Plant Cell Physiol. 42(9): 969–975 (2001)

Isolation and Purification of Tyrosine Hydroxylase from Callus Cultures of *Portulaca grandiflora*

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Tyrosine hydroxylase was separated from polyphenol oxidase activity and was highly purified from betacyanin producing callus cultures of *Portulaca grandiflora*. The purified enzyme catalyzed the formation of DOPA (L-3,4-dihydroxyphenylalanine) from tyrosine and required the pterin compounds (6-methyl-5,6,7,8-tetrahydropterin; 5,6,7,8-tetrahydrobiopterin; 6,7-dimethyl-5,6,7,8-tetrahydropterin) as coenzyme. The K_m values for tyrosine and 6-methyl-5,6,7,8-tetrahydropterin were 0.5 mM and 0.15 mM, respectively. This enzyme was activated by Fe^{2+} and Mn^{2+}, and inhibited by metal chelating agents.

Key words: Betacyanin — DOPA — *Portulaca grandiflora* — Polyphenol oxidase (EC 1.10.3.1) — Pterin — Tyrosine hydroxylase (EC 1.14.16.2).

Abbreviations: BH_4, 5,6,7,8-tetrahydrobiopterin; $DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; DOPA, L-3,4-dihydroxyphenylalanine; DTT, dithiothreitol; $6MPH_4$, 6-methyl-5,6,7,8-tetrahydropterin.

Introduction

The red-violet betacyanins and yellow betaxanthins are a class of water-soluble pigments (betalain) characteristic of certain species of the plant order Caryophyllales and some fungi, e.g. *Amanita muscaria*.

All betalains contain a betalamic acid moiety, which spontaneously condenses with either an amino acid or amine to form yellow betaxanthins, or with a *cyclo*-L-3,4-dihydroxyphenylalanine (DOPA) derivative to form red-violet betacyanins (Schliemann et al. 1999; see Fig. 1). Feeding experiments with labeled tyrosine demonstrated incorporation of the amino acid (including amino nitrogen) into *cyclo*-DOPA and the betalamic acid moiety of betanin by red beets (Liebisch 1969). Phenylalanine is not incorporated, which indicates that shows this amino acid could not be converted to tyrosine (Liebisch 1969). The first step in betalain biosynthesis is the hydroxylation of tyrosine to DOPA (Mabry 1980). A previous report has indicated that tyrosine hydroxylation is the rate-limiting step in the biosynthesis of betalain (Hirano and Komamine 1994).

Previously identified tyrosine hydroxylating enzymes include tyrosine hydroxylase (EC 1.14.16.2) in animals (Nagatsu et al. 1964) and tyrosinase (EC 1.14.18.1 and EC 1.10.3.1) in animals (Yamamoto and Brumbaugh 1984) and in fungi (Mueller et al. 1996). Tyrosine hydroxylase catalyzes the formation of DOPA from tyrosine, which requires iron and coenzyme tetrahydrobiopterin for catalytic activity (Nagatsu et al. 1964). Rat brain tyrosine hydroxylase exists in two distinct physical forms, a soluble and a membrane-bound form (Kuczenski and Mandell 1972). However, to our knowledge there has been no previous report on about the occurrence of tyrosine hydroxylase in higher plants. Tyrosinase is a copper-containing monooxygenase, and is well known as the key enzyme for melanin biosynthesis in pigment cells. It is a bifunctional enzyme that catalyzes both the hydroxylation of tyrosine to DOPA and the subsequent oxidation of DOPA to DOPA quinone. In plants, tyrosine hydroxylation has been ascribed to polyphenol oxidase (EC 1.10.3.1) (Wichers et al. 1984, Mayer 1987). Polyphenol oxidase is a widely distributed copper-containing protein, which is responsible for the darkening in damaged plant tissue. It catalyzes the hydroxylation of monophenols to *o*-diphenols, and the oxidation of *o*-diphenols to *o*-quinones (Wichers et al. 1984, Mayer 1987).

In a study by Joy et al. (1995), the coincidental appearance of polyphenol oxidase-mRNAs and betacyanins content in maturing fruits of *Phytolacca americana* suggested the involvement of a polyphenol oxidase-like enzyme in betacyanin biosynthesis. There have been reports that polyphenol oxidase shows monophenol hydroxylating activity in addition to diphenol oxidase activity (Wichers et al. 1984, Mayer 1987), and it is generally believed that either polyphenol oxidase or tyrosinase is involved in betacyanin biosynthesis in plants (Endress 1979, Mueller et al. 1996). Both polyphenol oxidase and tyrosinase can convert tyrosine to DOPA, and both enzymes have the ability to oxidize DOPA to DOPA quinone. More recently, tyrosinase was reported to be involved in betalain biosynthesis in some higher plants (Steiner et al. 1999). However, we had already found a tyrosine-hydroxylating enzyme that was different from tyrosinase in betacyanin-producing callus cultures of *P. grandiflora* in preliminary experiment.

In this paper, we report the isolation and kinetic characterization of the tyrosine hydroxylase. To our knowledge this is

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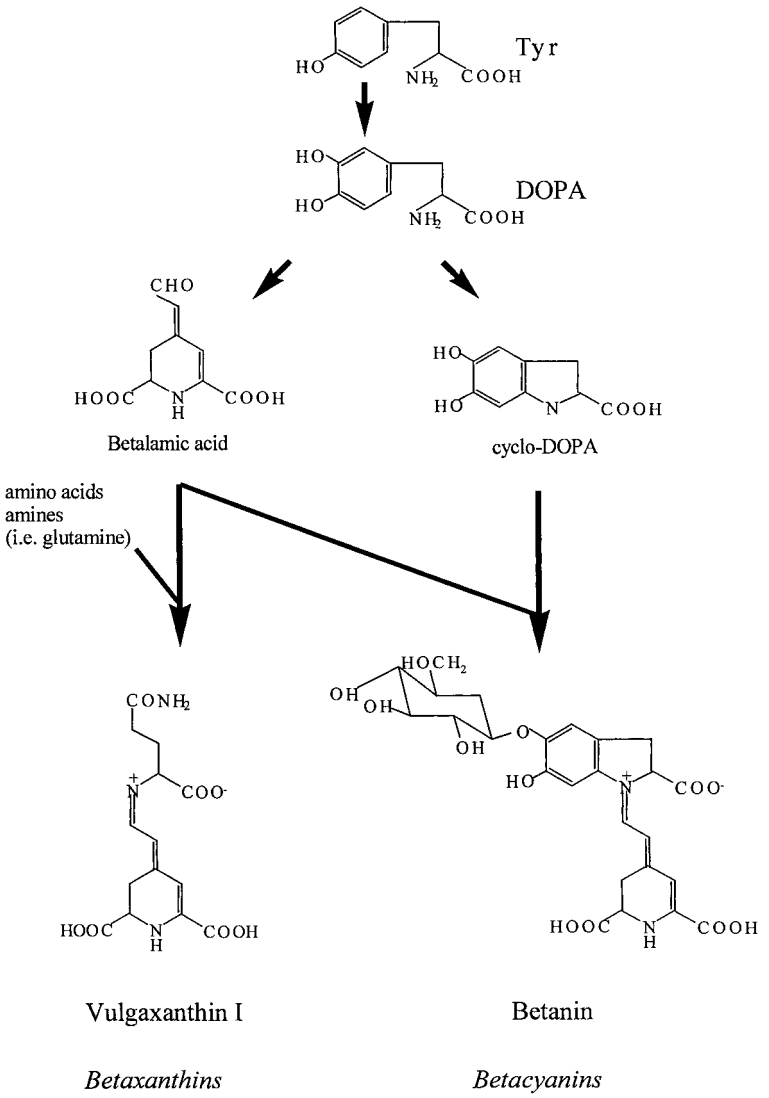

Fig. 1 Biosynthetic pathway of betalain.

the first report of tyrosine hydroxylase separation from polyphenol oxidase in higher plants.

Results

Evidence for the formation of DOPA from tyrosine in the enzyme system

The reaction mixture, consisting of the crude enzyme and [U-^{14}C]tyrosine, was incubated for 20 min at 30°C in the presence of 6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$). The reaction mixture included sodium ascorbate to inhibit DOPA oxidation. The relative location of the radioactivities and ninhydrin-positive areas on TLC are shown in Fig. 2. Two radioactive spots were found in the enzyme reaction mixture, and these coincided with the positions of authentic DOPA and tyrosine, respectively. In contrast, in the control, there was no spot corresponding to labeled DOPA (Fig. 2A). These results indicated that the enzyme product was possibly DOPA. Subsequently, the enzyme product was identified as [^{14}C-]DOPA by the recrystallization after the addition of unlabeled DOPA. The recrystallized compound showed a constant specific radioactivity after four recrystallization steps (Table 1).

Tyrosine hydroxylase activity was found mainly in the soluble protein fraction from the red callus and red suspension cultured cells of *P. grandiflora*, but was not detected in the white callus culture of *P. grandiflora* (data not shown).

Purification of enzyme

The soluble protein fraction from red callus was applied to a column of DEAE cellulose and eluted with a linear gradient

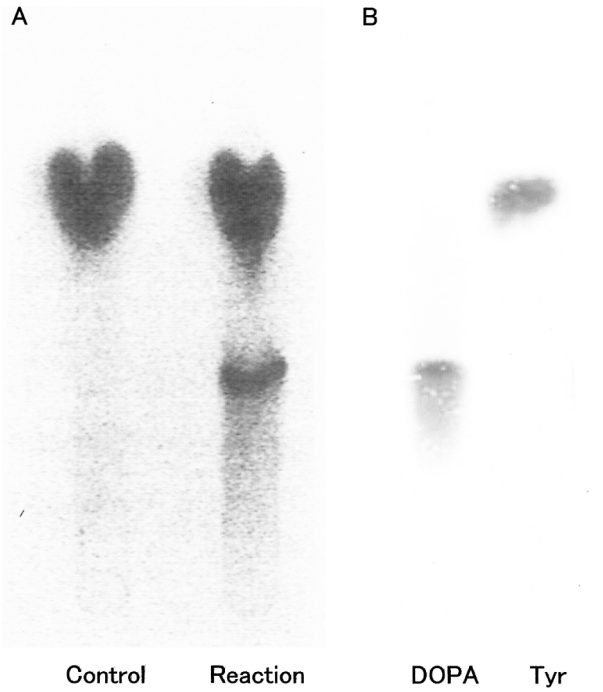

Fig. 2 Relative location of the radioactivities and ninhydrin-positive areas on a thin layer chromatogram. (A) Scanning patterns of radioactivities on TLC of the reaction products in reaction mixture. (B) The ninhydrin-positive areas of authentic DOPA and tyrosine.

of NaCl (Fig. 3A). Tyrosine hydroxylase was eluted as a single peak, and polyphenol oxidase was eluted as two peaks. The active hydroxylase fractions were chromatographed on a Sephacryl S-200 column, which clearly separated the polyphenol oxidase and tyrosine hydroxylase activities (Fig. 3B). The hydroxylase fractions were pooled and chromatographed on a Mono-Q column. The tyrosine hydroxylase fractions did not include polyphenol oxidase activity (Fig. 3C). Tyrosine hydroxylase was purified 140-fold by these procedures (Table 2).

Kinetics studies

The effect of tyrosine concentration on the activity of tyrosine hydroxylase is shown in Fig. 4A. The double-reciprocal plot gave a linear relationship. The apparent K_m value for tyrosine was 0.5 mM.

Pterin compounds are necessary for animal tyrosine hydroxylase activity. *Portulaca* enzyme also required the pterin compounds ($6MPH_4$, 5,6,7,8-tetrahydrobiopterin [BH_4], 6,7-dimethyl-5,6,7,8-tetrahydropterin [$DMPH_4$]) as coenzymes, but NADPH and NADH had no effect (Table 3). The effect of $6MPH_4$ concentration on enzyme activity is shown in Fig. 4B. The apparent K_m value for $6MPH_4$ was 0.15 mM.

Table 1 Recrystallization of radiolabeled DOPA

No. of recrystallizations	DOPA weight (mg)	Specific activity (dpm μmol^{-1})
1	13.72	440
2	9.62	484
3	6.09	462
4	2.91	451

The optimum pHs of tyrosine hydroxylase and polyphenol oxidase were pH 7.0 in 100 mM K-P_i buffer and pH 5.0 in 100 mM citrate buffer, respectively (data not shown).

Effect of metal ions on the enzyme reaction

The effects of the various metal ions and their chelators on the enzyme activity are given in Table 4. Among the metal ions examined, Fe^{2+} and Mn^{2+} were effective stimulators, showing activations of 186% and 136%, respectively. Cu^{2+} and Zn^{2+} showed somewhat inhibitory effects on the enzyme activity, and the chelators 2,2′-dipyridyl, 1,10-phenanthroline, tropolone, and mimosine inhibited the enzyme activity. Tropolone had a particularly strong inhibitory effect (3 to 10%) on the enzyme activity.

Estimation of molecular mass

The molecular masses of tyrosine hydroxylase and polyphenol oxidase were estimated to be 41 kDa and 53 kDa, respectively, by gel chromatography of Sephacryl S-200 columns.

Discussion

We found a tyrosine hydroxylating enzyme in betacyanin-producing callus cultures of *P. grandiflora*. In higher plants, the enzyme is considered to be a new enzyme, distinct from the reported enzymes based on the following evidence. It was reported that tyrosinase and polyphenol oxidase show tyrosine-hydroxylating activity additional to DOPA oxidation. In contrast, the enzyme reported in this paper was clearly separated from polyphenol oxidase activity. Our results differentiated the

Table 2 Purification of tyrosine hydroxylase from the red callus of *P. grandiflora*

Purification step	Protein (mg)	Total activity (pkat)	Specific activity (pkat (mg protein)$^{-1}$)	Purification (fold)	Yield (%)
80% $(NH_4)_2SO_4$	242.7	63.6	0.26	1.0	100
DEAE cellulose	3.42	8.8	2.56	9.8	14
Sephacryl S-200	0.07	1.6	23.58	90.7	2.5
Mono-Q	0.008	0.3	36.11	138.9	0.5

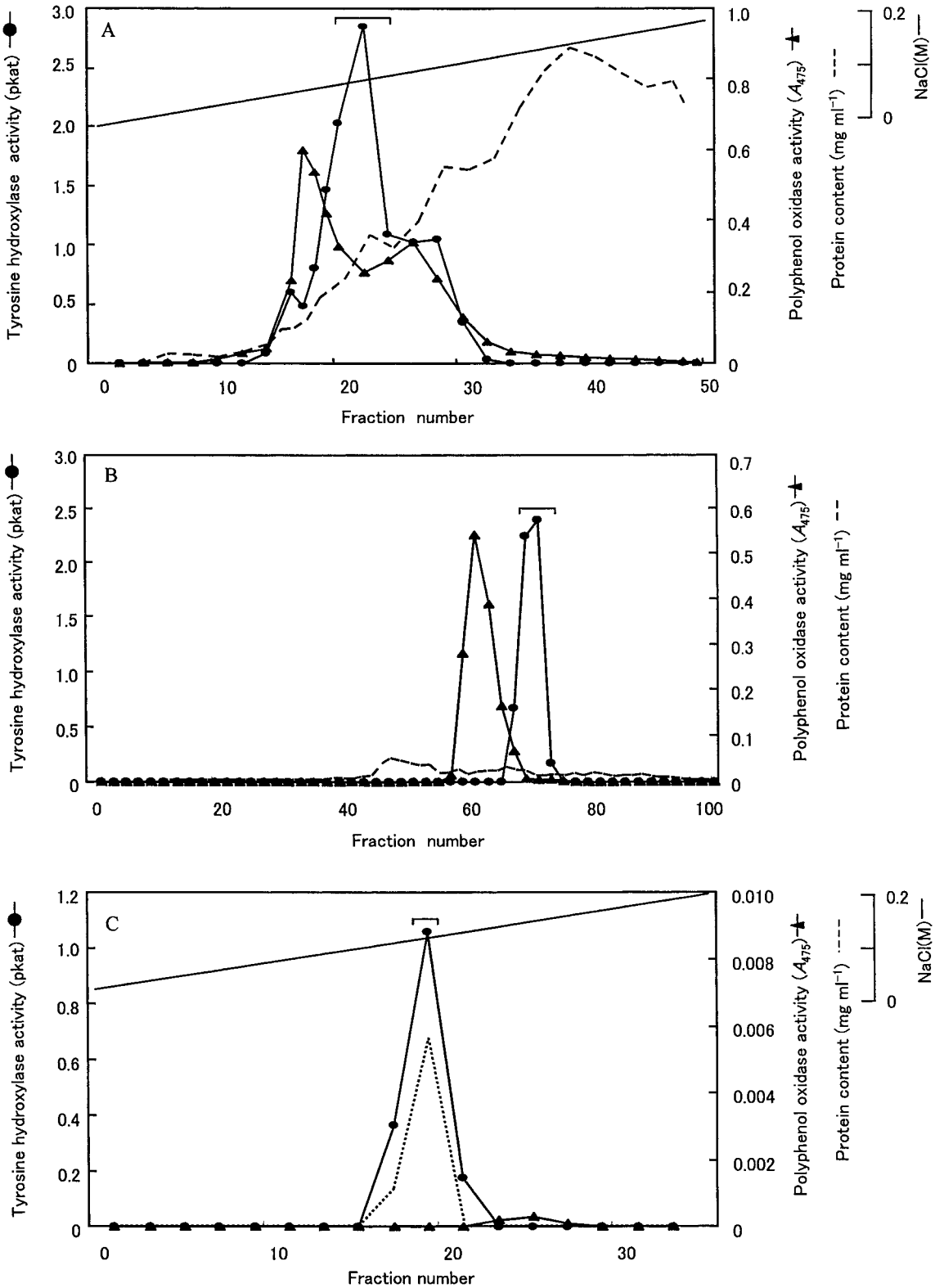

Fig. 3 Elution profiles of tyrosine hydroxylase and polyphenol oxidase on column chromatography. Closed circle, tyrosine hydroxylase activity; closed triangle, polyphenol oxidase activity; ---, protein content; —, NaCl gradient (panel A and C). Panel A; DEAE cellulose; panel B, Sephacryl S-200; panel C, Mono-Q column chromatography. Fractions indicated by bars were collected and pooled as enzyme fraction.

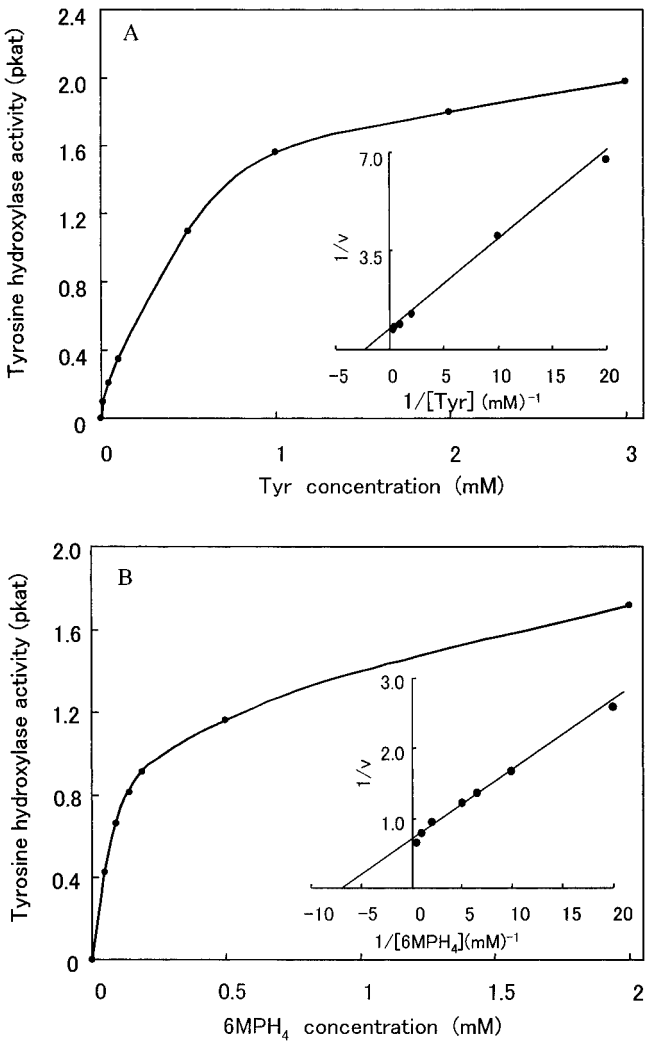

Fig. 4 The effect of tyrosine and $6MPH_4$ concentration on the enzymic reaction rate. The inset shows the double-reciprocal plots. The activity of tyrosine hydroxylase was carried out as described in Materials and Methods except for varying the concentrations of tyrosine (panel A) and $6MPH_4$ (panel B) in the reaction mixture.

Table 3 Electron donor specificity of tyrosine hydroxylase

Compounds	Tyrosine hydroxylase activity (pkat)
Control	0
NADH	0
NADPH	0
BH_4	1.5
$6MPH_4$	1.2
$DMPH_4$	0.7
Folic acid	0
Tetrahydrofolic acid	0

All compounds were tested at 2.0 mM in the standard assay mixture. Control was added the D.W. instead of compound.

Table 4 Effect of metal ions (A) and metal chelators (B) on the activity of tyrosine hydroxylase

A		
Metal ion	Conc (mM)	Relative activity (%)
None	—	100
Fe^{2+}	0.1	186
Cu^{2+}	0.1	71
Mg^{2+}	0.1	100
Zn^{2+}	0.1	73
Mn^{2+}	0.1	136
Co^{2+}	0.1	109
Al^{3+}	0.1	101
B		
Compound	Conc (mM)	Relative activity (%)
None	—	100
2,2′-Dipyridyl	1.0	69
	0.1	97
1,10-Phenanthroline	1.0	77
	0.1	100
Tropolone	1.0	3
	0.1	10
Mimosine	1.0	9
	0.1	41
EDTA	1.0	86
	0.1	100

enzyme from tyrosinase and polyphenol oxidase. Therefore, this enzyme is considered to be a new enzyme distinct from the polyphenol oxidase and tyrosinase reported in other higher plants (Wichers et al. 1984, Mayer 1987). The absence of tyrosine hydroxylase activity in betacyanin-less white callus indicates that this enzyme may be involve in betacyanin biosynthesis in *P. grandiflora*.

The use of a reaction mixture without the pterin compound resulted in complete loss of tyrosine hydroxylase activity. When the pterin compounds ($6MPH_4$, BH_4, $DMPH_4$) were added to the reaction mixture, the enzyme activity was detected (Table 3). Notably, when ammonium sulfate fraction was used as an enzyme source, in the first purification step, the tyrosine hydroxylase activity was detected without pterin compounds (data not shown). This inconsistency might be attributable to one of the following: the actual cofactor may have been bound to the enzyme in some manner, and/or the enzyme activity may have been lost due to dissociation of enzyme-pterin complex in the ion-exchange material. Consequently, addition of pterin compounds acted as an effective cofactor and recovered the tyrosine hydroxylase activity. Therefore, this enzyme is similar to mammalian tyrosine hydroxylase (Nagatsu et al. 1964, Markey et al. 1979, Oka et al. 1982).

The apparent K_m value of the purified enzyme was 0.5 mM for tyrosine when assayed in the presence of $6MPH_4$. This K_m value is higher than the value reported for mammalian tyrosine hydroxylase (Oka et al. 1982, Okuno and Fujisawa 1982). A K_m value of 0.076 mM was found for tyrosine hydroxylase from bovine caudate nucleus (Lloyd and Kaufman 1974); of 0.05 mM for that from bovine adrenal medulla (Oka et al. 1982); of 0.07 mM for that from rat adrenal gland (Okuno and Fujisawa 1982). The apparent K_m value of 0.15 mM for $6MPH_4$ of the purified tyrosine hydroxylase is in the range of those values found for tyrosine hydroxylase from other sources: a K_m value of 0.1 mM was found in that from rat adrenal (Ames et al. 1978); of 0.2 mM in that from pheochromocytoma PC-12 cells (Markey et al. 1979); of 0.26 mM in that from bovine adrenal medulla; and of 0.39 mM in that from bovine caudate nucleus (Oka et al. 1982).

With regard to some divalent cations on enzyme activity, it is of interest that this enzyme was markedly activated as well as mammalian tyrosine hydroxylase (Nagatsu et al. 1964, Petrack et al. 1968, Fitzpatrick 1989), by the addition of Fe^{2+}, and was inhibited by 2,2′-dipyridyl and 1,10-phenanthroline (Nagatsu et al. 1964). Pterin-dependent enzymes have been shown to require iron ions for activity (Nagatsu et al. 1964, Tong and Kaufman 1975). On the other hand, this enzyme was inhibited by Cu^{2+} and Zn^{2+}.

Very recently, Steiner et al. (1999) reported that tyrosine-hydroxylating enzyme had been partially purified from betacyanin-producing callus cultures of *P. grandiflora*, the same plant material used in this study. In the previous study, the enzyme was characterized as a tyrosinase by inhibition experiments and detection of concomitant *o*-diphenol oxidase activity (Steiner et al. 1999). In the present study, however, we were able to separate the activities towards tyrosine hydroxylation and DOPA oxidation that are catalyzed by polyphenol oxidase (Fig. 3), and properties of this enzyme was similar to mammalian tyrosine hydroxylase.

In conclusion, the tyrosine hydroxylase reported in this paper is a new enzyme in higher plants distinct from those reported earlier.

Materials and Methods

Cell culture

Callus cultures of red or white cell lines of *P. grandiflora* were maintained on a Murashige and Skoog medium (Murashige and Skoog 1962) containing 30 g liter^{-1} sucrose, 1.0 mg liter^{-1} 2,4-dichlorophenoxyacetic acid, 0.1 mg liter^{-1} kinetin and 0.3% (w/v) gellan gum. Cells were subcultured every 14 d by transfer onto 40 ml of fresh MS medium in 100 ml Erlenmeyer flasks. The cultures were kept at 25°C under white fluorescent light (33.3 μmol m^{-2} s^{-1}). Suspension cultures of *P. grandiflora* were grown at 25°C on a rotary shaker operating at 120 rpm. Subcultivation was carried out every 10 d by transferring cells into 100 ml of gellan gum free MS medium in 300 ml Erlenmeyer flasks.

Enzyme extraction

All preparatory operations were carried out at 2–4°C. Cultured cells were homogenized with Polytron for 2 min in 2 volumes (v/w) of cold 0.1 M KH_2PO_4/K_2HPO_4 (K-P_i) buffer (pH 7.0) that included 1.5 M NaCl, 15 mM sodium ascorbate, 0.5 mM dithiothreitol (DTT) and 1% (w/v) Polyclar AT. The homogenate was filtered through two layers of nylon cloth and centrifuged for 20 min at 33,000×*g*. The supernatant was loaded onto a column of Sephadex G-25 (3×15 cm), previously equilibrated with 10 mM K-P_i buffer (pH 7.0). The eluate was pooled and treated with solid ammonium sulfate. The protein fraction precipitated at 80% saturated ammonium sulfate for 90 min was collected by centrifugation for 20 min at 33,000×*g*, dissolved in a small amount of 10 mM Tris-HCl buffer (pH 8.0) and desalted using a Sephadex G-25 column (3×15 cm) equilibrated with the same buffer. The eluate was used as the soluble fraction. The precipitate fraction obtained by first centrifugation was washed with 10 mM Tris-HCl buffer (pH 8.0), suspended, sonicated, and then centrifuged for 20 min at 33,000×*g*. The resulting supernatant was loaded onto a column of Sephadex G-25 (3×15 cm), previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The eluate was used as the precipitate fraction.

Tyrosine hydroxylase activity

The standard assay mixture consisted of 15 μl of 0.1 M K-P_i buffer (pH 7.0), 30 μl of 0.1 mM L-tyrosine solution containing 5 mM sodium ascorbate, 2 mM $6MPH_4$ and [U-^{14}C]tyrosine (3.7 kBq in 30 μl of H_2O) and 45 μl of crude enzyme solution. The reaction was initiated by addition of enzyme solution and the mixture was incubated for 20 min at 30°C. The reaction was stopped by addition of 30 μl of 1 M HCl. As a control, the reaction was stopped at time zero. After centrifugation for 5 min at 10,000×*g*, 30 μl of supernatant were applied to a thin layer plate (Funacel SF, Funakoshi) and developed with solvent system (phenol : H_2O, 40 : 10, w/v). Radioactivities on the chromatograms were scanned with a bioimage analyser (BAS 1500, Fuji Photofilm). One unit of enzyme was defined as the amount which generated 1 μmol DOPA min^{-1} from tyrosine in the standard assay.

Polyphenol oxidase activity

Enzyme activities were determined spectrophotometrically by measuring the increase in absorbance at 475 nm for 5 min at 30°C with a spectrophotometer (V-550, JASCO). The reaction mixture consisted of 1.0 ml of citrate buffer (pH 5.0), 1.0 ml of 3 mM L-DOPA and 0.3 ml of enzyme solution. The reaction was carried out by addition of enzyme.

Recrystallization of radiolabeled DOPA

Radiolabeled DOPA eluted from a thin layer chromatogram was co-crystallized with an authentic specimen (20 mg) from *n*-propanol/water (6 : 4, v/v). The recrystallization was repeated until the compound showed a constant radioactivity.

Purification procedure of tyrosine hydroxylase

Cultured cells (300 g) were homogenized with Polytron for 2 min in 2 volumes (v/w) of cold 0.1 M K-P_i buffer (pH 7.0) including 1.5 M NaCl, 15 mM sodium ascorbate, 0.5 mM DTT and 1% (w/v) Polyclar AT. The homogenate was filtered through two layers of nylon cloth and centrifuged for 20 min at 33,000×*g*. The supernatant was loaded onto a column of Sephadex G-25 (3×15 cm) equilibrated with 10 mM K-P_i buffer (pH 7.0). The eluate was pooled and concentrated by ammonium sulfate precipitation (80% saturation). The precipitate was dissolved in a small amount of 10 mM Tris-HCl buffer (pH 8.0) and desalted by passing through it the Sephadex G-25 columns (3×15 cm) equilibrated with the same buffer. The enzyme solution was loaded onto a column of DEAE cellulose (2×35 cm) equilibrated with

10 mM Tris-HCl buffer (pH 8.0). After washing the column with same buffer, the protein was eluted with a linear gradient of 0 to 200 mM NaCl. The fractions showing tyrosine hydroxylase activity were pooled and concentrated by ultrafiltration (Centricell 20, Polysciences). The resulting concentrate was loaded onto a column of Sephacryl S-200 (3×100 cm) equilibrated with 10 mM K-P_i buffer (pH 7.0) containing 0.1 M NaCl. The fractions containing tyrosine hydroxylase activity were pooled and concentrated by ultrafiltration. The concentrate was loaded onto a column of Mono-Q 5/5 (Pharmacia Fine Chemicals) equilibrated with 10 mM K-P_i buffer (pH 7.0). The protein was eluted with a linear gradient of 0 to 300 mM NaCl.

Determination of protein

Protein concentration was determined by dye-binding method using bovine serum albumin as a standard (Bradford 1976).

Determination of molecular mass

The molecular mass of the purified protein was estimated by gel filtration chromatography on a Sephacryl S-200 column calibrated with the following globular proteins of known molecular mass: ribonuclease A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa) and bovine serum albumin (67 kDa). The molecular mass of the enzyme was calculated from a semi-log plot of marker proteins versus their relative migration distances.

Kinetics and other enzyme characterization

Kinetic constants were calculated from Lineweaver-Burk plots. K_m value and the effects of metal ions and chelators were assayed using the active fractions from the Sephacryl S-200.

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(Received February 23, 2001; Accepted July 2, 2001)