 °C, respectively, with catechol as substrate. Inhibitor studies were carried out and, of the 6 inhibitors tested, L-ascorbic acid, citric acid, L-cysteine, and potassium cyanide were the most effective against the PPOs of both the cultivars. The PPOs have both monophenol and polyphenol oxidase activities, with low K_m and high V_{max} values for catechol, p-cresol, and L-tyrosine, and high K_m and low V_{max} values for L-DOPA. The results suggest that the purified PPO forms isolated from 2 *Mucuna* species in the present study showed an affinity towards not only both catechol and p-cresol, but also L-tyrosine, confirming that the isolated PPO is tyrosinase and it might be responsible for the L-DOPA production in the *Mucuna* species. The comparative studies reveal that enzyme activity was slightly greater in crude extracts of *M. pruriens* compared to crude extracts of *M. prurita*, while the fold purity was greater in a partially purified fraction of *M. prurita* than it was in *M. pruriens*. The isolated enzyme can be further exploited for the overproduction of L-DOPA from in vitro cultures of the *Mucuna* species by biotechnology approaches.

Key words: *Mucuna pruriens, Mucuna prurita,* polyphenol oxidase, L-tyrosine, L-DOPA

Introduction

Polyphenoloxidases (monophenol dihydroxyphe nylalanine:oxidoreductase: E.C. 1.14.18.1; PPO) are widely distributed among prokaryotes and eukaryotes (1-6). This PPO is a copper containing metalloprotein that catalyzes the hydroxylation of o-monophenols to o-diphenols (E.C. 1.14.18.1; monophenol monooxygenase, tyrosinase, cresolase) and the oxidation of o-dihydroxyphenols to o-quinones (E.C. 1.10.3.2; diphenol oxygen oxidoreductase, diphenol oxidase, or catecholase). Structural and comparative studies have identified conserved regions in which

histidine residues bind 4 copper atoms located at 2 main sites that are involved in catalytic activity (7-9). PPO has been widely studied in various fruits and vegetables such as potato tuber (10), peach (11), apple (12,13), apricot (14), banana (15), grape (16), pear (17), green olive (18), strawberry (19), plum (20), kiwi (21), and mango (22). Tyrosinases (monophenol, L-DOPA:oxygen oxidoreductase) are polyphenol oxidases (PPOs) that belong to a group of non-blue copper proteins. The other important group of PPOs are the blue-copper proteins, named laccases (EC 1.10.3.2), because their first description was in the lacquer tree. Laccases are multicopper proteins characterized by the presence of 3 different types of copper in the molecule, whereas tyrosinases only have a pair of type III coppers (23).

Mucuna L. is an annual tropical legume, a climbing shrub with long vines that can reach over 15 m. It originated in southern China and eastern India, but is now found extensively all over the world. This plant has been mentioned in the treatises of ancient Indian texts such as the 'Charaka Samhiti' and the 'Susrutha Samhiti'. Its Sanskrit name is 'atmagupta', while in Hindi it is called 'kawach'. In southern India it is also known as 'naikurna'. It is also known by a multitude of common names, including velvet bean, cowitch, cowhage, kapikachu, nescafe, sea bean, kratzbohnen, konch, and yerepe. The name 'nescafe' is attributed to its usage as a coffee substitute in South America. It contains large amounts of L-DOPA, which is produced via the oxidation of tyrosine by the copper containing enzyme tyrosinase (24).

In the present investigation we have used in vitro regenerants of 2 species of *Mucuna* i.e. *Mucuna pruriens* L. and *Mucuna prurita* H. as sources for the purification and characterization of a polyphenol oxidase. Furthermore, it is hypothesized that the PPO has tyrosinase activity also. The 2 *Mucuna* species differ from each other by seed coat color: *M. pruriens* bears black seeds, whereas *M. prurita* bears white seeds. Our intention is to confirm whether or not the isolated PPOs have tyrosinase activity. The enzyme activity was slightly greater in the crude extracts of *M. pruriens* compared to the crude extracts of *M. prurita*, while the fold purity was greater in a partially purified fraction of *M. prurita* than it was in *M. pruriens*. The 2 PPOs were compared for their substrate and inhibitor specifity and also for their efficacy in both crude extracts and purified forms, respectively, from both *Mucuna* species. To our knowledge, no reports to date are available in the literature on characterization of PPO in *Mucuna prurita*.

Materials and methods

Preparation of explants and in vitro plant regeneration

Nodal explants (2.5-3.5 cm) were collected from ex vitro plants of both *Mucuna* species. The explants were excised, initially washed under running tap water to remove any coarse particles present and then subsequently washed with distilled water containing 2-4 drops of Tween-20 (commercially available) for 5 min, then rinsed 2-3 times with distilled water, and surface sterilized with 0.1% mercuric chloride (w/v) solution for 2-4 min. After rinsing 5 times with sterile distilled water, surface sterilized explants were trimmed into 1.5-2.0 cm pieces and placed on MS (25) medium supplemented with sucrose 3% (w/v), IAA (1.13 μM), and BAP (13.33 μM) (w/v) for regeneration and solidified with 0.8% agar-agar. The pH was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. The culture tubes were incubated at 16 h photoperiod provided by cool white fluorescent lamps (25 μmol m^{-2} s^{-1}) at 25 °C, and sub-cultured at 2 week intervals.

Extraction and assay of the PPO

The extraction and assay of the PPO were carried out as described by Jyotsnabaran Halder et al. (26) with appropriate modifications as required. All the steps were carried out at 4 °C to avoid enzyme denaturation. Fresh leaves were collected and homogenized in a 100 mL phosphate buffer 0.05 M with a pH of 7.0, containing 0.35M KCl and 0.5% Triton X-100. The homogenate was filtered through cheesecloth, the filtrate was centrifuged at 12,500 × *g* (REMI, India) for 20 min at 4 °C, and the supernatant was collected and used as an enzyme source.

Assay of the PPO activity

PPO activity was determined by measuring the increase in absorbance at 420 nm, every 15 s, for up to 5 min, using a Shimadzu UV-160 spectrophotometer

in the presence of atmospheric oxygen. The reaction mixture contained 0.5 mL of enzyme solution and 2.5 mL of 100 mM catechol in 0.1 M phosphate buffer with a pH of 7.0 at 25 °C. The reaction mixture without the enzyme extract served as a control and the buffer was used as blank. Enzyme activity was calculated from the linear portion of the curve. The amount of enzyme that caused an increase in absorbance of 0.001 per min was defined as 1 unit of PPO activity. Protein concentration was determined by Bradford's dye-binding (27) assay using bovine serum albumin as standard.

Partial purification and characterization of the PPO

The crude extract of the PPO was subjected to saturation with ammonium sulfate at 10%-90% concentration. The resulting fractionates were centrifuged at 12,500 × *g* (REMI, India) for 35 min at 4 °C, and the pellets were dissolved in a 0.05 M phosphate buffer with a pH of 7.0, and subjected to dialysis overnight at 4 °C using the same buffer. The PPO activity of the dialysates was assayed using catechol as substrate. The dialysates that showed PPO activity were subjected for separation by column chromatography using Sephadex G-100 (Sigma, St. Louis, USA), using a 0.05 M phosphate buffer with a pH of 7.0 as the eluent buffer. The eluent fractions showing maximum absorbance at 280 nm were also screened for PPO activity using catechol as substrate. Those showing maximum PPO activity were subjected to native-PAGE activity staining (4% stacking gel and 7.5% resolving gel) and SDS-PAGE (5% stacking gel and separation was done in 12% gels) (28). For native-PAGE and activity staining, approximately 8 µg of protein (supernatant) was loaded into each well. Both native PAGE and SDS-PAGE gels were stained for proteins using staining solution containing 0.025% Coomassie brilliant blue R-250 in 40% methanol, 10% acetic acid, and 50% water. Destaining was carried out in a solution containing the above mixture without dye. For PPO activity staining, native gels were used with 100 mM catechol in a 0.05 M phosphate buffer with a pH of 7.0 for 3 h (29).

Substrate specificity

Substrate specificity was determined using different substrates (catechol, L-tyrosine, L-DOPA, and p-cresol). Michaelis constant (K_M) and maximum velocity (V_{max}) were determined for all 4 substrates. Concentrations of all substrate solutions were maintained at 100 mM, and were prepared in a 0.1 M phosphate buffer with a pH of 7.0.

Effect of pH and temperature on the activity

The effect of pH and temperature on PPO activity was determined using 100 mM catechol at 25 °C. For pH experiments, different pH environments were maintained using appropriate buffers of 0.1 M concentration. The buffers used were glycine-HCl buffer (2.0 to 3.5), acetate buffer (4.0-5.5), phosphate buffer (6.0-7.5), and Tris-HCl buffer (8.0-9.0). The effect of temperature on PPO activity was as determined by performing the assay at a range of temperatures from 10 °C to 80 °C using catechol at pH optima (6.5-7.0).

Effect of inhibitors

PPO activity was measured using different inhibitors such as citric acid, ascorbic acid, cysteine, urea, potassium ferricyanide, and potassium cyanide at 7 different concentrations (20 mM to 100 mM in a 0.1 M phosphate buffer with a pH of 7.0) for each of the respective inhibitors, using catechol as substrate. In addition to these concentrations, the substances that showed inhibition such as cysteine, ascorbic acid, citric acid, and potassium cyanide were also tested at concentrations 0.05 mM, 1 mM, and 2 mM. The volumes of enzyme and inhibitor solutions used were 0.5 mL and 2.5 mL, respectively.

Result and discussion

Shoot induction in nodal explants

Explant swelling (bulging) was initially observed within 3 to 5 days of inoculation, and organogenesis was observed after 15 days of incubation (Figure 1A and B) on MS medium supplemented with IAA (1.13 µM) and BAP (13.33 µM) (w/v).

Purification and activity staining of the PPO

The results of the purification of the PPO in 2 cultivars of *Mucuna* are shown in Table 1. As the purification steps are continued with ammonium sulfate and Sephadex G-100, the activity of the enzyme in both the cultivar extracts was found to increase in each and every step (Table 1). In the crude

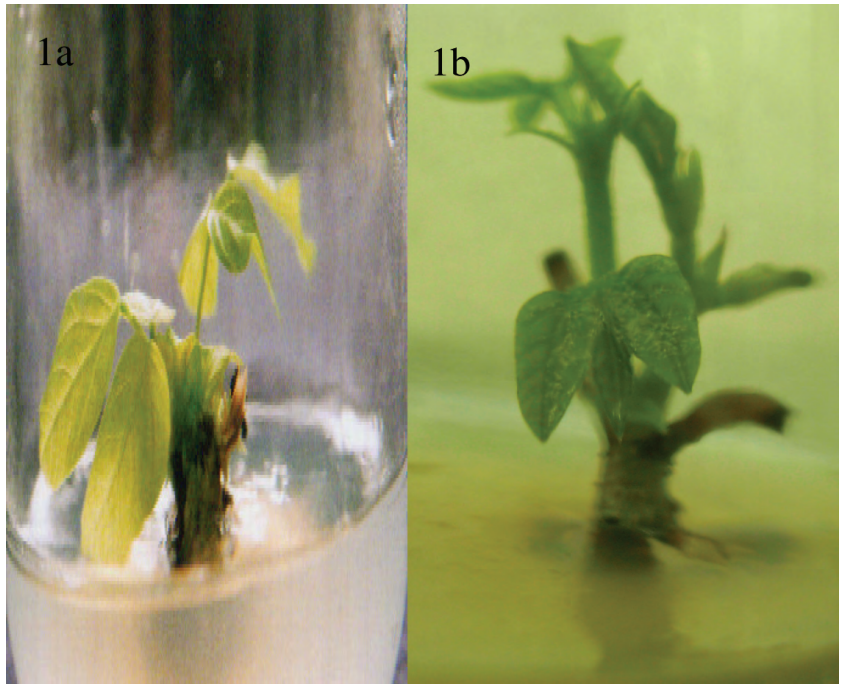

Figure 1. Micropropagation in 2 *Mucuna* species.
1a. Micropropagation in *M. pruriens*.
1b. Micropropagation in *M. prurita.*

Table 1. Partial purification of PPO from leaves of *M. pruriens* L. and *M. prurita* Hook.

Purification steps	Specific activity (units mg^{-1} protein)	
	Mucuna pruriens	*Mucuna prurita*
Crude extract	170	153
70% $(NH_4)_2SO_4$ fractionation	467 (2.747-fold)	452 (2.95-fold)
Dialysis	763 (4.48-fold)	742 (4.84-fold)
Sephadex G-100	1526 (9.27-fold)	1482 (9.66-fold)

extract of *Mucuna pruriens*, the activity was 170 units mg^{-1} protein, it was increased by 2.747-fold in 70% saturated ammonium sulfate fraction and increased by 9.27-fold in Sephadex G-100 eluted fraction. Similarly, in the crude extract of *Mucuna prurita,* the activity was 153 units mg^{-1} protein, it was increased by 2.95 fold in 70% saturated ammonium sulfate fraction and then increased by 9.66 fold in Sephadex G-100 eluted fraction. The enzyme activity was slightly greater in the crude extracts of *M. pruriens* compared to the crude extracts of *M. prurita*, while the fold purity was greater in the partially purified fraction of *M. prurita* than it was in *M. pruriens*.

Native PAGE analysis of the purified enzymes from both *M. pruriens* and *M. prurita* were resolved into single bands and that were confirmed by activity staining. SDS-PAGE analysis of purified PPO enzyme fractions revealed the presence of 4 bands (Figure 2a, b, and c) in each cultivar. From the results it could be predicted that the PPO enzyme of both *Mucuna* species is a tetrameric protein.

Substrate specificity

The substrate specificity for the PPO of both *Mucuna* cultivars was found to be similar; hence, the values are represented in a single table. The K_M values for catechol, L-DOPA, L-tyrosine, and p-Cresol are 4.12×10^{-3}, 16.4×10^{-3}, 6.12×10^{-3}, and 5.01×10^{-3}, respectively. Catechol was the best suited substrate, having the lowest K_M value and highest V_{max} value when compared to other 3 substrates (Table 2).

Effects of pH and temperature on PPO activity

The optimum pH for enzyme catalyzed oxidation of catechol in phosphate buffer was found to occur at a pH of 6.5 and 7.0 in both of the *Mucuna* cultivars. There was much less activity above and below this pH range (Figures 3 and 4). For both sources, the optimum temperature for PPO activity, with catechol as substrate and at a pH of 7.0 was between 30 °C and 40 °C. Here, as well, the activity was much less above and below this temperature range (Figures 5 and 6). In both of the *Mucuna* cultivars, the optimum pH for enzyme catalyzed oxidation of catechol in a phosphate buffer was found to occur at a pH of 6.5 and 7.0. The optimum pH of 6.5 and 7.0 was similar to the optimum pH of PPOs in royal cherry (30), guava (31), banana (32), and ferula (33). The optimum temperature for the maximum PPO activity with

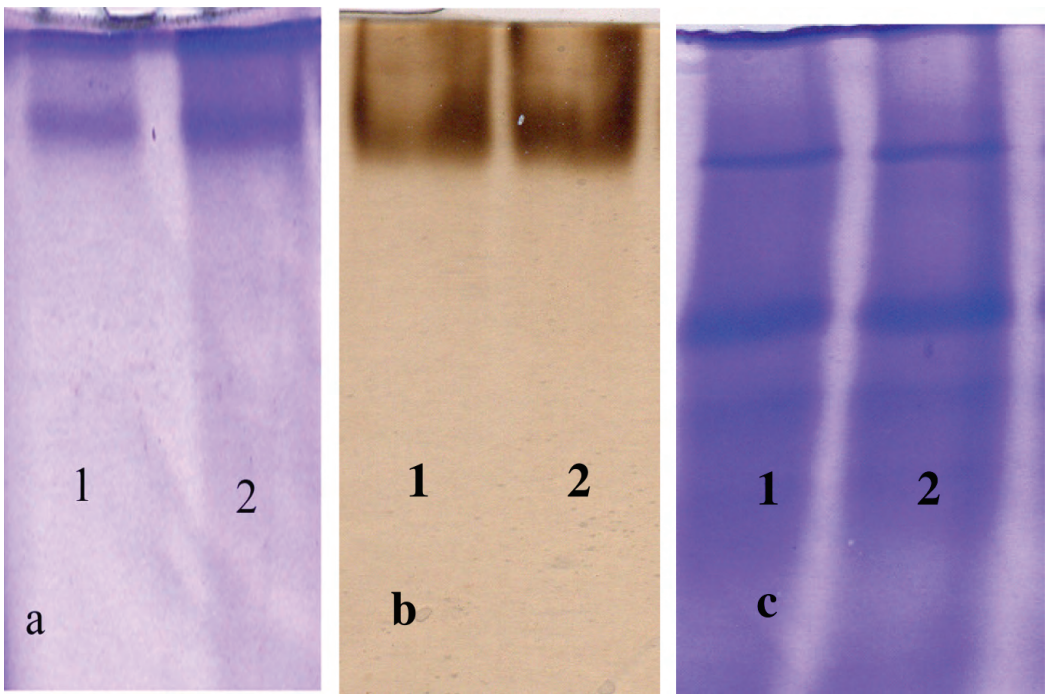

Figure 2. a. Native PAGE.
b. Activity (Native PAGE) staining for the PPO enzyme.
Lane 1. *Mucuna pruriens* L. Lane 2 *Mucuna prurita* Hook.
c. SDS-PAGE analysis of PPO.
Lane 1. Polypeptide subunits of *Mucuna pruriens* L.
Lane 2. Polypeptide subunits of *Mucuna prurita* Hook.

Table 2. Substrate specificity of PPO enzyme from *M. pruriens* L. and *M. prurita* Hook.

Substrate	Specific activity (units mg^{-1} protein)	K_m (μM)	V_{max} (abs min^{-1})
Catechol	2300	4.12×10^{-3}	3020
L-DOPA	430	16.40×10^{-3}	990
L-Tyrosine	1930	6.12×10^{-3}	2930
p-Cresol	2110	5.01×10^{-3}	2983

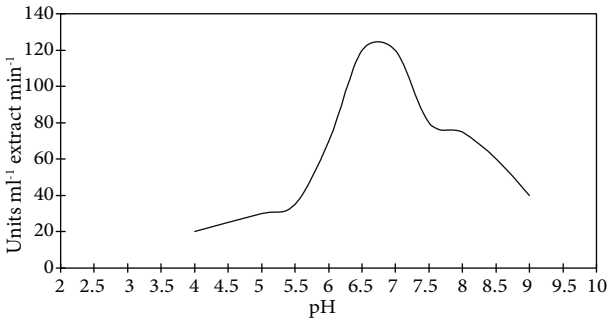

Figure 3. Effect of pH on PPO of *M. pruriens*.

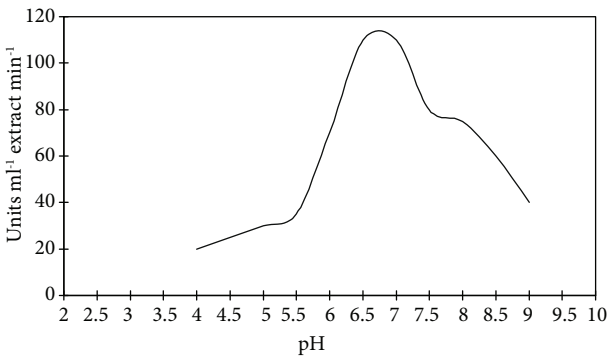

Figure 4. Effect of pH on PPO of *M. prurita*.

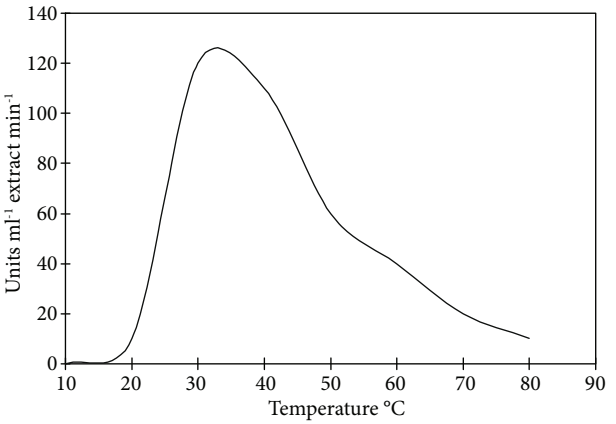

Figure 5. Effect of temperature on PPO of *M. pruriens*.

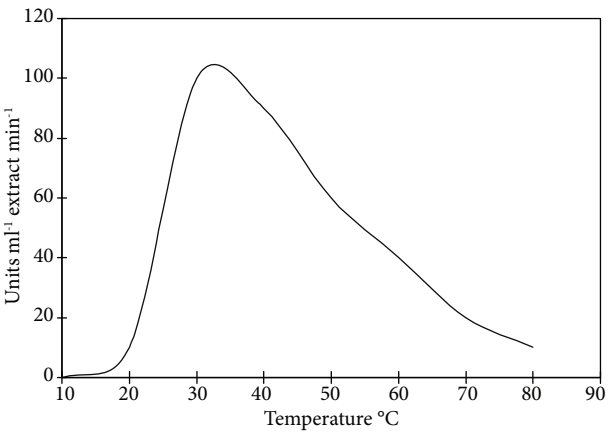

Figure 6. Effect of temperature on PPO of *M. prurita*.

catechol as substrate at a pH of 7.0 was between 30 °C and 40 °C, which is similar to the PPO of garland chrysanthemum (34).

Effect of inhibitors

The effects of various compounds on the PPO activity were provided in Tables 3 and 4. Enzyme activity was markedly inhibited by potassium cyanide, citric acid, cysteine, and ascorbic acid. However, potassium ferricyanide did not have any effect on the activity of PPO. Urea also showed the inhibitory effect, but only at a higher concentration (100 mM). Inhibition efficiency increased with the

Table 3. Effect of inhibitors on PPO enzyme activity.

Inhibitors (concentration in mM)	% inhibition			
	20	40	80	100
Ascorbic acid	100	100	100	100
Citric acid	100	100	100	100
Cystine	100	100	100	100
Potassium cyanide	100	100	100	100
Potassium ferricyanide	00	00	00	00
Urea	00	20	60	80

Table 4. Effect of inhibitors on PPO enzyme activity.

Inhibitors (concentration in mM)	% inhibition		
	0.05	1.0	2.0
Ascorbic acid	100	100	100
Citric acid	100	100	100
Cystine	80	90	100
Potassium cyanide	100	100	100

increase in concentration, from 20 mM to 100 mM. Ascorbic acid and potassium cyanide showed an inhibitory effect at even lower concentration (0.05 mM). Citric acid and cysteine also had the same effect, but not as much as that of ascorbic acid and potassium cyanide (Table 4). The effect of inhibitors on PPO activity of both plant varieties was found to be similar, though the PPOs were isolated from 2 individual sources; hence, the results are shown for 1 form of the enzyme (Tables 3 and 4). The enzyme was markedly inhibited by potassium cyanide, citric acid, cysteine, and ascorbic acid. According to Mayer (2), these inhibitors might inhibit the enzyme reaction by reacting with the enzyme molecule. Potassium cyanide might interact with the cofactor of the enzyme as a chelating agent. Ascorbic acid can react with amino groups in close proximity to the active site(s) of the enzyme through Strecker degradation. Cysteine on the other hand, may react directly with sulfhydryl groups with the reduction of o-quinone.

Polyphenol oxidases (PPOs) are a group of copper enzymes that are able to catalyze the oxidation of aromatic compounds in the presence of oxygen. The enzyme has monophenolase and diphenolase activities (35). Polyphenol oxidases were isolated from different sources like bacteria, fungi, and plants. Plant PPOs are involved in the oxidation of secondary metabolites like polyphenols (phenylpropanoids). In addition to these 2 other kinds of PPOs identified in plants, tyrosinases are involved in L-DOPA production by oxidizing L-tyrosine and laccases are involved in the production of melanin. Experimentally, tyrosinases and laccases have been classically differentiated on the basis of substrate specificity and sensitivity to inhibitors, although they can oxidize an overlapping range of diphenolic compounds. The most important difference is that only tyrosinases exhibit cresolase activity and only laccases can oxidize methoxy-phenols such as syringaldazine (36). Laccases have been found to be abundantly distributed in plants and many fungi, where their involvement in formation of melanin and a variety of different, and sometimes contradictory, physiological functions

have frequently been proposed (36). The PPO forms isolated from 2 *Mucuna* species in the present study oxidize catechol, a polyphenol, which confirms it as a PPO. In addition, the PPO has an affinity similar to that of catechol towards L-tyrosine and p-cresol, a monophenol (Table 2). This confirms that the isolated PPO is a tyrosinase, as it has shown cresolase activity, and it might be responsible for the L-DOPA production in *Mucuna* species. The tyrosinase activity of the PPO isolated in the present study is in concurrence with the previous findings reported for tyrosinase activity (37).

In conclusion, the isolated PPOs from 2 *Mucuna* species are tyrosinases and are tetrameric proteins and behave similarly where their substrate specificity, pH and, temperature optima are concerned. The enzyme is a tyrosinase and is sensitive to potassium cyanide, ascorbate, and citrate. Future studies are warranted so that the purified enzyme can be exploited for the overproduction of L-DOPA by in vitro cell culture and biotechnology approaches.

Corresponding author:

Raghavendra SATHYANARAYANA

Department of Biochemistry, P.G. Center,

Shivagangothri, Kuvempu University,

Tholahunsae, Davangere, Karnataka - INDIA

E-mail: raghu_rsn2004@rediffmail.com

References

1. Martins LO, Soares CM Pereira MM et al. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural Component of the *Bacillus subtilis* endospore coat. Journal of Biological Chemistry 277: 18849-18859, 2002.
2. Mayer AM, Staples RC. Laccase: new functions for an old enzyme. Phytochemistry 60: 551-565, 2002.
3. Claus H. Laccases and their occurrence in prokaryotes. Archives of Microbiology. 179:145-150, 2003.
4. Valderrama B, Oliver P, Medrano-Soto A et al. Antonie Van Leewenhoek 84: 289-299, 2003.
5. Claus H. Extracellular enzymes and peptides of lactic acid bacteria: significance for vinification. Micron. 25: 93-96, 2004.
6. Ruijssenaars HJ, Hartmans S. A cloned Bacillus halodurans multicopper oxidase exhibiting alkaline laccase activity. Applied Microbiology Biotechnology 65: 177-182, 2004.
7. Antorini M, Herpoël-Gimbert I, Choinowski T et al. Purification, crystallisation and X-ray diffraction study of fully functional laccases from two ligninolytic fungi. Biochem. Biophys. Acta. 1594: 109-114, 2002.
8. Hakulinen N, Kiiskinen LL, Kruus K et al. Crystal structure of a laccase from *Melanocarpus albomyces* with an intact trinuclear copper site. Natural Structural Biology 9: 601-605, 2002.
9. Piontek K, Antorini M, Choinowski T. Crystal structure of a laccase from the fungus *Trametes versicolor* at 1.90-Å resolution containing a full complement of coppers. Journal of Biological Chemistry 277: 37663-37669, 2002.
10. Patil SS, Zucker M. Potato phenolases: purification and properties. Journal of Biological Chemistry 240: 3938, 1965.
11. Jen JJ, Kahler KR. Characterization of polyphenol oxidase in peaches, grown in the Southeast. Horticulture Science 9: 590, 1974.
12. Flurkey WN, Jen JJ. Peroxidase and polyphenol oxidase activities in developing peaches. Journal of Food Biochemistry 4: 29, 1980.
13. Oktay M, Küfrevioglu OI, Kocaçalişkan I et al. Polyphenoloxidase from Amasya apple. Journal of Food Science 60: 494, 1995.
14. Janovitz-Klapp AH, Richard FC, Goupy PM et al. Inhibition studies on apple polyphenol oxidase. Journal of Agricultural Food Chemistry 38: 1437, 1990.
15. Kahn V. Effects of proteins, protein hydrolyzates, and amino acids on o-dihydroxy phenolase activity of polyphenol oxidase of mushroom, avocado and banana. Journal of Food Science 50: 11, 1985.
16. Galeazzi MA, Sgarbieriv C. Effects of proteins, protein hydrolyzates, and amino acids on o-dihydroxyphenolase activity of polyphenol oxidase of mushroom, avocado and banana. Journal of Food Science 46: 1404-1406, 1981.
17. Valero E, Varon R, Garcia-Carmona F. Characterization of polyphenol oxidase from Airen grapes, Journal of Food Science 53: 1482, 1988.
18. Sapers GM, Miller RL. Browning inhibition in fresh-cut pears. J. Food Sci. 63: 342-346, 1998.
19. Ben-Shalom N, Kahn V, Harel E et al. Catechol oxidase from green olives: properties and partial purification. Phytochemistry 16: 1153, 1977.
20. Ebelling W, Montgomery MW. Strawberry polyphenol oxidase: purification and characterization. Journal of Food Science 55: 1315, 1990.
21. Siddiq M, Sinha NK, Cash JN. Characterization of polyphenol oxidase from Stanley plums. Journal of Food Science 57: 1177, 1992.
22. Park EY, Luh BS. Polyphenoloxidase of kiwifruit. Journal of Food Science 50: 678, 1985.

23. Messerschmidt A, Huber R. The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. European Journal of Biochemistry 187: 341-352, 1990.

24. Ding Y, Kinjo J, Yang CR et al. Triterpenes from *Mucuna birdwoodiana*. Phytochemistry 30: 3703-3707, 1991.

25. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plant 15: 473-497, 1962.

26. Halder J, Tamuli P, Bhaduri AN. Isolation and characterization of polyphenol oxidase from Indian tea leaf (Camellia sinensis). Nutritional Biochemistry 9: 75-80, 1998.

27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.

28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680, 1970.

29. Vanloon LC. Tobacco polyphenol oxidases: a specific staining method indicating non-identity with peroxidases. Phytochemistry 10: 503-507, 1971.

30. Benjamin ND, Montgomery MW. Polyphenol oxidase of royal ann cherries: purification and characterization. Journal of Food Science 38: 799-806, 1973.

31. Augustin MA, Ghazali HM, Hashim H. Polyphenoloxidase from guava (*Psidium guajava* L.). Journal of the Science of Food and Agriculture 36: 1259-1265, 1985.

32. Yang CP, Fujita S, Ashrafuzzaman MD et al. Purification and characterization of polyphenol oxidase from banana (*Musa sapientum* L.) pulp. Journal of Agricultural Food Chemistry 48: 2732-2735, 2000.

33. Erat M, Sakiroglu H, Kufrevioglu I. Purification and characterization of polyphenol oxidase from Ferula sp. Food Chemistry 95: 503-508, 2006.

34. Nkya E, Kouno C, Li YJ et al. Purification and characterization of polyphenol oxidase from garland chrysanthemum (*Chrysanthemum coronarium* L.). Journal of Agricultural Food Chemistry 51: 5467-5471.

35. Vámos Vigyázó L. Polyphenol oxidase and peroxidase in fruits and vegetables. CRC Crit. Rev. Food Sci. Nutr. 15: 49, 1981.

36. Thurston CF. The structure and function of fungal laccases. Microbiology 140: 19-26, 1994.

37. Yoshida H. Chemistry of lacquer (*Urushi*) part I. Journal of Chemical Society 43: 231-237, 1883.