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BETAXANTHIN PATTERN OF HAIRY ROOTS FROM *BETA VULGARIS* VAR. *LUTEA* AND ITS ALTERATION BY FEEDING OF AMINO ACIDS*

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Key Word Index—*Beta vulgaris* var. *lutea*; Chenopodiaceae; hairy roots; betaxanthins; betalamic acid; amino acids; *in vivo* condensation; biosynthesis.

Abstract—Betaxanthins from hairy root cultures of *Beta vulgaris* var. *lutea* showed two predominating substances, portulaxanthin II and vulgaxanthin I, and several minor components in HPLC profiles. The presence of muscaaurin VII, indicaxanthin, dopaxanthin, vulgaxanthin II, III and IV were suggested, the latter two as new natural compounds. Administration of nine L-amino acids to individual hairy root strains led to an increase in concentration or the appearance of one betaxanthin in each case. These betaxanthins structurally corresponded to the substances applied, except of that from glutamic acid. It was vulgaxanthin I instead of vulgaxanthin II and might be formed due to the function of glutamine synthetase. Also, the D-isomers of amino acids were incorporated into corresponding betaxanthins not only by hairy root cultures but also by seedlings of *B. vulgaris* var. *lutea*. The feeding experiments substantiate arguments for a spontaneous condensation of betalamic acid with an amino acid or amine in the course of betaxanthin biosynthesis. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Betalamic acid (Fig. 1) derives from L-3,4-dihydroxyphenylalanine (L-DOPA) via 4,5-secodopa [1] and is the characteristic precursor of all betalains [2]. Its condensation with cyclodopa leads to the red–violet betacyanins of higher plants; that with other amino acids or amines results in yellow betaxanthins. While most betacyanins are glycosides or acylglycosides the betaxanthins lack glycoside groups [2] (Fig. 1).

The biosynthetic steps of this pathway are still poorly understood at the enzyme level. DOPA 4,5-dioxygenase that initiates the transformation of its substrate into betalamic acid was isolated only from fly agaric, *Amanita muscaria* [1, 3], but not from higher plants. Even manifold experiments with a betaxanthin-producing plant cell culture [4] and hairy root culture [5] were unsuccessful in detecting this enzyme (H. Böhm, unpubl. results). It is still unclear whether betalamic acid condenses enzymically (D. Strack, pers. commun.) or spontaneously [1] with the second precursor of betalains *in vivo*. Two enzymes transferring glucose to the 5- or 6-position of betanidin were characterized in *Dorotheanthus bellidiformis* cell cultures [6, 7]. This result supports the assumption that glycosylation of cyclodopa in the course of betacyanin biosynthesis can take place prior to and after its condensation with betalamic acid [8].

Since we could show that betaxanthin formation was influenced by DOPA feeding in plants of several betalain-containing species [9, 10] we tried to alter the betaxanthin pattern of cultured hairy roots from *Beta vulgaris* var. *lutea* [5] by the administration of various amino acids. These experiments have brought further insights into the mechanism of condensation leading to the betalain skeleton *in vivo*.

RESULTS AND DISCUSSION

Hairy roots of 12 strains derived from seedlings of yellow beet (*Beta vulgaris* var. *lutea*) grew well in liquid MS0 and B50 medium. They produced betaxanthins and formed small amounts of betacyanins as indicated by a maximum at 470 nm and a shoulder at 540 nm in the absorption spectrum of the extracted betalains.

* Dedicated to Professor Mamoru Tabata on the occasion of his 65th birthday.

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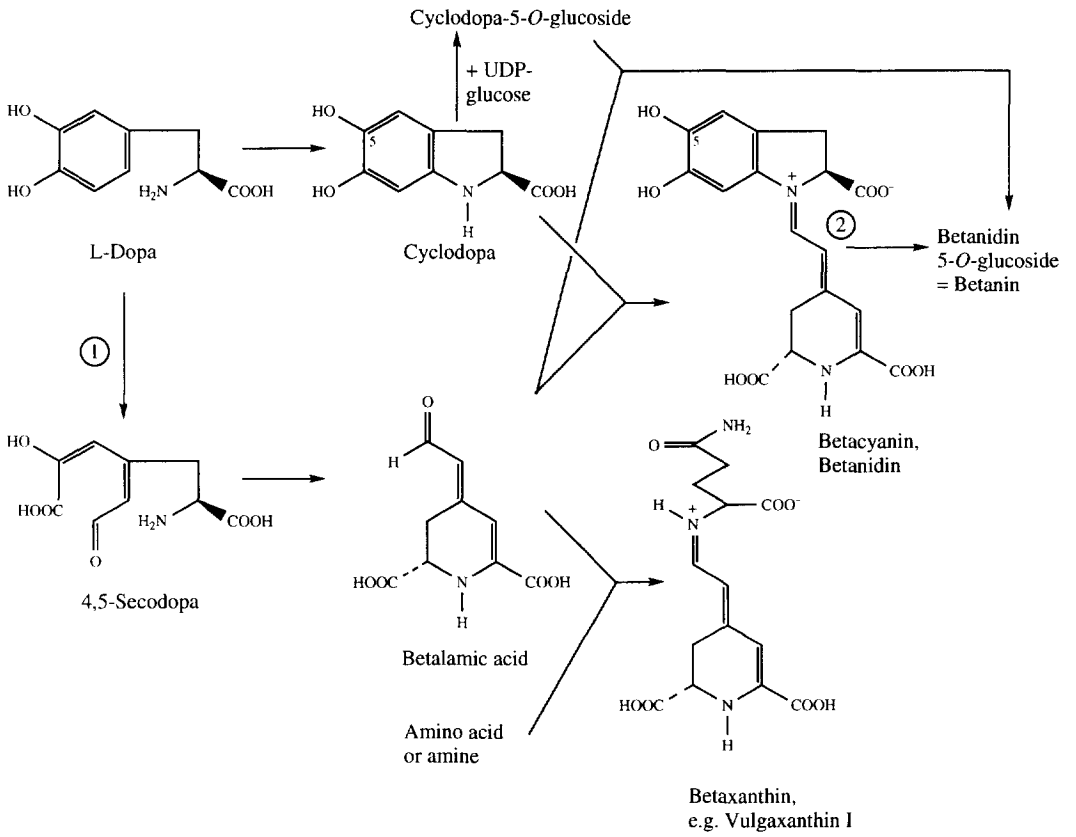

Fig. 1. Scheme of the biosynthetic pathways to betalains—betacyanins and betaxanthins—indicating especially the condensation steps and characterized enzymes. 1, DOPA 4,5-dioxygenase; 2, UDP-glucose: betanidin 5-*O*-glucosyltransferase.

A mixture of the plant material was collected from several subcultures and analysed in order to obtain a representative pattern ('standard pattern') of the betaxanthins. High pressure liquid chromatography of the combined methanolic and aqueous extracts resulted in a profile that showed two major components, portulaxanthin* II as the main betaxanthin and vulgaxanthin I (Fig. 2(a), Table 1). They were identified by comparing their retention times and absorption spectra with those of semi-synthetic substances. Chromatographic comparison was performed in separate runs and after coinjection. Interestingly, the main betaxanthin found in hairy roots of *B. vulgaris* var. *lutea* differs from that identified as vulgaxanthin I in beets of the same subspecies (Fig. 2(b)). This indicates an intense formation of tyrosine in the hairy roots since the B50 medium is free from amino acids and MS0 contained a very low tyrosine concentration (0.63 mg/100 mg protein). Characterized minor betaxanthins of the standard pattern are numbered in the HPLC profile (Fig. 2(a)) and described in Table 1. Two of them, compounds **2** and **8**, were probably part of a mixture with other betaxanthins from the cap skin of *A. muscaria* (named muscaaurin V) but were not structurally identified [14]. Their trivial names we proposed are given in quotation marks. Further minor compounds of the standard spectrum could not be characterized by comparison of the HPLC profile with those of semi-synthetic betaxanthins. Portulaxanthin I (R_t = 10.2 min) is obviously absent and reference substances containing glycine (R_t = 10.5 min), L-valine (R_t = 28.3 min) or L-phenylalanine (R_t = 37.9 min) were not unequivocally congruent with certain HPLC peaks. One has to realize, however, in this connection that a betaxanthin which is lacking in the standard pattern might be featured by a single hairy root strain and *vice versa*.

Feeding experiments were carried out with hairy roots of five individual strains cultured in B50 medium since it contains no organic nitrogen source. After the administration of the L-isomers of nine amino acids—Val, Leu, Asn, Glu, Gln, Phe, Pro, Hyp or His—in each case the increase in concentration or the appearance of one betaxanthin could be detected as exemplified in Fig. 3(a–c). Except for the feeding of glutamic acid, these betaxanthins structurally correspond

* We favour this term to which Piattelli changed (e.g. refs [11, 12]) after his first description of portulacaxanthin in the Italian language [13].

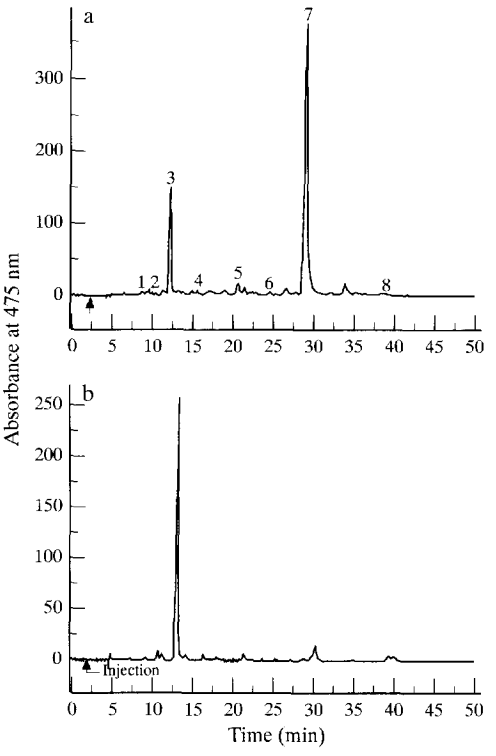

Fig. 2. HPLC profile of betaxanthins (a) from hairy roots of *B. vulgaris* var. *lutea* (standard pattern, see Table 1) and (b) from beets of *B. vulgaris* var. *lutea*.

to the administered substances. The identification was performed by HPLC comparison of feeding product and semi-synthetic betaxanthin derived from the same amino acid.

Supplementation of cultured hairy roots with glutamic acid always led to an increase of vulgaxanthin I instead of vulgaxanthin II. This finding might be caused by the function of glutamine synthetase which catalyses the reaction between glutamic acid and ammonia as a key enzyme of the assimilation processes in higher plants [19]. In order to examine the assumption we tried to reduce or even inhibit the activity of glutamine synthetase. Of the known enzyme inhibitors L-methionine sulfoximine [20] was chosen and added to feeding solutions to final concentrations of 125 and 250 μM. Under both conditions practically no vulgaxanthin I was formed but the HPLC peak of vulgaxanthin II did not enlarge. In another attempt to lower the glutamine synthetase activity we omitted ammonia, magnesium and manganese from the culture medium of feeding experiments. The nitrogen containing molecule is not only a substrate but also stimulates the enzyme activity in roots of sugarbeet [21]. The elements are known as cofactors of glutamine synthetase [22]. However, hairy roots of *B. vulgaris* var. *lutea* formed as much vulgaxanthin I in depleted medium as under normal conditions after the feeding of glutamic acid. Correspondingly, they showed no increased vulgaxanthin II concentration in the absence of the medium components discussed. In view of these results more detailed experiments will be necessary to achieve the incorporation into vulgaxanthin II of glutamic acid fed to hairy roots of *B. vulgaris* var. *lutea*.

Since natural betaxanthins contain amino acids with L-configuration [2] the possible enzymes responsible for the condensation with betalamic acid should be stereo-chemically specialized. It might therefore be revealing to follow the metabolic fate of D-amino acids administered to our *in vitro* system. As Fig. 3(d) demonstrates, the feeding of D-phenylalanine led to the formation of a major betaxanthin which was identified by HPLC as a derivative of this amino acid and separated from the compound containing the L-phenylalanine residue. Similar results could be obtained in experiments with D-valine and D-norvaline (not shown).

In order to exclude that these findings are due to the transformed character of hairy roots, seedlings of *B. vulgaris* var. *lutea* were fed with L- and D-phenylalanine in 5 mM solutions. Both isomers were incorporated into the corresponding betaxanthins which appeared in nearly identical concentrations (Fig. 4). Consequently, the condensation of betalamic acid with a D-amino acid coming from outside is a true process in betaxanthin synthesizing plants.

As can be seen in Fig. 3 (c and d) and Fig. 4(b) pairs of betaxanthins were present after the feeding of L-

Table 1. Characterized betaxanthins of the standard pattern in hairy roots of *B. vulgaris* var. *lutea* (numbers correspond to those of Fig. 2(a))

No.	Retention time (min)	Absorption maximum (nm)	Trivial name	Amino acid moiety	Ref.
1	6.5	470	Muscaaurin VII	L-His	[14]
2	7.9	469	'Vulgaxanthin III'	L-Asn	
3	10.0	468	Vulgaxanthin I	L-Gln	[15]
4	13.5	468	Vulgaxanthin II	L-Glu	[15]
5	18.6	478	Indicaxanthin	L-Pro	[16]
6	22.7	470	Dopaxanthin	L-DOPA	[17]
7	27.0	467	Portulaxanthin II	L-Tyr	[18]
8	36.7	467	'Vulgaxanthin IV'	L-Leu	

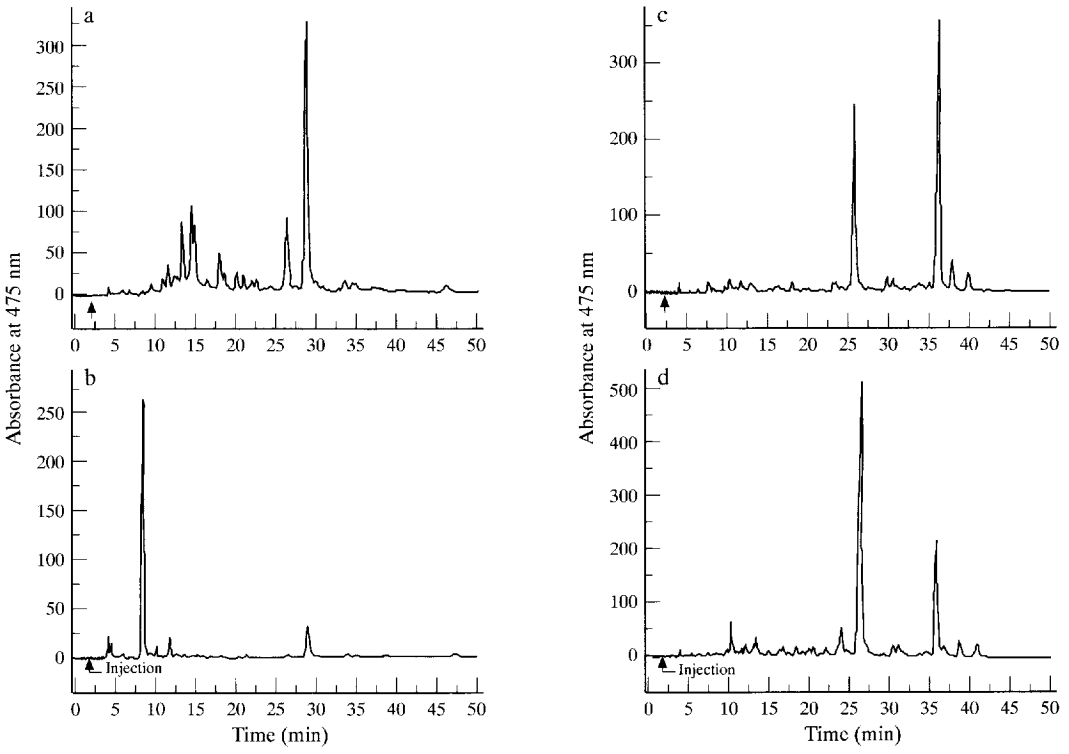

Fig. 3. HPLC profile of betaxanthins (a) of the hairy root strain 5E from *B. vulgaris* var. *lutea*, (b) after feeding of L-histidine, (c) after feeding of L-phenylalanine and (d) after feeding of D-phenylalanine.

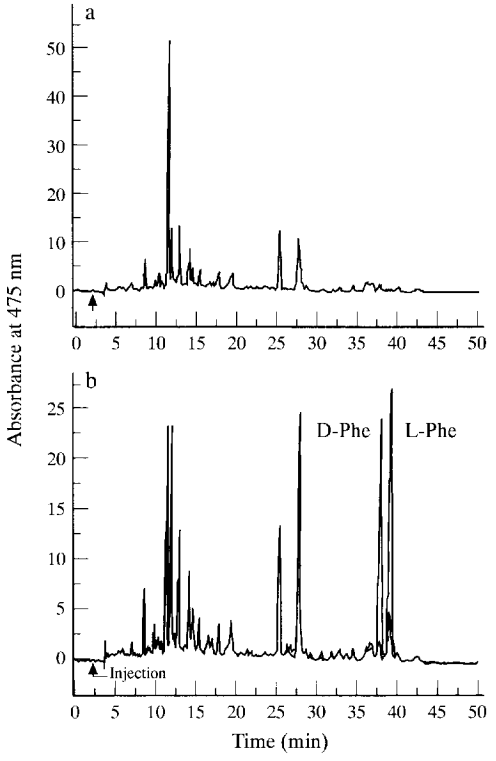

Fig. 4. HPLC of betaxanthins from *B. vulgaris* var. *lutea* seedlings: (a) profile of the control, (b) profiles after feeding of L- and D-phenylalanine, respectively.

and D-phenylalanine. According to their HPLC characteristics they consisted of a major compound corresponding to the amino acid applied and a minor compound which was derived from the respective isomer. Semi-synthetic preparations of betaxanthins from L- and D-phenylalanine also resulted in a main product accompanied by a small amount of an isomeric derivative in either case. Respective findings were obtained with L- and D-isomers of valine and norvaline *in vivo* and *in vitro*. Apparently, under these conditions a partial isomerization of the amino acids took place.

The absence of dopaxanthin in violet flowers of *Portulaca grandiflora* after feeding of DOPA [9, 10] seems to be in contrast to the results reported here. However, it could be shown, in accordance with general findings (Fig. 1), that DOPA was incorporated into the betalamic acid moiety of all additionally formed betaxanthins and probably consumed in this way. The fate of DOPA in hairy roots from yellow beet could not be followed due to a high polyphenol oxidase activity of the plant material which blackens the cultures within some hours after administration.

HPLC profiles of betaxanthins from several feeding experiments showed reduced peaks compared with the control besides the predominating compound derived from the amino acid applied. This situation is exemplified in Fig. 3(b) and might be caused by the preferential condensation of available betalamic acid with the excess amino acid taken in from the medium of the hairy root cultures of *B. vulgaris* var. *lutea*. It

could be explained also by a process in which the applied amino acid substitutes for amino acid residues of native betaxanthins.

The results of our feeding experiments imply that the *in vivo* condensation of betalamic acid with an amino acid or amine is a spontaneous but not an enzymatic process. This also may be true of the condensation in the course of betacyanin formation and consequently in the biosynthesis of all betalains.

EXPERIMENTAL

Plant material. Seeds of *B. vulgaris* var. *lutea* (yellow beet) were provided by Dr H. Pyysalo, Helsinki, and sown in the open ground as well as under sterile conditions on solid Knop's medium. Beets measuring 4–6 cm in diameter were harvested for betaxanthin analysis after the first growing season. The *in vitro* seedlings were used for the initiation of hairy roots as reported [5] and for feeding experiments. Each hairy root gave rise to an individual strain which was named after the number of the parent plant and a capital in case several hairy roots were formed at one seedling. Hairy roots free from *Agrobacterium rhizogenes* were established in liquid media after Murashige and Skoog [23] and Gamborg *et al.* [24] without phytohormones, named MS0 and B50. They were cultivated in 100 ml Erlenmeyer flasks containing 30 ml medium on a gyratory shaker (110 rpm) under 14 hr day^{-1} light (*ca* 3000 lux, white fluorescent tubes) at $26 \pm 1°$. Every 7th day a leading root tip (*ca* 3 cm length) was transferred into fresh medium and the residues of hairy roots were collected in order to determine their 'standard pattern' of betaxanthins. Due to different growth intensity the individual strains were not equally represented in the resulting mixt.

Extraction, purification and analysis of betaxanthins. Lyophilized and pulverized plant material was successively shaken with petrol, MeOH, and H_2O for about 30 min each. Both the latter extracts were combined and evaporated to dryness under vacuum. Their residue was used for visible absorption spectroscopy in MeOH (Uvikon 930, Kontron Instruments) and for HPLC after its treatment with solvent A (see below), centrifugation and filtration (Chromafil A-45, 0.45 μm; Macherey-Nagel, Düren).

HPLC was routinely carried out with an apparatus from Gerätebau H. Knauer, Berlin, at room temp. 50–100 μl betaxanthin soln were injected into a 250×4 mm Eurospher 80-C18 (5 μm) column protected by a guard column (5×4 mm) with the same stationary phase. Elution was performed at a flow rate of 1.0 ml min^{-1} with solvent A = 2% MeOH in 0.1% phosphoric acid for 2 min, then a 0–30% gradient of solvent B = MeOH within 30 min and further isocratic treatment for 18 min. Eluent absorption was followed at 475 nm (Variable Wavelength Monitor). In order to measure the absorption spectra (250–500 nm) of individual betaxanthins an HPLC Merck-Hitachi (Darmstadt) equipment with a diode array detector was used. The stationary phase was Li Chrospher 100 RP-18 endcapped (5 μm) in a 250×4 mm column and a 5×4 mm guard column. Elution was performed as described before with the difference that solvent B consisted of 0.1% phosphoric acid in MeOH.

Preparation of reference betaxanthins [4, 10]. Routinely 150 mg residue of the methanolic-aq. extract from yellow beets were dissolved in 42 ml H_2O and hydrolysed by addition of 5 ml conc. NH_3. After 30 min 50 mg of an amino acid were added. The mixt. was kept under weak vacuum at 30° and its pH adjusted to 3 by 2N HCl after 45 min. When its absorption maximum at about 470 nm showed no further increase the reaction was stopped by evaporation to near dryness. The excess of amino acid was separated from the formed betaxanthin by Avicel (Macherey-Nagel, Düren) CC with iso-ProOH–H_2O of proportions between 4:1 and 49:1.

Feeding experiments. The soln of an amino acid was added by sterile filtration to the B50 medium of a hairy root culture up to 3 and sometimes 1 mM concentration on the 4th day of subcultivation. After 3 days the hairy roots were separated, washed with H_2O and lyophilized. The feeding of a certain amino acid was paralleled by its own control and repeated at least once. L-Methionine sulfoximine was simultaneously added with glutamic acid under sterile conditions to final concentrations of 125 and 250 μM. The B50 medium with reduced nutrients, to which hairy roots were transferred 4 days before feeding of glutamic acid, was free from $(NH_4)_2SO_4$, $MgSO_4$ and $MnSO_4$.

5 mM solns of L- and D-phenylalanine were administered to hypocotyls with cotyledons and one pair of leaves each from sterile seedlings of *B. vulgaris* var. *lutea* (7 weeks old) in small glass tubes under continuous light (*ca* 1000 lux) at 20°. The feeding experiment, including control, was finished after 2 days by lyophilization of the plant material.

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