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Purification and Characterization of Polyphenol Oxidase from Callus Cultures of *Portulaca grandiflora*

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Abstract

Three isoforms of polyphenol oxidase (I-a, I-b and II) were purified from betacyanin-producing callus cultures of *Portulaca grandiflora*, by ammonium sulfate precipitation followed by successive column chromatographies of DEAE cellulose, Sephacryl S-200, hydroxyapatite and Mono-Q. The molecular masses of the monomeric polyphenol oxidase I-a and I-b were calculated to be 66 and 65 kDa, respectively, when analyzed by SDS-PAGE. Polyphenol oxidase II appeared to be a heterodimer of two subunits with molecular masses of 25 and 27 kDa. The optimal pH for polyphenol oxidase activity of I-a and I-b was 5.0, and for II was 6.0. These enzymes were markedly inhibited by sulfhydryl binding reagents, ascorbate, kojic acid, copper-chelating reagents and Fe^{2+}. L-DOPA (L-3,4-dihydroxyphenylalanin) was a good substrate for these enzymes, although no activity with tyrosine and hydroquinone was observed. The apparent *K*m values of polyphenol oxidase I-a, I-b and II for DOPA were 2.0, 2.2 and 3.5 mM, respectively. No monophenol oxidase (tyrosine hydroxylation) activity by these enzymes was observed.

Keywords: betacyanin, polyphenol oxidase, *Portulaca grandiflora*, tyrosine hydroxylation

Introduction

Polyphenol oxidase are also known as catechol oxidase, phenolase and *o*-diphenol oxygen oxidoreductase (EC 1.10.3.1) and catalyze the oxidation of *o*-diphenols to *o*-diquinones. Although the enzymatic properties have been studied in detail (Tolbert, 1973; Mayer and Harel, 1979; Vaughn and Duke, 1984), little information on its physiological roles is available.

Wichers *et al.* (1984) reported that DOPA (L-3,4-dihydroxyphenylalanin) was synthesized by the hydroxylation of tyrosine by phenol oxidase in cultured cells of *Mucuna pruriens*. Nagatsu *et al.* (1972) also reported that the formation of catecholamines in banana plants might be catalyzed by a phenol oxidase, which is different from animal tyrosine hydroxylase. It is generally believed that either polyphenol oxidase or tyrosinase is involved in betacyanin biosynthesis in plants (Mueller *et al.*, 1996; Steiner *et al.*, 1999). Some polyphenol oxidases as well as tyrosinase can convert tyrosine to DOPA, and have the ability to oxidize DOPA to DOPA quinone. Tyrosinase was reported to be involved in betalain biosynthesis in higher plants (Steiner *et al.*, 1999).

Recently, we described the separation of tyrosine hydroxylase activity from polyphenol oxidase activity and the purification of tyrosine hydroxylase from betacyanin producing callus cultures of *Portulaca grandiflora* (Yamamoto *et al.*, 2001). However, it remains a possibility that polyphenol oxidase in *P. grandiflora* may be able to hydroxylate L-tyrosine to L-DOPA, as well as to oxidize L-DOPA to DOPA quinone.

The purpose of the present study is to examine the properties of some polyphenol oxidase purified from betacyanin producing callus cultures of *P. grandiflora*.

Materials and Methods

Cell culture

Callus cultures of red cell lines of *P. grandiflora* were maintained on a MS medium (Murashige and

Skoog, 1962) containing 30 g l^{-1} sucrose, 1.0 mg l^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 mg l^{-1} kinetin and 0.3% (w/v) gellan gum. Cells were subcultured every 14 days by transfer onto 40 ml of fresh MS medium in 100 ml Erlenmeyer flasks. The cultures were kept at 25 ℃ under white fluorescent light (33.3 μmol m^{-2} s^{-1}).

Purification procedure of polyphenol oxidase

Cultured cells (300 g) were homogenized with Polytron for 2 min in 2 volume (v/w) of cold 0.1 M K Pi 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 2 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 x 15 cm) equilibrated with 10 mM K-Pi 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 the Sephadex G-25 column (3 x 15 cm) equilibrated with the same buffer. The enzyme solution was loaded onto a column of DEAE cellulose (2 x 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 polyphenol oxidase activity were pooled and concentrated by ultrafiltration (Centricell 20, Polysciences). The resulting concentrate was applied to a column of Sephacryl S-200 (3 x 100 cm) equilibrated with 10 mM K-Pi buffer (pH 7.0) containing 0.1 M NaCl. The fractions containing polyphenol oxidase activity were pooled and concentrated by ultrafiltration. The concentrate was applied to a column of hydroxyapatite (1 x 5 cm) equilibrated with 10 mM K-Pi buffer (pH 7.0). The protein was eluted with a linear gradient of 10 to 500 mM K-Pi buffer. The fractions containing polyphenol oxidase activity were pooled and concentrated by ultrafiltration. The concentrate was applied to a column of Mono-Q 5/5 (Pharmacia Fine Chemicals) equilibrated with 10 mM K-Pi buffer (pH 7.0). The protein was eluted with a linear gradient of 0 to 200 mM NaCl.

Polyphenol oxidase activity

Enzyme activities were determined spectrophotometrically by measuring the increase in absorbance at 475 nm for 5 min at 30 ℃ with a spectrophotometer (V-550, JASCO). The reaction mixture consisted of 1.0 ml of 100 mM 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. An increase in absorbance of 0.1 min^{-1} was taken as 1 unit of enzyme activity. The activity of tyrosine hydroxylation was assayed according to the method of Yamamoto *et al.* (2001).

Substrate specificity

The following substrates were used to monitor polyphenol oxidase activity spectrophotometrically: L- and D-DOPA (475 nm), catecol (410 nm), L- and D-tyrosine (475 nm), pyrogallol (400 nm), hydroquinone (490 nm), methyhydroquinone (490 nm). All substrates were used at a concentration of 3 mM in 100 mM citrate buffer (pH 5.0).

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 partially purified protein was estimated by gel filtration chromatography on a Sephacryl S-200 column calibrated with the following globular proteins of known molecular masses: 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.

Polyacrylamide gel electrophoresis

The purity of active fractions from column eluates was monitored by SDS-PAGE according to Laemmli (1970). The apparent molecular masses of the denatured proteins were calculated using a standard protein kit (Pharmacia) and found to be as follows: phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumnin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa). Electrophoresis was conducted in polyacrylamide gel at 20 mA gel^{-1} and the gel was silver-stained.

Kinetics and other enzyme characterization

Kinetic constants were calculated from Lineweaver-Burk plots. *Km* value and the effects of metal ions and chelators were assayed using the active fractions from the Mono-Q.

Results and Discussion

Purification of polyphenol oxidase I and II

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

Table 1 Purification of polyphenol oxidase I-a and I-b.

Purification step	Protein mg	Total activity unit	Specific activity unit/mg protein	Purification fold	Yield %
80%$(NH_4)_2SO_4$	242.7	1102	5	1	100
DEAE cellulose	4.4	73	17	3	6.6
Sephacryl S-200	0.7	20	28	6	1.8
Hydroxyapatite					
I-a	0.1	6	48	10	0.5
I-b	0.2	6	41	9	0.5
Mono-Q					
I-a	0.01	1.2	123	27	0.11
I-b	0.013	1.5	113	25	0.14

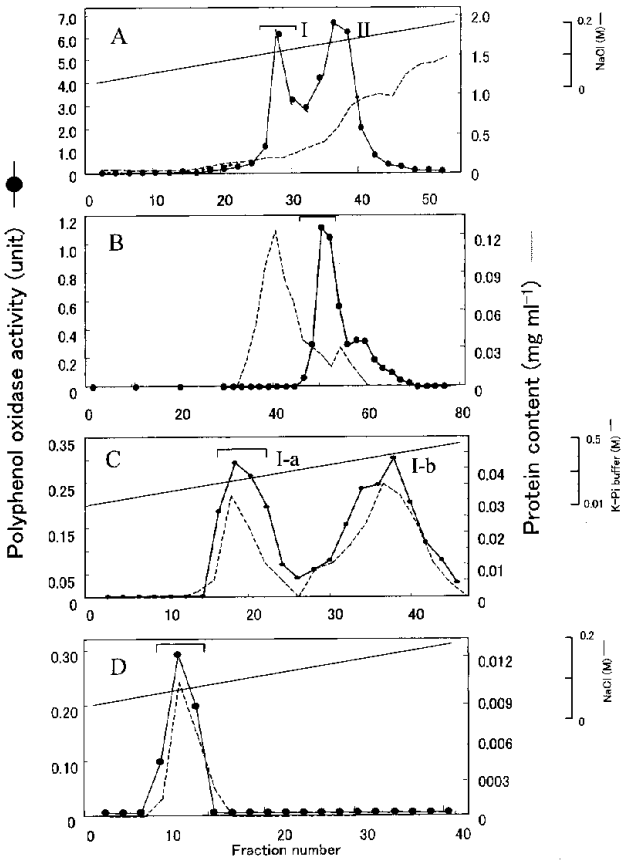

Fig. 1 Elution profiles of polyphenol oxidase I on column chromatographies. (●) polyphenol oxidase activity (---) protein content (—) NaCl gradient (A and D) K-Pi buffer (C). (A) DEAE cellulose (B) Sephacryl S-200 (C) Hydroxyapatite (D) Mono-Q

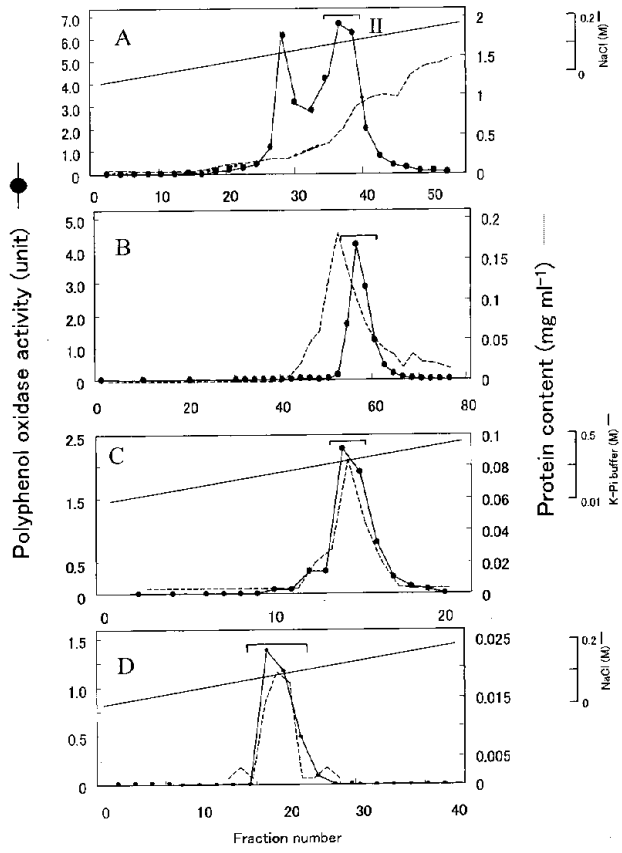

Fig. 2 Elution profiles of polyphenol oxidase II on column chromatographies. (●) polyphenol oxidase activity (---) protein content (—) NaCl gradient (A and D) K-Pi buffer (C). (A) DEAE cellulose (B) Sephacryl S-200 (C) Hydroxyapatite (D) Mono-Q.

with a linear gradient of NaCl (**Fig. 1A**). Polyphenol oxidase was eluted as two peaks. Peak I was chromatographed on a Sephacryl S-200 column (**Fig. 1B**). The active fractions of Peak I were pooled and chromatographed on a hydroxyapatite with a linear gradient of K-Pi buffer, whereupon the polyphenol oxidase was separated into two peaks (**Fig. 1C**). Fractions under individual activity peaks (designated I-a and I-b, respectively) were pooled, adsorbed on a Mono-Q column and eluted by increasing concentrations of NaCl. Typical data on the purification process are summarized in **Table 1**. Purifications of up to 27-fold and 25-fold were accomplished by these procedures for I-a and I-b, respectively.

The soluble protein fraction was applied to a column of DEAE cellulose and eluted with a linear gradient of NaCl (**Fig. 2A**). Fractions containing

Table 2 Purification of polyphenol oxidase II.

Purification step	Protein mg	Total activity unit	Specific activity unit/mg protein	Purification fold	Yield %
80% $(NH_4)_2SO_4$	282.6	1800	6	1	100
DEAE cellulose	9.3	216	33	5	12
Sephacryl S-200	1.5	105	69	10	5.8
Hydroxyapatite	0.8	42	136	21	2.3
Mono-Q	0.3	21	242	37	1.2

Table 3 Effect of metal ions on the activities of polyphenol oxidase. Metal ions were preincubated with the enzyme protein for 5 min at 30 ℃ before the addition of the substrate into the reaction mixture.

Compounds	Relative activity (%)		
	I-a	I-b	II
Control	100	100	100
Ca^{2+}	105	101	83
Mg^{2+}	98	101	88
Fe^{2+}	12	32	20
Cu^{2+}	93	119	89
Zn^{2+}	88	81	79
Mn^{2+}	92	99	88
Co^{2+}	85	97	89
Al^{3+}	99	92	91

Table 4 Effect of metal chelators on the activities of polyphenol oxidase . These compounds (1 mM) were preincubated with the enzyme protein for 5 min at 30 ℃ before the addition of the substrate into the reaction mixture.

Compounds	Relative activity (%)		
	I-a	I-b	II
Control	100	100	100
2,2'-Dipyridyl	91	96	97
1,10-Phenathroline	97	100	99
Tropolone	0	0	0
Mimosine	12	21	18
EDTA	95	100	98

enzyme II were pooled and applied to a Sephacryl S-200 column. The elution profile is shown in **Fig. 2B**. The active fractions were pooled and applied to hydroxyapatite (**Fig. 2C**). The enzyme protein from hydroxyapatite column chromatography was adsorbed on a Mono-Q column and eluted by increasing concentrations of NaCl. Enzyme II eluted at around 0.1 M NaCl (**Fig. 3D**). Typical data on the purification process are summarized in **Table 2.** Purification of up to 38-fold was accomplished by the procedure for enzyme II.

Table 5 Effect of various compounds on the activities of polyphenol oxidase . These compounds (1 mM) were preincubated with the enzyme protein for 5 min at 30 ℃ before the addition of the substrate into the reaction mixture.

Compounds	Relative activity (%)		
	I-a	I-b	II
Control	100	100	100
NEM	83	75	84
Glutathione	2	5	4
PCMB	4	6	5
Iodoacetate	54	61	69
Kojic acid	0	0	0
Ascorbate	0	0	0

pH optimum

The optimum pH of polyphenol oxidase I-a and I-b was pH 5.0, and that of II was pH 6.0 in 100 mM citrate buffer, respectively (data not shown). Differences in polyphenol oxidase pH optima with various substrates were reported (Wichers *et al.*, 1984; Fujita *et al.*, 1991; Rocha and Morais, 2001) varying from 4.0 to 7.8, depending on the origin of the material, the extraction method and substrate.

Effects of metal ions, metal chelators and sulfhydryl binding agents on the enzyme reaction

The effects of the various metal ions and their chelators on the enzymes are summarized in **Tables 3** and **4**. The enzymes were markedly inhibited by the addition of Fe^{2+} as well as longan fruit enzyme (Jiang, 1999) and by copper-chelating reagents (tropolone and mimosine) as well as other sources (Halder *et al.*, 1998). The enzymes were also inhibited by sulfhydryl binding reagents such as PCMB (p-chloromercuribenzoate), iodoacetate, NEM (N-

ethylmaleimide) and glutathione, suggesting the possibility that a sulfhydryl group may be involved in the reaction (**Table 5**). Furthermore, these enzymes were also inhibited by kojic acid and ascorbate. A poor inhibitor of these enzymes by EDTA was similar to the response to EDTA by polyphenol oxidase of eggplant (Fujita and Tono, 1988) and head lettuce (Fujita *et al.*, 1991).

Estimation of molecular mass

The molecular mass of I-a and I-b in the denatured state were calculated to be 66 and 65 kDa, respectively (**Fig. 3**). Comparing the elution volumes of I-a and I-b from the gel filtration column of Sephacryl S-200 with those of known molecular standards, the molecular masses of I-a and I-b in the native state were each calculated to be approximately 65 kDa. Thus, I-a and I-b are considered to be monomers in the native state. II showed a native molecular mass of 53 kDa, whereas under denaturing conditions molecular masses of 27 and 25 kDa were found (**Fig. 3**). Therefore, the native enzyme II probably occurs as a heterodimer. The molecular mass of *Mucuna* enzyme was found to be 90 kDa and dimeric (Wicher *et al.*, 1984), that of spinach enzyme to be 158 kDa and tetrameric (Golbeck and Cammarata, 1981) and that of potato enzyme to be 59 kDa (Kowalski *et al.*, 1992). A broad range of molecular mass has thus been reported for the polyphenol oxidase of higher plants.

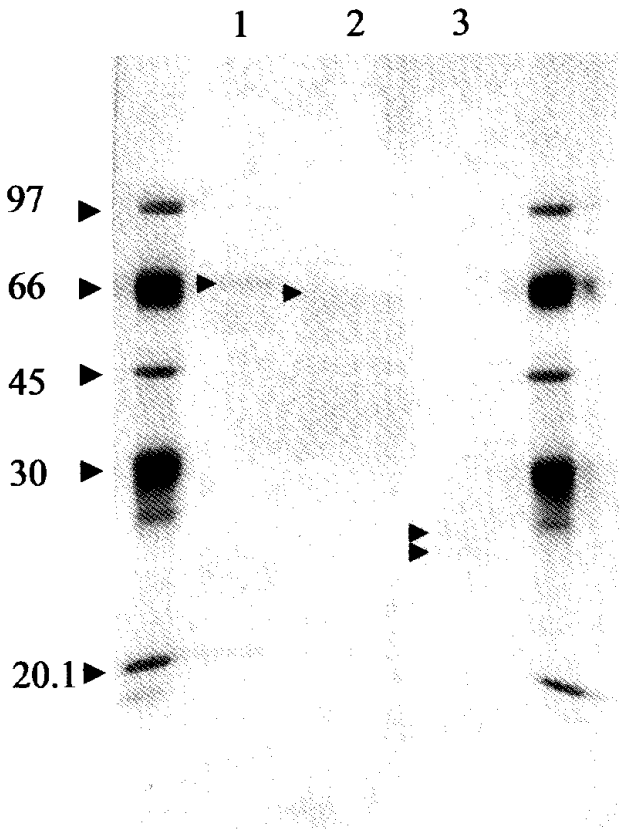

Fig. 3 SDS-PAGE protein patterns of purified polyphenol oxidase.
Lane 1, enzyme I-a; lane 2, enzyme I-b; lane 3, enzyme II. Molecular masses of standard proteins are indicated on the left and right in kDa. Gel was stained with silver nitrate.

Substrate specificity

The activity of polyphenol oxidase with DOPA, tyrosine, catecol, pyrogallol and hydroxyquinone was examined (**Table 6**). All three of the polyphenol oxidases oxidized L-DOPA, D-DOPA and catechol. The activity of enzyme I-a toward catechol was higher than that of I-b or II. All three enzymes had very low activity levels toward pyrogallol. No activity toward tyrosine, hydroquinone or methylhydroquinone was observed.

Double reciprocal plots of the effects of DOPA concentrations on polyphenol oxidase activities show a linear relationship (**Fig. 4**). The apparent *K*m values of polyphenol oxidase I-a, I-b and II with DOPA as substrate were 2.0, 2.2 and 3.5 mM, respectively. These *K*m values are lower than the values reported for other plant polyphenol oxidases: Shin *et al.* (1997) reported a *K*m value of 24 mM for the enzyme from mung bean leaf, Partington and Bolwell (1996) a *K*m of 4.3 mM for the enzyme from potato tuber and Tolbert (1973) a *K*m of 7 mM for the enzyme from spinach leaf. The Vmax (unit min^{-1}) of I-a, I-b and II for DOPA was 0.8, 0.76 and 1.01, respectively.

Wichers *et al.* (1984) reported that phenol oxidase isolated from *Mucuna pruriens* was able to hydroxylate L-tyrosine to L-DOPA, and Nagastu *et al.* (1972) pointed out that phenol oxidase extracted from banana takes part in the formation of catecho-

Table 6 Substrate specificity of polyphenol oxidase I-a,b and II. The mixture of the enzyme and substrates (3 mM) was incubated for 5 min at 30 ℃. Rate of oxidation of L-DOPA was taken as 100%.

Substrate	Relative activity (%)		
	I-a	I-b	II
L-DOPA	100	100	100
D-DOPA	54	60	48
Catecol	90	43	42
L-Tyrosine	0	0	0
D-Tyrosine	0	0	0
Pyrogallol	5	8	15
Hydroquinone	0	0	0
Methylhydroquinone	0	0	0

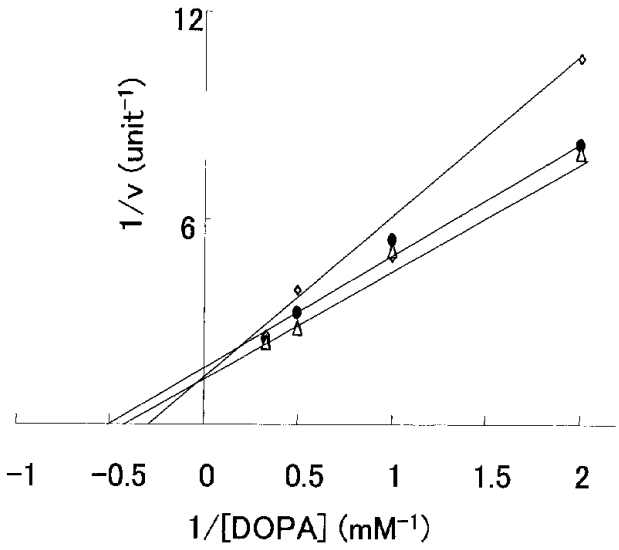

Fig. 4 Lineweaver-Burk plots of L-3,4-dihydroxyphenylalanin (DOPA) concentration. The activity of polyphenol oxidases was carried out as described in Materials and Methods except for varying the concentration DOPA in the reaction mixture. (●) enzyme I-a (△) enzyme I-b (◇) enzyme II.

lamines. However, the three polyphenol oxidases (I-a,b and II) purified from *P. grandiflora* did not catalyze the hydroxylation of tyrosine. In other words, these enzymes had no monophenol oxidase activity (tyrosine hydroxylating activity). Therefore, these enzymes seem to be different from the enzymes of the *Mucuna* plant and the banana. In addition, since these enzymes did not oxidize *p*-diphenol (hydroquinone and methylhydroquinone), the possibility of their being a laccase is excluded. As a result, it is concluded that the purified polyphenol oxidases from *P. grandiflora* are not involved in tyrosine hydroxylation but in oxidation of o-diphenols.

Schliemann *et al.* (1998) reported that DOPA oxidation to DOPA quinone is involved in betacyanin biosynthesis. Therefore, since the purified polyphenol oxidases from *P. grandiflora* catalyze the oxidation DOPA, it is possible that they may play a part in betacyanin biosynthesis.

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