JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Characterization of the Activity of Tyrosinase on Betaxanthins Derived from (*R*)-Amino Acids

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The activity of tyrosinase (EC 1.14.18.1) on selected (*R*)-betaxanthins is characterized in depth, demonstrating that the activity of the enzyme is not restricted to betaxanthins derived from (*S*)-amino acids. Conversion of (*R*)-tyrosine-betaxanthin [(*R*)-portulacaxanthin II] to the pigment (*R*)-dopaxanthin and its further oxidation to a series of products is described. Compound identity was studied by high performance liquid chromatography and electrospray ionization-mass spectrometry. The reaction rate on the (*R*)-isomer of dopaxanthin is 1.9-fold lower than that obtained for the (*S*)-isomer in previous studies. Tyrosinase showed stereospecificity in its affinity toward betaxanthins. The characterization of the activity of tyrosinase on (*R*)-betaxanthins reinforces the role of the enzyme in the biosynthetic scheme of betalains.

KEYWORDS: Stereospecificity; tyrosinase; metabolic pathway; betalains; betaxanthins

INTRODUCTION

Tyrosinase or polyphenol oxidase (monophenol, *o*-diphenol: oxygen oxidoreductase; EC 1.14.18.1) is a copper-containing enzyme that catalyzes two different reactions using molecular oxygen: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of the *o*-diphenols to *o*-quinones (diphenolase activity) (*1*, *2*). This enzyme is widely distributed in plants, microorganisms, and animals where tyrosinase is responsible for melanization. Different tyrosinases obtained from several biological sources have similar structural and functional characteristics (*3*, *4*). A catalytic stereospecificity has been reported for tyrosinases from several sources, including mushroom (*5*−*7*), acting on chiral *o*-diphenols, which are substrates for tyrosinase.

Tyrosinase is an important enzyme in the food industry because during the processing of fruits and vegetables any wounding may cause cell disruption and lead to quinone formation. The enzymatic browning implies a considerable economic loss in the commercial production of fruits and vegetables. The appearance of food and beverages may be affected, as may the taste and its nutritional value, often decreasing the quality of the final product (*8*, *9*).

A wide variety of plant tyrosinase behavior has been described and reviewed (*10*, *11*). Although the physiological function of tyrosinase in higher plants is yet to be fully determined (*12*), its implication in the secondary metabolism of betalains has been proposed. Betalains are water-soluble nitrogen-containing secondary metabolites, which are present in plants belonging to the order Caryophyllales (*13*) and in the fungal genera *Amanita* (*14*) and *Hygrocybe* (*15*). Betalains are pigments and contain a betalamic acid as chromophore. Depending upon the nature of the betalamic acid addition residue, betalains are classified in betacyanins or betaxanthins. Betacyanins contain a *cyclo*-DOPA (usually glycosylated) residue and exhibit a violet coloration, while betaxanthins contain different amino acids or amines and exhibit yellow coloration.

Interest in betalains has grown since their antiradical activity was characterized (*16*−*18*), and they are widely used as additives in the food industry because of their natural colorant properties and the absence of toxicity even at high concentrations (*19*).

In the biosynthetic pathway of betalains, the hydroxylation of Tyr to dopa (3,4-dihydroxyphenylalanine) and the further oxidation of dopa, both catalyzed by tyrosinase, were considered to be the first steps in the biogenesis of betalamic acid and betacyanins (*13*, *20*). However, new possibilities are open following the demonstration of the direct activity of tyrosinase on betaxanthins (*21*) and the characterization of the reaction products. The enzyme tyrosinase has been purified from betalain-containing extracts of *Amanita muscaria* (*22*), *Portulaca grandiflora* (*23*), and *Beta vulgaris* (*24*). In addition, a tyrosine hydroxylase has been preliminary reported in callus cultures of *Portulaca grandiflora* (*25*).

Natural betaxanthins contain only amino acids with (*S*)-configuration (*26*), and so the enzymes involved in their metabolism should be stereochemically specialized. However, through feeding experiments conducted by Hempel and Böhm (*27*), and later by Schliemann et al. (*28*), the (*R*)-isomers of amino acids were incorporated into the corresponding betaxanthins, being proposed as a spontaneous condensation reaction of betalamic acid with an amino acid or amine in the course of

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10.1021/jf0514120 CCC: $30.25
Published on Web 10/13/2005

betaxanthin biosynthesis. The effect of the stereoisomerism of betaxanthins on the activity of the enzymes involved in the metabolism of these pigments has not been reported to date.

In this work, betaxanthins derived from (*R*)-amino acids are obtained and purified in sufficient quantities to work with. The capacity of tyrosinase to catalyze the oxidation of selected (*R*)-betaxanthins is investigated for the first time.

MATERIALS AND METHODS

Chemicals. Mushroom tyrosinase (2590 units/mg solid, lot 092K70491), chemicals, and reagents were purchased from Sigma (St. Louis, MO). Solvents were from Merck Chemicals Ltd. (Dorset, England). HPLC-grade acetonitrile was purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA).

Betaxanthins (Dopaxanthin and Tyr-Betaxanthin) Semisynthesis. (*R*)-Betaxanthins were obtained as immonium condensation products of betalamic acid with (*R*)-forms of the amino acids DOPA and Tyr as described by Wyler et al. (*29*), with some modifications. Briefly, betanin was used as a source of betalamic acid. Basic hydrolysis (pH 11.4) of 0.15 mM betanin solutions was carried out with NH_3. The betalamic acid released (*30*) was condensed with the amino acid after reaching pH 5.0 with acetic acid under ice-cooling. The medium contained 700 μmol amino acid/μmol betalamic acid, and the corresponding betaxanthin was obtained, accompanied by a color change from pale yellow (betalamic acid, $\lambda_m = 424$ nm) to deep yellow (betaxanthins, $\lambda_m = 480$ nm). Immediately, an FPLC system was used for purification to remove the excess of substrates and secondary products affecting stability (described below).

Betanin was obtained from commercial red beet. Extraction was performed in 10 mM phosphate buffer, pH 6.0 in a model 230 Omnimixer (Sorvall Inc., Norwalk, CT) at maximum speed for 10 s. The homogenate was filtered through cheesecloth and centrifuged at 120000*g*. The supernatant was then filtered using a YM-10 membrane (Millipore, Bedford, MA) to remove proteins. All steps were carried out at 4 °C. The pigment was then purified according to the method described by Escribano et al. (*16*). A Sephadex G-25 (Sigma) gel was conditioned and used in a 30 mL column. Elution was performed with a volume of 40 mL of water, with 1 mL fractions being collected. The elution process was followed at 536 and 480 nm. Fractions containing purified betanin were pooled.

Absorbance Spectroscopy. A Kontron Uvikon 940 spectrophotometer was used for absorbance spectroscopy.

Quantification of Betalains. Pigment concentration was evaluated through absorbance, taking a molar extinction coefficient of $\epsilon = 48\,000$ M^{-1} cm^{-1} at 480 nm for betaxanthins (*28*, *31*), and $\epsilon = 65\,000$ M^{-1} cm^{-1} at 536 nm for betanin (*32*). Measurements were made in water at 25 °C.

Tyrosinase Assays. Unless otherwise stated, the reaction medium (1.0 mL) contained 50 mM sodium phosphate buffer, pH 6.0, and 25 μg/mL of the enzyme. Other conditions are detailed in the text and in the figure legends. All experiments were performed in triplicate using cuvettes of 1 cm of light path. Additionally, cuvettes of 0.5 cm were used for the determination of the kinetic parameters, allowing higher substrate concentrations.

The apparent molar extinction coefficient (ϵ_{app}) corresponding to (*R*)-dopaxanthin oxidation can be defined as the difference between the extinction coefficient of the substrate and the extinction coefficient of the oxidation product mixture. It was determined at 480 nm by an end-point method, with a set of experiments carried out at initial 10, 17.5, and 25 μM concentrations, and at sufficiently high enzyme concentration and incubation time, allowing the reaction to proceed until all substrate had been converted to product. The data thus obtained were fitted by least-squares linear regression.

FPLC Purification. Anionic exchange chromatography of synthetic betaxanthins was performed in an Äkta purifier apparatus (Amersham Biosciences Inc., Uppsala, Sweden). The equipment was fully operated via PC using Unikorn software version 3.00. Elutions were followed at 280, 480, and 536 nm.

Solvents used were 2,2-bis-(hydroxymethyl)-2,2′,2″-nitrilotriethanol (BisTris) 20 mM, pH 6.0 (solvent A) and BisTris 20 mM, pH 6.0 with NaCl 2 M (solvent B). A 25 × 7 mm, 1 mL Q-Sepharose Fast Flow column (cross-linked agarose with quaternary ammonium as exchanger group, 90 μm of particle size) purchased from Amersham Biosciences Inc. was used. After sample injection, the elution process was as follows: 0% B from beginning to 7.5 mL (for analytical purposes) or to 15 mL (for semipreparative elution); after washing, a linear gradient was developed from 0% B to 35% B in 20 mL, with 1 mL fractions being collected. Cleaning (7 mL, 50% B) and reequilibration (7 mL, 100% A) steps were performed between each elution. Injection volume for analytical purposes was 50 μL (flow 0.4 mL/min). For semipreparative use, 1 mL was injected (flow 0.5 mL/min).

Desalting on a SPE Cartridge. For desalting betaxanthins, 1 mL of C-18 cartridges (Waters, Milford, MA) was conditioned with 5 mL of methanol followed by 10 mL of purified water. Aqueous solutions of betaxanthins were injected and bound to the mini-column. Salts and buffers eluted first and were washed off by rinsing the column with water. Dopaxanthin and Tyr-betaxanthin were eluted isocratically with water, and samples were freeze-dried. Compounds were obtained as powders and stored at −80 °C until use.

HPLC Analysis. *PDA Detection.* A Shimadzu LC-10A apparatus equipped with a SPD-M10A photodiode array detector was used for analytical HPLC. Reversed phase chromatography was performed with a 250 × 4.6 mm Kromasil 100 C-18 column packed with 5 μm particles (Teknokroma, Barcelona, Spain). Gradients were formed between two helium degassed solvents. Solvent A was water acidified with 0.05% trifluoroacetic acid (TFA), and solvent B was composed of acetonitrile (AcN) with 0.05% TFA. Linear gradient was performed in 20 min from 0% B to 28% B. The flow rate was 1 mL/min, operated at 25 °C. The injection volume was 20 μL.

HPLC-MS. HPLC-ESI-MS analyses were performed in an Agilent VL 1100 apparatus equipped with a LC/MSD Trap (Agilent Technologies Inc., Palo Alto, CA). Elution conditions were as described above using a Zorbax SB-C18 (30 × 2.1 mm, 3.5 μm) column (Agilent Technologies Inc.) with a flow rate of 0.3 mL/min. Vaporizer temperature was 350 °C, and voltage was maintained at 3.5 kV. Sheath gas was nitrogen operated at a pressure of 35 psi. Samples were ionized in positive mode. Ion monitoring mode was full scan in the range *m*/*z* 60−600. For detection, the electron multiplier voltage was 1350 V.

Before analysis, all samples derived from enzymatic activities were ultra-filtrated trough YM-10 membranes (Millipore) to remove the catalyst.

RESULTS AND DISCUSSION

Semisynthesis of (*R*)-Betaxanthins: Dopaxanthin and Tyr-Betaxanthin. Semisyntheses of (*R*)-Tyr-betaxanthin and (*R*)-dopaxanthin (structures shown in **Figure 1**) were started from aliquots of a purified betanin:isobetanin mixture (95:5) obtained from red beet extracts (*16*). After the synthesis of each betaxanthin, a purification protocol was carried out. The purification process was aimed at obtaining the betaxanthin free from the corresponding amino acid and without *cyclo*-DOPA-glucoside, which is able to reverse the reaction to the formation of the original betanin.

Anionic exchange chromatography was applied, and (*R*)-betaxanthins were able to interact with the quaternary ammonium ligand, binding to the matrix, while (*R*)-Tyr, (*R*)-DOPA, and *cyclo*-DOPA-glucoside were totally washed out in the unbound fraction. (*R*)-Amino acid samples were injected into the column to ascertain their elution in the unbound fraction, and this was followed by absorbance at 280 nm.

Elution volumes of the pigments under the protocol described in Materials and Methods were obtained by injecting 50 μL of the reaction mixtures. To purify larger amounts of (*R*)-betaxanthins, the injection volumes of samples and the length of the washing module were increased. The yields obtained for betaxanthins synthesis are shown in **Table 1**. Data correspond

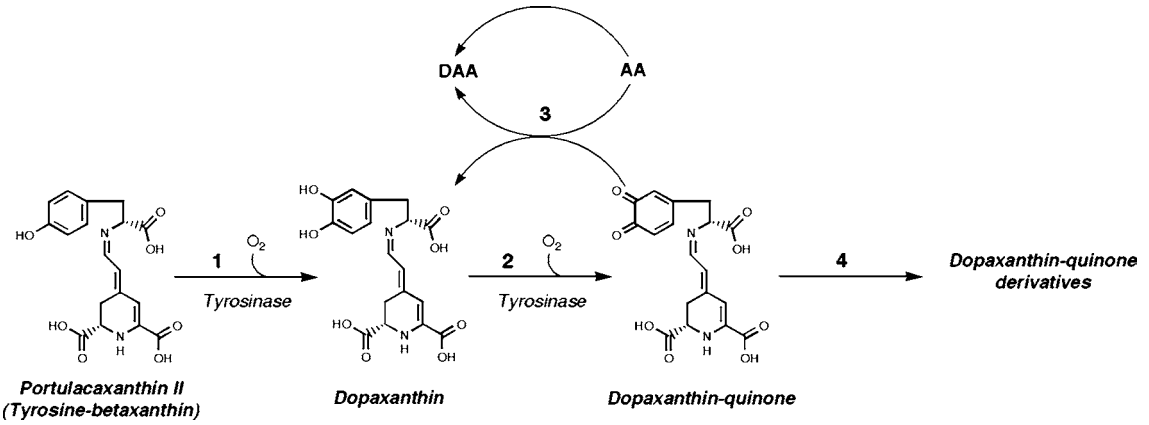

Figure 1. Scheme for the activity of tyrosinase on (*R*)-betaxanthins. DAA: dehydroascorbic acid.

Table 1. FPLC Elution Volumes, Purification Yields, and Data Obtained by HPLC Analysis for (*R*)-Betaxanthins

		synthesis and purification yield (%)		HPLC Rt (min)			
betaxanthin	FPLC EV (mL)	analysis	preparative	2*R*/*S*	2*R*/*R*	λmax (nm)	mass[a] [M + H]$^+$
(*R*)-dopaxanthin	15.64	80	49	14.16	14.46	472	391
(*R*)-tyrosinexanthin	15.00	38	28	16.08	16.43	472	375

[a] Masses are in accordance with the proposed structures. Errors are below 0.1 *m*/*z*.

to the combined process involving synthesis and purification, because the solutions of synthesized betaxanthins are not stable until *cyclo*-DOPA-glucoside is removed by chromatography.

According to HPLC analysis, the presence of betalamic acid was detected together with (*R*)-dopaxanthin (163 mg/g) and (*R*)-Tyr-betaxanthin (391 mg/g). This is due to coelution in the FPLC protocol, which could not be avoided, and to the low solubility of DOPA (1.65 mg/mL) and, especially, Tyr (0.453 mg/mL). The solubility of these compounds is also responsible for the low yields of the corresponding betaxanthins when compared to other betaxanthins derived from more soluble amino acids (results not shown). HPLC retention times of (*R*)-Tyr-betaxanthin and (*R*)-dopaxanthin are also listed in **Table 1**. The HPLC protocol is able to separate the diastereoisomeric forms of both pigments. As was previously stated, there is 5% of betanin present in the isomeric form isobetanin in the starting material, and because isobetanin is processed in the same way as betanin, the synthesized and purified betaxanthins also have 5% in the "iso" form (2*R*/*R*).

Figure 2A,**B** shows the chromatographic profiles obtained for the HPLC analysis of the synthesized pigments. In both cases, the existence of the isomers (2*R*/*S*) (major peaks) and (2*R*/*R*) (minor peaks) can be seen. Positions for the (2*R*/*S*) and (2*R*/*R*) isomers are the opposite of those occupied by the counterpartners derived from (*S*)-amino acids, (2*S*/*S*) and (2*S*/*R*) (*13*, *21*). Absorbance spectra are shared by both diastereoisomers in each case, and maximum wavelengths (shown in **Table 1**) are equal to those previously reported for the (*S*)-forms of the betaxanthins. The HPLC protocol described under Materials and Methods was used with a photodiode array detector (PDA), and also with a mass spectrometry (MS) detector. Mass values determined for the semisynthetic compounds were as expected for the corresponding protonated molecular ions [M + H]$^+$ (**Table 1**).

Monophenolase Activity of Tyrosinase. Conversion of (*R*)-Tyr-Betaxanthin to (*R*)-Dopaxanthin. As demonstrated above, (*R*)-Tyr is condensed spontaneously with betalamic acid to obtain Tyr-betaxanthin. The Tyr-betaxanthin purified by FPLC had a high NaCl content, which had to be removed to avoid the inhibition of tyrosinase by chloride (*33*). Thus, C-18

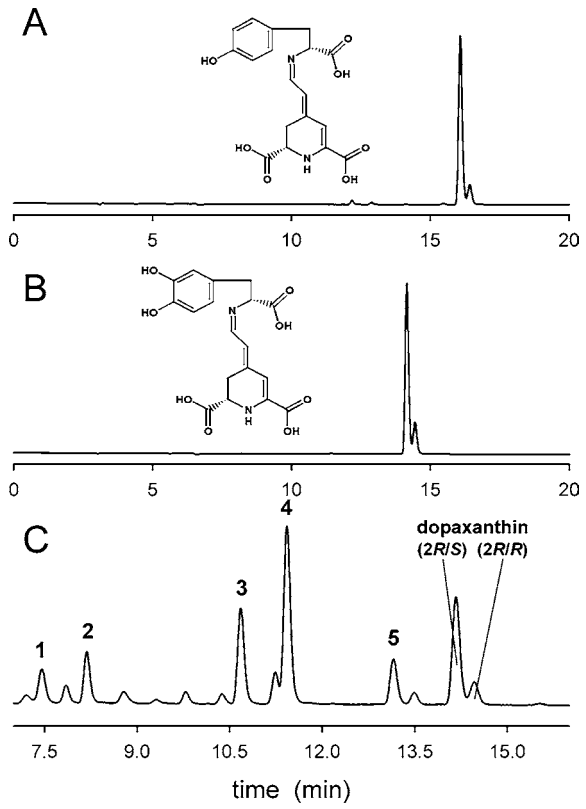

Figure 2. HPLC elution profiles for (*R*)-Tyr-betaxanthin (**A**), (*R*)-dopaxanthin (**B**), and the products derived from enzymatic action of tyrosinase (**C**). (2*R*/*S*)-pigments (main peaks) are accompanied by the (2*R*/*R*)-diastereoisomers (minor peaks). 20 μL of 20 μM solutions were injected. Full scales are A_{480} = 0.11 absorbance units for (**A**) and (**B**) and A_{480} = 0.03 absorbance units for (**C**). In (**C**), the reaction was started with 25 μg/mL of tyrosinase and stopped after 15 min.

Table 2. Retention Times and HPLC-PDA and MS Data of the Oxidation Products of (*R*)-Dopaxanthin

compound	Rt (min)	λmax (nm)	$[M + H]^+$
oxidation product 1	7.44	488	389
oxidation product 2	8.17	480	389
oxidation product 3	10.68	464	389
oxidation product 4	11.42	464	389
oxidation product 5	13.15	488	389

cartridges were used for removing the salt content, and the target betaxanthins were recovered with yields of 92%.

The synthesized and purified (*R*)-Tyr-betaxanthin was stable in solution under pH 6.0 at 25 °C (**Figure 2A**). However, when the enzyme tyrosinase was added (25 μg/mL) and ascorbic acid (AA) was initially present in the medium (20 mM), the pigment content evolved and the peaks corresponding to Tyr-betaxanthin in HPLC analysis disappeared, and two new peaks appeared. The peaks derived from the activity of tyrosinase on (*R*)-Tyr-betaxanthin were assigned to those corresponding to (*R*)-dopaxanthin by coelution and comparison of spectral properties with (*R*)-dopaxanthin directly synthesized from betalamic acid shown in **Figure 2B**. The enzyme from mushroom is extensively used as a model for studies at molecular and kinetic levels (*34*). For comparative purposes, the commercial mushroom tyrosinase was used as an enzyme source.

This is the first time interconversion between betaxanthins derived from (*R*)-amino acids is described (**Figure 1**). The obtaining of the *o*-diphenol is in agreement with the general behavior described for tyrosinase acting on monophenols under reducing conditions (*35*).

(*R*)-Dopaxanthin as Substrate of the Diphenolase Activity of Tyrosinase. HPLC Analysis. The ability of tyrosinase to hydroxylate the (*R*)-form of Tyr-betaxanthin to (*R*)-dopaxanthin opened up the possibility of obtaining the (*R*)-isomer of the corresponding *o*-quinone. When monophenolase activity took place without AA, or after its total depletion, or the diphenolase activity of tyrosinase was assayed on (*R*)-dopaxanthin, a series of previously undescribed products, showing a complex HPLC profile, was obtained (**Figure 2C**).

All of the new products obtained by the action of tyrosinase on (*R*)-dopaxanthin had similar maximum absorbance wavelengths, as is summarized in **Table 2**. Moreover, the reaction medium was analyzed by HPLC-MS, and all of the products shared the same mass: 389 *m*/*z* (**Table 2**). Masses obtained for the reaction products of (*R*)-dopaxanthin corresponded to that of betanidin (structural unit of most betacyanins), indicating the presence of five isomers with similar absorption maxima (**Table 2**). However, betanidin exhibits a maximum wavelength of 544 nm under the HPLC system and was not detected.

The presence of these five compounds agrees with the formation of quinone derivatives as discussed for the evolution of the (*S*)-form of dopaxanthin (*21*). AA was added to the medium (20 mM) after the action of tyrosinase and once the enzyme had been removed by ultrafiltration. No change in the chromatographic profile was detectable, and, as AA is able to reverse *o*-quinones to the diphenols, it is demonstrated that the original quinone was converted into other species, not susceptible to AA reduction (**Figure 1**).

Masses and insensitivity to AA treatment indicate that dopaxanthin-quinone evolves to more stable species by intramolecular cyclization rather than by intermolecular condensation, because masses corresponding to dimers or advanced oligomers were not detected. Thus, the only way to obtain such a variety of nonquinoidal products derived from dopaxanthin-quinone is

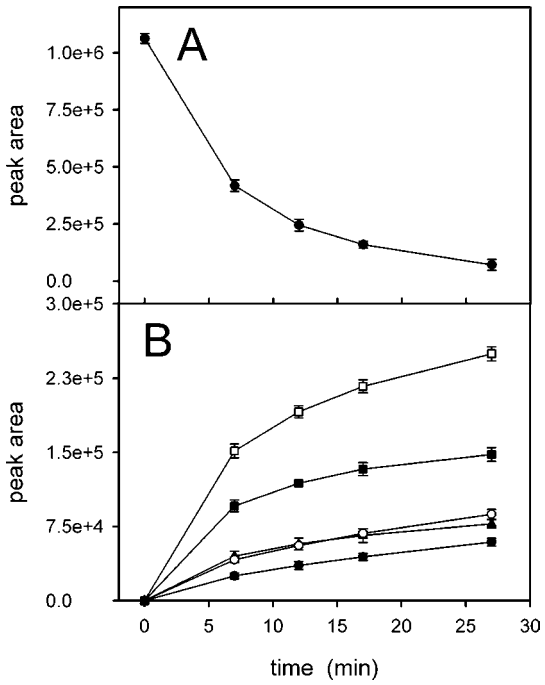

Figure 3. Progress curves for (2*R*/*S*)-dopaxanthin oxidation by tyrosinase. (**A**) Depletion of the substrate. (**B**) Appearance of reaction products. Products are identified by means of retention times as follows: (●) 7.44 min, (○) 8.17 min, (■) 10.68 min, (□) 11.42 min, and (▲) 13.15 min. The initial dopaxanthin concentration was 20 μM, and the reaction was started with 25 μg/mL of tyrosinase. The experiment was performed in triplicate, and the mean and standard deviations were plotted.

by following the same process experienced by DOPA-quinone when it is converted into leuko-DOPA-chrome: internal nucleophilic cyclization (*2*, *36*). In dopaxanthin-quinone, the number of active sites susceptible to giving internal cyclization with positions 5 or 6 of the quinoidal ring (*37*) is increased to four on account of the resonance system present in the betalamic acid moiety of the molecule.

The results are similar to those reported for the oxidation of (*S*)-dopaxanthin (*21*) where five main peaks were associated with the oxidation of (2*S*/*S*)-dopaxanthin and other minor peaks were assigned to the oxidation of the (2*S*/*R*)-isomer. Inversion of (2*R*/*S*) and (2*R*/*R*) isomer positions of peaks 3, 4, and 5 occurred for the products of the enzymatic activity with respect to (2*S*/*S*) and (2*S*/*R*) products, as described above for the substrates.

The depletion of the substrate dopaxanthin (**Figure 3A**) and the appearance of the reaction products (**Figure 3B**) were followed by HPLC. **Figure 3** shows the evolution of the peak areas corresponding to the (2*R*/*S*) isomer. It can be seen that a hyperbolic-type curve was obtained for the depletion of the substrate.

Spectroscopic Analysis of (*R*)-Dopaxanthin Oxidation by Tyrosinase. (*R*)-Dopaxanthin oxidation by tyrosinase was monitored spectrophotometrically by observing changes in the UV−vis spectrum over time (**Figure 4**). The control solution was stable at pH 6.0 at 25 °C. Maximal spectral changes were observed at 476 nm (decrease in absorbance), and an isosbestic point existed at 500 nm. Another lower maximum at 514 nm (increase in absorbance) could be detected better by subtracting the first recording from the rest (differential spectra, **Figure 4A**, inset).

The Coleman et al. (*38*) matrix analysis was performed for the oxidation of (*R*)-dopaxanthin (**Figure 4B**) and predicted the

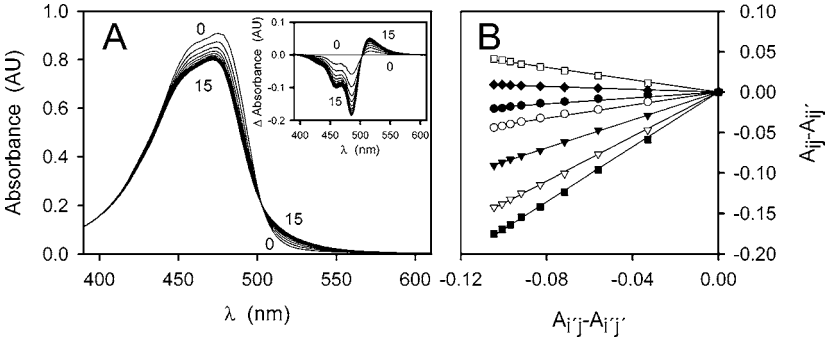

Figure 4. (**A**) Consecutive scan spectra for (*R*)-dopaxanthin oxidation by tyrosinase. The pigment content in the assay medium was 18.5 μM. The reaction was started by addition of the enzyme (100 μg/mL). Scan speed was 1000 nm/min at 1 min intervals. Inset: differential spectra derived from the previous scans subtracting the first recording from the rest. (**B**) Coleman graphic analysis of the consecutive spectra shown in (**A**). The test of two species with restrictions was applied. In this analysis, A_{ij} is the absorbance at a wavelength *i* obtained during tracing *j*, where $i' = 480$ nm, $i =$ ● (426 nm), $i =$ ○ (444 nm), $i =$ ▼ (470 nm), $i =$ ▽ (478 nm), $i =$ ■ (487 nm), $i =$ □ (523 nm), $i =$ ◆ (555 nm), and $j' =$ first tracing.

existence of two absorbing species in the analyzed solution. Moreover, the existence of an isosbestic point is often considered proof of the presence of only two absorbing compounds. These observations are apparently in conflict with the presence of at least five different reaction products, as demonstrated by HPLC. However, they can be justified in the particular case when there is a fixed linear relation between the concentrations of the multiple components in a solution (*39*). The appearance of linearly related compounds makes the existence of an isosbestic point possible. At a spectral level, the result of the reaction can be considered as a single product. Thus, the convergence of straight lines at the origin of the coordinate axis in the Coleman analysis is in relation to the concomitant appearance of the compounds in the solution, as shown in **Figure 3**.

(*R*)-Dopaxanthin oxidation rate by tyrosinase was studied and showed dependence on substrate concentration (results not shown). No kinetic parameters could be determined as the lower affinity of the enzyme for the (*R*)-form of the substrate avoided reaching saturating concentrations and, therefore, proper K_m determination. However, the rate of the reaction was estimated at fixed concentrations and compared to the data previously obtained for the (*S*)-form (*21*). The transformation rate is 1.9 times lower than that calculated for (*S*)-dopaxanthin, measured at a concentration of substrate 22 μM. Thus, it can be deduced that the affinity of tyrosinase for (*S*)-forms of betaxanthins is higher than that for (*R*)-forms.

The activity of mushroom tyrosinase on (*R*)-forms of simple diphenols resulted in high K_m values when compared to the (*S*)-forms, as described by Espín et al. (*5*). These authors determined that (*R*)-DOPA, the amino acid from which (*R*)-dopaxanthin is derived, yields a $K_m = 4.5$ mM, while the value for (*S*)-DOPA is $K_m = 0.8$ mM. Tyrosinase extracted from betalain-producing callus cultures of *Portulaca grandiflora* exhibited similar K_m for both isomers of DOPA, 2.4 and 2.8 mM for the isomers (*S*) and (*R*), respectively, although the oxidation rate was 5.8 times lower for (*R*)-DOPA (*23*). A higher K_m value for the enzyme extracted from avocado (*40*) has been reported for the (*R*)-isomer of DOPA than for (*S*)-DOPA, together with a reduction in the oxidation rate (1.3-fold).

Activity on (*R*)-dopaxanthin was calculated taking into account that the concentrations of the products derived from dopaxanthin-quinone are linearly related (as supported by the Coleman analysis and the existence of the isosbestic point) and by calculating the corresponding apparent molar extinction coefficient (ϵ_{app}) at 480 nm for the transformation of (*R*)-dopaxanthin as described under Materials and Methods. The ϵ_{app} relates the increments of absorbance at a given wavelength during the enzymatic reaction to the concentration of transformed substrate that causes it. The oxidation rate can thus be evaluated. The value was $\epsilon_{app} = 9580\ M^{-1}\ cm^{-1}$, the same as that obtained for the (*S*)-isomer (*21*).

This work shows that tyrosinase can express monophenolase and diphenolase activities on (*R*)-Tyr-betaxanthin and (*R*)-dopaxanthin, respectively. The results with nonphysiological betaxanthins are in agreement with the first published data concerning the activity of this enzyme on betaxanthins derived from (*S*)-amino acids and support the role of tyrosinase in the biosynthetic scheme of betalains (*21*). The existence of a limited stereospecificity in the action of tyrosinase on (*R*)-betaxanthins implies that, although the (*S*)-isomers are favored, pigments derived from (*R*)-amino acids can be obtained by the action of the enzyme at the proposed level. This suggests that the existence of a specific isomer of a betalain must be determined upstream, in the biogenesis of the precursor amino acid.

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Received for review June 14, 2005. Revised manuscript received September 9, 2005. Accepted September 15, 2005. The present work has been supported by two grants from MCYT (Spain) and FEDER (AGL2003-05647) and the Fundación Séneca, Consejería de Agricultura, Agua y Medio Ambiente (Spain), and FEDER (Project AGR/11/FS/02). F.G.-H. holds a fellowship from the Fundación Séneca (Spain).

JF0514120