Tyrosinase involved in betalain biosynthesis of higher plants

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Abstract. A tyrosine-hydroxylating enzyme was partially purified from betacyanin-producing callus cultures of Portulaca grandiflora Hook. by using hydroxyapatite chromatography and gel filtration. It was characterized as a tyrosinase (EC 1.14.18.1 and EC 1.10.3.1) by inhibition experiments with copper-chelating agents and detection of concomitant o-diphenol oxidase activity. The tyrosinase catalysed both the formation of L-(3,4-dihydroxyphenyl)-alanine (Dopa) and cyclo-Dopa which are the pivotal precursors in betalain biosynthesis. The hydroxylating activity with a pH optimum of 5.7 was specific for L-tyrosine and exhibited reaction velocities with L-tyrosine and D-tyrosine in a ratio of 1:0.2. Other monophenolic substrates tested were not accepted. The enzyme appeared to be a monomer with an apparent molecular mass of ca. 53 kDa as estimated by gel filtration and SDS-PAGE. Some other betalain-producing plants and cell cultures were screened for tyrosinase activity; however, activities could only be detected in red callus cultures and plants of P. grandiflora as well as in plants, hairy roots and cell cultures of Beta vulgaris L. subsp. vulgaris (Garden Beet Group), showing a clear correlation between enzyme activity and betacyanin content in young B. vulgaris plants. We propose that this tyrosinase is specifically involved in the betalain biosynthesis of higher plants.

Key words: Beta – Betacyanin – Betalain – Polyphenol oxidase – Portulaca – Tyrosinase

Introduction

The classical tyrosinases belong to a group of copper proteins, polyphenol oxidases (PPOs), that not only exhibit monophenol monooxygenase activity (EC 1.14.18.1) leading, for example, from tyrosine to L-(3,4-dihydroxyphenyl)-alanine (Dopa; tyrosine hydroxylase activity), but also exhibit o-diphenol oxidase activity (EC 1.10.3.1.), leading to benzoquinone structures, e.g. from Dopa to Dopaquinone (Dopa oxidase activity). Dopaquinone undergoes a spontaneous cyclization to form cyclo-Dopa which might polymerise via Dopachrome to form protecting melanin in the skin of animals. A likewise protective function might be ascribed to its role in most plants in defence reactions of plants against insects and microbial pathogens; however, this has yet to be proven.

The function of tyrosinase in forming Dopa as an endproduct, which occurs in various plant tissues (Tera-moto and Komamine 1988), or as an intermediate metabolite, e.g. in the biosynthesis of benzylisoquinoline alkaloids (Rueffer and Zenk 1987), has been shown. The involvement of both hydroxylation and oxidation in plants during the biosynthesis of low-molecular-weight natural products such as betacyanins, has been assumed, but has not yet been shown conclusively. Constabel and Haala (1968) observed in betanin-producing callus cultures of Beta vulgaris L. var. crassa higher phenol oxidase activities than in non-producing cultures. The effects of enzyme cofactors and inhibitors on betacyanin accumulation in callus cultures of Portulaca grandiflora (Endress 1979) suggested the involvement of a tyrosinase in betacyanin biosynthesis. This assumption has been supported by the detection of PPO transcripts which correlates with betacyanin accumulation in fruits of Phytolacca americana (Joy et al. 1995) as well as by the description of a tyrosinase from the betalain-accumulating pileus of Amanita muscaria (Mueller et al. 1996). Both tyrosinase reactions might have a function in betalain biosynthesis, with Dopa as pivotal precursor of the red to red-violet betacyanins and the yellow betaxanthins, i.e. the water-soluble pigments from members of certain
families of the plant order Caryophyllales (Steglich and Strack 1990). This role has been deduced from feeding experiments in the early days of research on betalain biosynthesis (e.g. Höhrhammer et al. 1964; Minale et al. 1965). Enzymatic Dopa oxidation to Dopaquinone followed by spontaneous cyclization leads to cyclo-Dopa, which reacts with betalamic acid to form betanidin.

Furthermore, Dopa undergoes an extradiol cleavage (Fischer and Dreiding 1972; Impellizzeri and Piattelli 1972) leading via 4.5-seco-Dopa to betalamic acid, which likewise reacts with various amino acids and amines to form the betaxanthins. The Dopa dioxygenase involved in the reaction has been partially characterized (Girod and Zryd 1991a; Terradas and Wyler 1991) and cloned in the reaction has been partially characterized (Girod and Zryd 1991a; Terradas and Wyler 1991) and cloned (Hinz et al. 1997) from the fly agaric (Amantia muscaria), but the corresponding enzyme has not yet been detected in higher plants.

Recently, we found tyrosine-hydroxylating activities in callus cultures and seedlings of Portulaca grandiflora by using an optimised HPLC method applying fluorescence detection (Steiner et al. 1996). In continuation of this study we here report on the characterization of tyrosinase activities involved in betalain biosynthesis of P. grandiflora and Beta vulgaris L. subsp. vulgaris (Garden Beet Group).

Materials and methods

Plant and cell cultivation. Callus cultures of Portulaca grandiflora Hook., line K64 (red) and W63 (white), were maintained on a modified LS medium (Linsmaier and Skoog 1965) containing 20 g l\(^{-1}\) sucrose, 2 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid (2,4-D), 0.2 mg l\(^{-1}\) kinetin and 8 g l\(^{-1}\) agar (Bacto agar, Difco, Detroit, Mich., USA). Cells were subcultured every 14 d by transferring cells onto 30 ml of solidified LS medium in 100-ml Erlenmeyer flasks. The cultures were kept at 25 °C under a 16-h-d regimen (65 μmol m\(^{-2}\) s\(^{-1}\)). Intact P. grandiflora plants were grown from seeds (Erfurter Samen- und Pflanzenzucht GmbH, Erfurt, Germany) in a greenhouse under natural daylight conditions and then transferred into the culture (IPB).

Suspension cultures from Beta vulgaris L. subsp. vulgaris ‘Rote Kugel’ (Garden Beet Group: nomenclature according to Lange et al. 1998), a variety with a red-coloured hypocotyl, were grown at 25 °C under a 16-h-d regimen (65 μmol m\(^{-2}\) s\(^{-1}\)) on a shaker operating at 120 rpm. Subcultivation was carried out every 14 d by transferring cells into 30 ml of modified hormone-free MS medium (Murashige and Skoog 1962) containing 21.1 mg l\(^{-1}\) Na\(_{2}\)EDTA and 15.7 mg l\(^{-1}\) FeSO\(_4\) \(\cdot\) 7H\(_2\)O in 250-ml Erlenmeyer flasks.

Furthermore, from B. vulgaris L. subsp. vulgaris (Garden Beet Group) plants a betacyanin-producing hairy-root culture, established by I. Kuzovkina, was grown in the dark at 25 °C on a shaker operating at 120 rpm. Subcultivation was carried out every 14 d by transferring three root tips (2 cm) into 30 ml of modified hormone- and glycine-free half-strength MS medium containing 20 g l\(^{-1}\) sucrose, 660 mg l\(^{-1}\) CaCl\(_2\), 2H\(_2\)O, 80 mg l\(^{-1}\) myo-inositol, 0.1 mg l\(^{-1}\) pyridoxine \(\cdot\) HCl, and 0.1 mg l\(^{-1}\) thiamine \(\cdot\) HCl in 100-ml Erlenmeyer flasks.

Intact red beet plants [B. vulgaris L. subsp. vulgaris ‘Cylinda’ (Garden Beet Group) or ‘Renova’ (Garden Beet Group)] were grown from seeds in a greenhouse under natural daylight conditions. Seed material of the cultivar ‘Cylinda’ was obtained from Dom Samen (Kevelaer, Germany) and of the cultivar ‘Renova’ from Saatzucht Quedlinburg (Quedlinburg, Germany).

Hairy-root cultures from B. vulgaris L. subsp. vulgaris ‘Golden Beet’ (Garden Beet Group), a variety with yellow-coloured hypocotyls, lines 5A and 7, were grown at 25 °C under a 16-h-d regimen (65 μmol m\(^{-2}\) s\(^{-1}\)) on a shaker operating at 120 rpm. Subcultivation was carried out every week by transferring three root tips (2 cm) into 30 ml of modified 2,4-D-free B5 medium (Gamborg et al. 1968) containing 30 g l\(^{-1}\) sucrose, 18.6 mg l\(^{-1}\) Na\(_{2}\)EDTA, and 13.8 mg l\(^{-1}\) FeSO\(_4\) \(\cdot\) 7H\(_2\)O in 100-ml Erlenmeyer flasks.

Cell-suspension and callus cultures of Dorotheanthus bell-idiformis (Burm. f.) N.E.Br. (Heuer et al. 1996), Chenopodium rubrum L. (Bokern et al. 1991) and Phytolacca americana L. were cultivated as described earlier (Schliemann et al. 1996).

Chemicals. l-Tyrosine, l-Dopa, l-ascorbate and Polyclar AT were obtained from Serva, catalase, dopamine, caffeic acid, chlorogenic acid, Bradford dye-binding method (Bradford 1976) using human serum albumin as a standard. For protein purification with hydroxyapatite chromatography as the first step, the crude extract was desalted in 20 mM KPi (pH 6.5) while for protein purification starting with Celite 535 the extract was desalted in 10 mM KPi (pH 7.0). Before column chromatography on hydroxyapatite and Celite, the crude extracts were diluted to 10 mM and 5 mM KPi, respectively.

Cell culture material and plant organs from B. vulgaris and P. grandiflora were homogenized in the extraction buffer with 10% Polyclar AT (w/v) and 50 mM ascorbate. Plant material was homogenized in a Waring Blender with the exception of roots from P. grandiflora and hypocotyls from seedlings (4–12 d old) of B. vulgaris which were homogenized in a mortar in the presence of liquid nitrogen.

Enzyme purification. All steps were carried out at 4 °C except those using the Äkta Explorer (10 °C). Portulaca grandiflora callus material (250 g, 14 d old, line K64), stored at −80 °C, or P. grandiflora root material (8 g, from 6-week-old plants grown in the greenhouse) were used for preparation of the crude extract.

Protocol I. Hydroxyapatite chromatography. Chromatography on hydroxyapatite and gel filtration were performed using an automated liquid chromatography system (Äkta Explorer; Pharmacia Biotech, Freiburg, Germany; software Unicorn Version 2.10). The crude extract was centrifuged and applied to ceramic hydroxyapatite (25 ml) equilibrated in 20 mM KPi (pH 6.5). After washing off any unbound protein at 0.8 ml min\(^{-1}\), the enzyme was eluted with a linear gradient (160 ml) from 20 to 800 mM KPi (flow rate 2.0 ml min\(^{-1}\)). The fractions containing tyrosine hydroxylase activity of the tyrosinase (TOH) were pooled and concentrated by ultrafiltration. For gel filtration, the concentrated peak fractions were applied to a Superdex G-75 HiLoad 16/60 column and the protein was eluted with 50 mM KPi (pH 6.5) containing 150 mM NaCl at a flow rate of 0.4 ml min\(^{-1}\).