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Fluorescence Detection of Tyrosinase Activity on Dopamine-Betaxanthin Purified from *Portulaca oleracea* (Common Purslane) Flowers

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Tyrosinase or polyphenol oxidase (EC 1.14.18.1) is one of the key enzymes for the biosynthesis of natural pigment betalains. These are an important class of water-soluble pigments, characteristic of plants belonging to the order Caryophyllales. In this work, dopamine-betaxanthin (also known as miraxanthin V) is reported as the pigment responsible for the bright coloration in yellow flowers of *Portulaca oleracea* (common purslane). The natural pigment is purified, and used as a substrate for the catecholase (diphenolase) activity of the enzyme tyrosinase. A new, continuous method to follow the activity is developed based on the fluorescent properties of the betaxanthin. Fluorescence of the enzyme activity derived products is reported for the first time. Relevance of the fluorescent phenomenon is discussed based on fluorescence images and the description of a physiological inner filter effect present in flowers of *P. oleracea*. The first description of the betalain content in flower pistils is also provided.

KEYWORDS: Tyrosinase; betalain; betaxanthin; *Portulaca oleracea*; fluorescence

INTRODUCTION

Tyrosinase, or polyphenol oxidase (monophenol, *o*-diphenol: oxygen oxidoreductase; EC 1.14.18.1), which is widely distributed in higher plants, animals, and microorganisms, is a binuclear copper containing enzyme that catalyzes two different reactions by using molecular oxygen: hydroxylation of monophenols to *o*-diphenols (monophenolase activity), and oxidation of the *o*-diphenols to the corresponding *o*-quinones (diphenolase activity) (*1, 2*). *o*-Quinones, are usually unstable in aqueous solutions and can undergo nonenzymatic reactions, such as cyclization to amine-chrome following a Michael intramolecular 1,4 addition. Furthermore, the quinone molecules can react with other substances, forming a variety of brown or black compounds. This affects the appearance of the vegetable as well as its taste and nutritional value, often decreasing the quality of the final product.

The wide literature on the catalytic properties of tyrosinase has been properly reviewed (*3, 4*), and different tyrosinases from several biological sources have similar structural (*5, 6*) and functional characteristics. The enzyme from mushroom is used as a model for studies at molecular and kinetic levels (*7*).

Betalains are water-soluble nitrogen-containing pigments of growing interest in the food industry. They are present in plants belonging to the order Caryophyllales (*8, 9*) and in the fungal genera *Amanita* (*10*) and *Hygrocybe* (*11*). They replace anthocyanins (*12*) and contain betalamic acid as structural unit and chromophore. Depending upon the nature of the betalamic acid addition residue, betalains can be classified as either betacyanins or betaxanthins. Betacyanins contain a *cyclo*-DOPA (usually glycosylated) residue and exhibit a violet coloration, while betaxanthins contain different amino acid or amine side chains and exhibit yellow coloration. Betaxanthins have been reported to give color to flowers of a great variety of plant genera, such as *Mirabilis* (*13*), *Glottiphylum* (*14*), and *Portulaca* (*15*).

The involvement of the enzyme tyrosinase in the biosynthetic pathway of betalains is a matter of discussion. It has been proposed at two different stages. It catalyzes the hydroxylation of tyrosine to DOPA (3,4-dihydroxyphenylalanine) before its transformation to betalamic acid (*8*). More interestingly, the enzyme can also transform monophenolic betaxanthins to diphenolic betaxanthins, and the latter to quinoidal compounds related to betacyanins (*16*). **Figure 1** shows an overview of the steps catalyzed by tyrosinase in the biosynthesis of betalains.

In this paper, tyrosinase activity is followed using a new continuous protocol on a natural betaxanthin purified from *Portulaca oleracea* (common purslane). The analysis of the betalain content in this species reveals a new source for a noncommercial compound and extends the knowledge on the distribution of these pigments. The pigment content justifies the bright coloration of flower petals and gives new insights into the fluorescent phenomena in plants.

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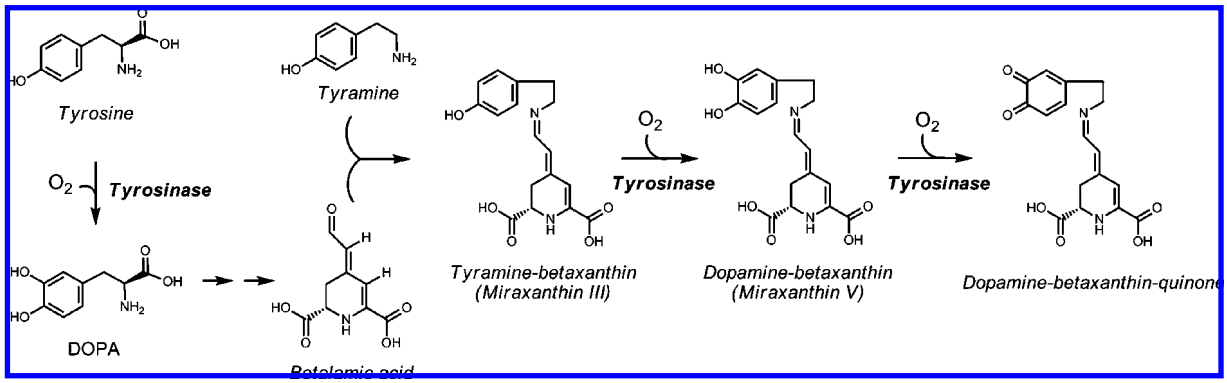

Figure 1. Tyrosinase catalyzed steps in the biosynthesis of betalains derived from tyramine.

MATERIALS AND METHODS

Chemicals. Mushroom tyrosinase (5,370 units mg^{-1} solid, lot 076K7034), other enzymes, 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).

Plant Material and Sample Preparation. *Portulaca oleracea* plants were obtained from a local commercial supplier (La Generala, Murcia, Spain), and grown by the authors in Murcia (Spain). All plants were grown side by side under the same conditions. Flower samples were carefully collected, and the petals were removed. Pigments were extracted in 10 mM phosphate buffer pH 6.0, containing 10 mM ascorbic acid (AA) in a Polytron homogenizer (Kinematica AG, Littau, Switzerland) (5 s, 2 pulses, at medium speed). The homogenate was filtered through nylon cloth and centrifuged at 120000*g* for 40 min. The supernatant was then filtered through Amicon YM-10 membrane (Millipore, Bedford, MA) to remove proteins, and the filtrate was used for pigment analysis or further purification. Pigment extractions from pistils were performed manually in a 10 mM AA solution of 50% methanol in water. Extracts were used for pigment analysis after centrifugation on bench centrifuge. The whole process was carried out at 4 °C.

Standard Betalains. Betanin was obtained from commercial red beet. Extraction was performed in 10 mM phosphate buffer, pH 6.0 in a bench blender model Osterizer (Jarden Corporation, Rye, NY) 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. (*17*). 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, and 1 mL fractions were collected. The elution process was followed at 536 and 480 nm. Fractions containing purified betanin were pooled.

Betanidin, the structural unit of betacyanins, was obtained enzymatically from purified betanin through β-glucosidase (EC 3.2.1.21; β-D-glucoside glucohydrolase) treatment. A 4 mM betanin solution was incubated for 30 min with 14 units/mL of β-glucosidase (Sigma) in 50 mM acetate buffer, pH 5.0, at 25 °C. The enzyme was removed by ultrafiltration through YM-10 membranes.

Synthetic betaxanthins used as standards for pigment identification were obtained as immonium condensation products of betalamic acid with amines or (*S*)-forms of amino acids, as described previously (*18, 19*). In short, betanin purified from red beet was used as starting material. Basic hydrolysis (pH 11.4) of 0.15 mM betanin released betalamic acid, which was condensed with the amine or amino acid after reaching pH 5.0. 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). Standard pigments were characterized spectrophotometrically, chromatographically, and by electrospray ionization mass spectrometry (ESI-MS) (*19*). Once synthesis was achieved, an automated system was used for purification to remove the excess of substrates and secondary products affecting stability.

FPLC Purification. Anionic exchange chromatography of synthetic standards and natural dopamine-betaxanthin was performed in an Äkta purifier apparatus (General Electric Healthcare, Milwaukee, WI). The equipment was completely operated via a 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; after washing, a linear gradient was developed from 0% B to 26% B in 15 mL, with 1 mL fractions being collected. Injection volume was 1 mL, and the flow rate was 1.0 mL min^{-1}.

C-18 Solid Phase Extraction. One mL C-18 cartridges (Waters, Milford, MA, USA) were conditioned with 5 mL of methanol followed by 10 mL of purified water. Salts and buffers from the samples were washed off by rinsing the column with water. Dopamine-betaxanthin was eluted with acetone and then concentrated to dryness under vacuum. The residue was redissolved in water for further use or stored at −80 °C.

HPLC Analysis. *PDA Detection.* A Shimadzu LC-10A apparatus equipped with a SPD-M10A photodiode array detector was used for analytical HPLC separations. 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 with 0.05% trifluoroacetic acid, and solvent B was composed of acetonitrile with 0.05% trifluoroacetic acid. Linear gradient was performed during 20 min from 0% B to 28% B. The flow rate was 1 mL min^{-1}, at 25 °C. Injection volume was 20 μL. Pigments from natural samples and standards had the same retention times and superimposable spectra.

Fluorescence Detection. For HPLC-fluorescence analyses, the same apparatus equipped with an RF-10AXL fluorescence detector was used. Excitation was carried out at 460 nm and emission was followed at 510 nm. Before analysis, samples derived from enzymatic activity were ultrafiltrated trough YM-10 membranes (Millipore) in order to remove the catalyst and stop the reaction.

Absorbance Spectroscopy. A Jasco V-630 spectrophotometer (Jasco Corporation, Tokyo, Japan) was used for absorbance spectroscopy.

For the quantification of betalains, pigment concentration was evaluated taking a molar extinction coefficient of $\varepsilon = 48{,}000\ M^{-1}\ cm^{-1}$ at 480 nm for betaxanthins (*20, 21*) and $\varepsilon = 65{,}000\ M^{-1}\ cm^{-1}$ and $\varepsilon = 54{,}000\ M^{-1}\ cm^{-1}$ at 536 nm for betanin and betanidin, respectively (*22*). Measurements were made in water at 25 °C.

Fluorecence Spectroscopy. Fluorescence spectroscopy was performed in an LS50B apparatus (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA), using quartz cuvettes of 10 mm path length, at 25 °C. Excitation and emission slits were set at 10 nm. Excitation spectra were recorded by following emission at the maximum wavelength. Emission spectra were obtained by exciting at the corresponding maximum.

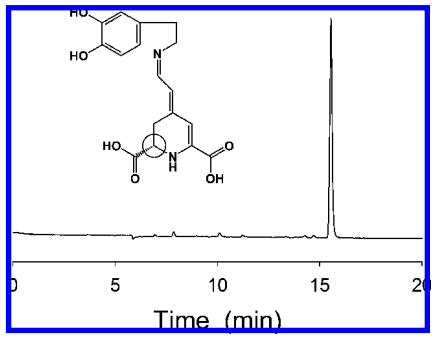

Figure 2. Chromatographic profile for the analysis of pigment composition in yellow *Portulaca oleracea* flowers. Inset: dopamine-betaxanthin structure, showing the chiral carbon circled. Elution was followed at 480 nm. Full scale is $A = 0.060$ absorbance units. Twenty microliters of an extract containing dopamine-betaxanthin 12 μM was injected.

For tyrosinase assays, unless otherwise stated, the reaction medium contained 50 mM sodium phosphate buffer, pH 6.0, and 6.0 μg mL^{-1} of the enzyme. Other conditions and reagents are detailed in the text and in the figure legends. A 105.250-QS quartz cuvette (Hellma GmbH & Co., Müllheim, Germany) was used for kinetic parameters determination. A linear signal was found for fluorescence in the ranges of concentration 0−50 μM ($r = 0.990$) and 50−100 μM ($r = 0.991$). Kinetic data analysis was carried out by using linear and nonlinear regression fitting (*23*), using the SigmaPlot Scientific Graphing for Windows version 8.0 (2001, SPSS Inc.).

Photography. Fluorescence photographs were obtained by using an excitation filter to limit the flash output between 360 and 480 nm, as described previously (*15*). A yellow barrier filter blocked off the reflected blue light under 490 nm, transmitting only the wavelengths emitted by the fluorochromes. Both filters were performed and supplied by Physical Sciences Inc. (Andover, MA).

Microscopy. Confocal microscopy images were obtained in a Leica TCS SP confocal microscope (Leica Microsystems, Wetzlar, Germany), equipped with a Kripto-Argon ion laser. Images of fresh samples were obtained by excitation at 488 nm with the laser beam. Emission was recorded at 500−525 nm.

RESULTS AND DISCUSSION

Pigment Analysis in *P. oleracea*. Portulaca is a genus of plants native to South America belonging to the family Portulacaceae. The existence of betalains in the genus *Portulaca* has been known since the description of these pigments in four different *P. grandiflora* phenotypes (*24*). Later studies confirmed and extended the number of structures present in their petals (*15*). In yellow flowers, the following betaxanthins were found: dopaxanthin, vulgaxanthin I, portulacaxanthin II and miraxanthin V. Complex pigment mixtures are frequently reported for betaxanthins in natural extracts (*13*, *25*).

Four different phenotypes of *P. oleracea* were studied in order to extend the knowledge on the distribution of betalains in plants of the Portulacaceae. Plants with white, yellow, pink, and red flowers were used to analyze the pigment content of their petals. An HPLC system suitable for the analysis of betalains (*19*) was applied, and in contrast to the multiplicity of pigments found in *P. grandiflora*, dopamine-betaxanthin was found to be the only pigment in *P. oleracea* yellow flowers. The structure and chromatogram are shown in **Figure 2**. Identity was determined by retention time and absorbance spectrum, and confirmed by coelution with synthetic dopamine-betaxanthin standard. The pigment content was calculated to be above 0.2 mg/g fresh petal weight.

Although simple compositions are not common in the distribution of betalains, they have been reported in other species. This is the case of the bright yellow flowers of three *Glottiphylum* species, and *Lampranthus productus*, which all belong to the Aizoaceae family and contain dopaxanthin as the only pigment (*14*, *16*). This is the first time dopamine-betaxanthin has been reported as a single pigment, and it is possible to use *P. oleracea* yellow flowers as a source of noncommercial dopamine-betaxanthin. Furthermore, betaxanthin producing cell lines from this phenotype could be established, as previously done for other betalain containing plant materials (*26*). The pigment has also been found in *Mirabilis jalapa* (*13*), and in hairy root cultures from yellow beet (*20*). Furthermore, a group of betacyanins, the 2-descarboxy-betacyanins, are derived from dopamine and not from DOPA, and were first described in flowers of *Carpobrotus acinaciformis* (*27*). 2-Descarboxy-betacyanins can be derived from dopamine-betaxanthin through the activity of the enzyme tyrosinase (*20*, *28*).

According to HPLC analysis, coloration of pink flowers is due to the presence of the betacyanins betanin (betanidin-5-*O*-β-glucoside), isobetanin, betanidin, and isobetanidin. **Table 1** summarizes the pigment content detected in each phenotype of *P. oleracea*. As expected, a mixture of the betacyanins and betaxanthins found respectively in the pink and yellow shades was detected in the red petals. White flowers can be considered a control when studying the pigment content of the colored ones. In this case only trace amounts of betanin were detected.

Purification and Characterization of Natural Dopamine-Betaxanthin. In order to obtain pure dopamine-betaxanthin from a natural source, it was purified from extracts of yellow flower petals. The pigment was extracted from the petals in buffer at pH 6.0 as described in Materials and Methods. After proteins had been removed by ultrafiltration, dopamine-betaxanthin was submitted to purification in an automated system based on anionic exchange chromatography (*19*). The recovery for natural dopamine-betaxanthin after chromatographic purification was 75% of the starting pigment. In order to remove the NaCl used during the elution process, pooled fractions were submitted to C-18 solid phase extraction. The yield for the last process was 95% and the final sample was concentrated to dryness, redissolved in water and used for further characterization.

The existence of structural isomers is frequently reported in betalains (*29*). It corresponds to the two possibilities in the chiral carbon C-15 present in the betalamic acid moiety of the structure. In *P. oleracea*, the betacyanins described in pink and red flowers contain both isomers: betanin (2*S*/*S*) and isobetanin (2*S*/*R*), and betanidin (2*S*/*S*) and isobetanidin (2*S*/*R*), as reported on **Table 1**.

The HPLC system used in this work is able to separate both isomers for most of the diastereoisomeric betaxanthins (*19*). However, dopamine-betaxanthin is a chiral molecule and cannot be resolved into the two plausible forms (*S*) or (*R*). In order to determine the chiral configuration of the pigment responsible for the yellow coloration, a diastereoisomeric betaxanthin derived from (*S*)-methionine was synthesized from extracted and purified dopamine-betaxanthin. Methionine-betaxanthin was not found in *P. oleracea* extracts. The substitution of the amine moiety was achieved by a Schiff condensation reaction, as described under Materials and Methods, but using extracted dopamine-betaxanthin as starting material instead of betanin. As a control, methionine-betaxanthin was obtained as well from a racemic 50:50 betanin:isobetanin mixture. HPLC recordings for both semisynthetic betaxanthins obtained from these starting materials are shown in **Figure 3**. As can be seen, the compound obtained from the flower petals corresponds to a mixture of both isomers, and mainly contains the isomer (2*S*/*S*), which accounts for 94% of the total pigment. This is similar to the ratios

Table 1. Pigment Content in Petals of Different Phenotypes of *P. oleracea*

	betalains (mg/g fresh weight)				
phenotype	dopamine-betaxanthin	betanin	isobetanin	betanidin	isobetanidin
white		0.003 ± 0.002			
yellow	0.218 ± 0.005				
red	0.321 ± 0.006	0.840 ± 0.006	0.030 ± 0.002	0.389 ± 0.006	
pink		0.067 ± 0.003	0.003 ± 0.001	0.066 ± 0.004	0.002 ± 0.001

obtained in red and pink flowers for betanin and betanidin (around 96%). Therefore dopamine-betaxanthin from *P. oleracea* is not present as a stereopure form with (*S*) configuration, as was found for the DOPA derived betaxanthin in *Glottiphylum*, and *Lampranthus* species (*16*). There is a 6% of dopamine-betaxanthin in the chiral form (*R*).

Oxidation of Dopamine-betaxanthin by Tyrosinase. Dopamine-betaxanthin is a fluorescent molecule, a phenomenon common to all betaxanthins, due to the presence of the electron resonance system supported by the condensation of betalamic acid with an amino group (*15*). To study the effect of the transformation of dopamine-betaxanthin by the action of the enzyme tyrosinase on the molecule fluorescence, excitation and emission spectra were recorded during the course of the reaction.

Figure 4A shows the evolution of the fluorescence as the reaction progresses, with a decrease in the fluorescence intensity being exhibited for both spectra. For the substrate, maximum wavelengths are $\lambda_{exc} = 465$ nm, and $\lambda_{ems} = 512$ nm. All the substrate was consumed according to HPLC analysis (results not shown), and, as can be seen, the reaction mixture keeps fluorescent properties. For this fluorescence, maximum wavelegth obtained for excitation is $\lambda_{exc} = 492$ nm, and the maximum for emission is $\lambda_{ems} = 540$ nm. The corresponding Stokes shift is 48 nm. The final reaction medium contains a mixture of products derived from the activity of tyrosinase, all with the same mass of *m*/*z* 345 ($[M + H]^+$) (*28*). The quinone resulting from the activity of tyrosinase on the hydroxyl groups of dopamine-betaxanthin evolves to stable "leuko" species through internal cyclization. The observed fluorescence is explained by the fact that the resulting compounds maintain the same electron resonance system regardless of the cycle created. Fluorescence in this kind of molecule has not been reported before.

In order to follow tyrosinase activity on dopamine-betaxanthin, a continuous method based on fluorescence spectroscopy can be developed. Since all products derived from the activity of tyrosinase come from the evolution of the quinone, their concentrations are linearly related and can be considered as a single compound from the spectroscopic point of view. The decrease in substrate concentration can be followed by its fluorescence at the corresponding maximum wavelengths ($\lambda_{exc} = 465$ nm, and $\lambda_{ems} = 512$ nm). A hyperbolic-type curve was obtained for the depletion of natural dopamine-betaxanthin in the presence of tyrosinase. **Figure 4B** shows the time courses obtained for the enzymatic reaction at different rates. A fluorescence based method can be useful for low levels of activity, pointing to future screening applications in the search for enzymatic activities on betaxanthins. Oxidation rates were also calculated. In this way, kinetic parameters can be deter-

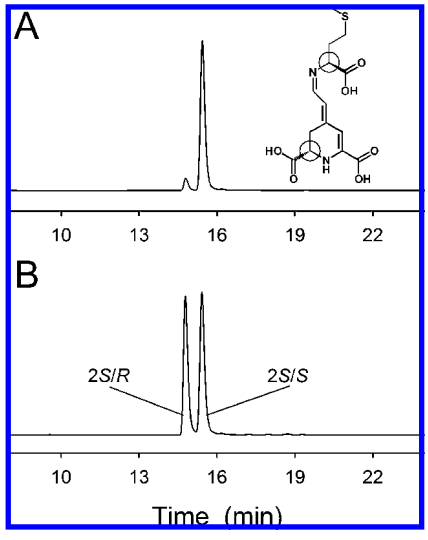

Figure 3. Fluorescence HPLC chromatogram for diastereoisomeric methionine-derived betaxanthin, obtained by semisynthesis from natural dopamine-betaxanthin extracted from *P. oleracea* (**A**). Inset: Methionine-betaxantin structure, showing chiral carbons circled. Structural standards were obtained from a 50:50 racemic mixture of (2*S*/*S*):(2*S*/*R*) betanin (**B**). Elutions were followed at $\lambda_{ems} = 510$ nm, with excitation at $\lambda_{exc} = 460$ nm.

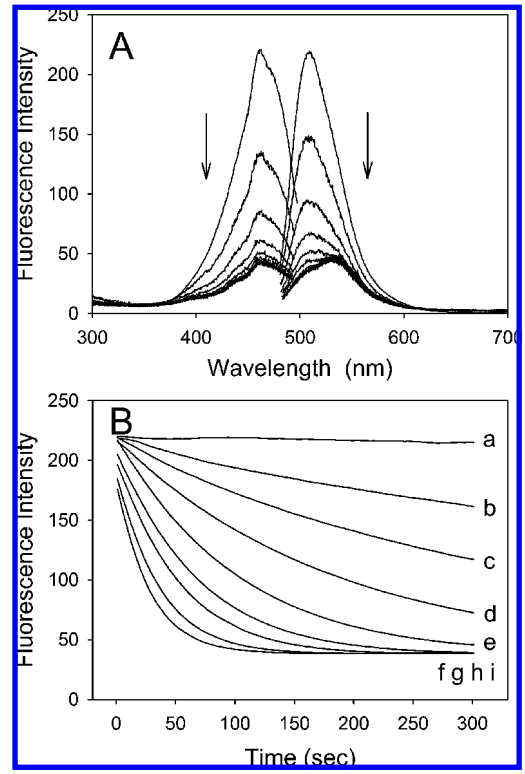

Figure 4. Excitation and emission consecutive spectra for the evolution of dopamine-betaxanthin reaction mixture. Ten scans were performed at 1,200 nm/min at 12 s intervals (**A**). Progress curves for dopamine-betaxanthin oxidation by tyrosinase, following fluorescence at $\lambda_{ems} = 512$ nm, with excitation at $\lambda_{exc} = 465$ nm (**B**). Six μM pigment solutions were used in phosphate buffer 50 mM, pH 6.0. 6.0 μg/mL enzyme was added in **A**, and the following concentrations in **B**: 0.0 μg/mL (a), 0.2 μg/mL (b), 0.5 μg/mL (c), 1.0 μg/mL (d), 2.0 μg/mL (e), 3.0 μg/mL (f), 4.0 μg/mL (g), 6.0 μg/mL (h), and 8.0 μg/mL (i) enzyme.

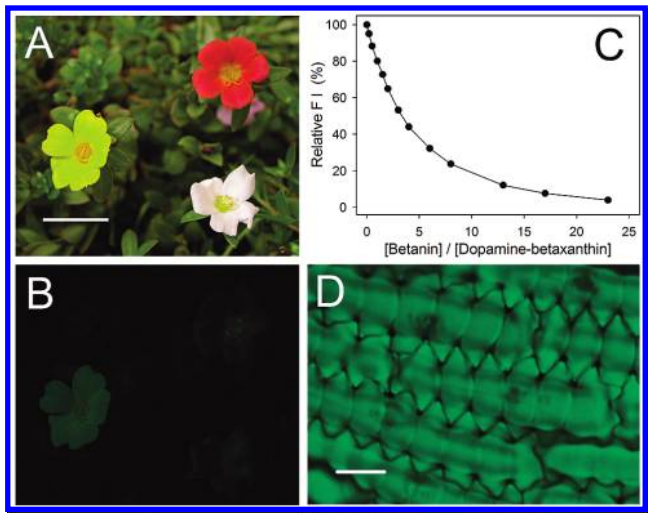

Figure 5. Betaxanthin fluorescence in *P. oleracea* petals. **A** and **B** show entire flowers, respectively illuminated by white light, or excited by blue light (scale bar, 2 cm). **C** shows the IFE on the visible fluorescence of dopamine-betaxanthin produced by the presence of increasing concentrations of betanin. Betaxanthin concentration was 1.9 μM. **D** is a confocal microscope image of yellow petal cells obtained through the excitation with the 488 nm line of a krypton−argon ion laser (scale bar, 20 μm).

mined within the linear range of concentration for the fluorescence signal, even with the presence of a complex mixture of reaction products. This allowed the determination of the following values: $V_m = 110.8 \pm 11.0$ μM min^{-1}, and $K_m = 123.2 \pm 19.0$ μM. Previously, the kinetic parameters were calculated in a similar manner through absorbance measurements for a different tyrosinase lot as $V_m = 74.4$ μM min^{-1}, $K_m = 94.7$ μM (*28*).

Fluorescence Visualization in *P. oleracea* Flowers. Due to the presence of fluorescent dopamine-betaxanthin in the flower petals of *P. oleracea*, these structures can be considered as fluorescent items. Fluorescence is evidenced by the use of a filter system especially designed for green fluorescence visualization (*15*). When illuminated by white light, a yellow flower containing dopamine-betaxanthins appears yellow because of the reflectance of the nonabsorbed radiation combined with the light emitted via the fluorescence (**Figure 5A**). The fluorescent phenomenon is shown when flowers are illuminated by filtered blue light. As can be seen, only the yellow flower (containing dopamine-betaxanthin as a single pigment) is fluorescent, while the white flower (used as a control) is not visible (**Figure 5B**). The search for novel classes of colors or intensities in flowers is of great importance in the flower industry, and has led other researchers to express Green Fluorescent Protein in ornamental plants (*30*). Dopamine-betaxanthin gives the yellow coloration to the petals of *P. oleracea*. Its fluorescence characteristics make it possible for the entire flower to emit light in the visible range of the electromagnetic spectrum.

As shown in **Table 1**, red flowers contain dopamine-betaxanthin in a higher quantity than that found in the yellow phenotype. However, it is not visible in the fluorescence image of **Figure 5**. In this flower, fluorescent dopamine-betaxanthin is accompained by betacyanins, mainly betanin, as described in **Table 1**, and it has been demonstrated that betacyanins have the ability to absorb the light emitted by betaxanthins (*31*). The overlapping between the betanin absorbance spectrum and the emission one of dopamine-betaxanthin may lead to a high degree of absorbance of the light emitted by the fluorophore when they are present together, and avoids the petal appearing under fluorescence conditions. The effect of the addition of increasing concentrations of betanin to a dopamine-betaxanthin solution was studied to test the possible attenuation of dopamine-betaxanthin fluorescence by betacyanins. A reduction of the fluorescence was obtained as seen in **Figure 5C**, due to the inner filter effect (IFE) in the two component fluorophore−chromophore system. The decrease in fluorescence comes as a the result of reabsorption of the emitted radiation.

This decrease in the intensity of fluorescence, F_{IFE}, can be given by an expression similar to that described for absorbance by Lambert−Beer's law (*32*):

$$F_{IFE} = F_0 \cdot e^{-kxc} \quad (1)$$

where F_0 is the fluorescence intensity measured in the absence of the absorber, c is the absorber concentration, x is fixed by the position of the cell ($x = 0.5$ cm), and k is related to the capacity of the absorber to attenuate the fluorescence. The exponential expression given by equation (1) can be fitted to the data of **Figure 5C**, yielding a k value for the system dopamine-betaxanthin/betanin of $k = 200{,}000$ M^{-1} cm^{-1}. This value is higher than that determined for the pair dopaxanthin/betanin ($k = 150{,}000$ M^{-1} cm^{-1}) (*31*), which is the only previous case described in betalain's physiological IFE. The attenuating capacity of betanin on dopamine-betaxanthin fluorescence is higher, and may account for a general phenomenon on all betacyanin and betaxanthin containing solutions and tissues, based on the similar spectroscopical properties of betaxanthins.

Pink flowers of *P. oleracea* do not present light emission and they appear dark in fluorescence photographs when the described filter system is used (results not shown), as do white flowers. Analogous fluorescence images can be obtained by using a microscopy system similar to the one used for Green Fluorescent Protein analysis (*33*). **Figure 5D** shows cells of a yellow petal, containing dopamine-betaxanthin, visualized under a confocal microscope. Light emission from cells is obtained with laser excitation, using the 488 nm line of an argon laser. The big petal vacuole contains the fluorescent pigments (*34*), and occupies almost the whole cell, allowing its visualization under physiological conditions. Controls were performed with white flower petals, revealing a dark field when fluorescence conditions were used.

Pigment Content in Flower Pistils. It can be observed from **Figures 5A** and **5B** that the yellow flower pistil appears bright yellow in white light image, and emits green fluorescence under blue light stimulation. Analogously, for the red flower, the pistil is yellow in color, and exhibits fluorescence. In contrast, the white flower pistil appears white and without fluorescence. HPLC analyses on pistil pigment extractions were performed to identify the pigments responsible for the coloration and fluorescence of pistils. As can be seen in **Figure 6**, white flower pistils contain no pigments. Yellow flower pistils contain dopamine-betaxanthin at 0.12 mg/g. In the case of the pistils excised from red flowers, the pigment content is mainly dopamine betaxanthin (0.30 mg/g), with betanin (0.011 mg/g) and betanidin (0.010 mg/g) also detected. For pistils derived from pink flowers, the pigment content is mainly formed by the betacyanins betanin (0.040 mg/g) and betanidin (0.028 mg/g), but also a small amount of dopamine-betaxanthin (0.015 mg/g) was found. These pistils are pinkish in color (**Figure 6** inset) and do not fluoresce under the conditions studied. Fluorescence in petals, and pistils containing large amounts of dopamine-betaxanthin can be of physiological relevance in creating an attractive pattern for pollinators (*35, 36*). To the best of our knowledge, this is the first evaluation of the betalain content in pistils.

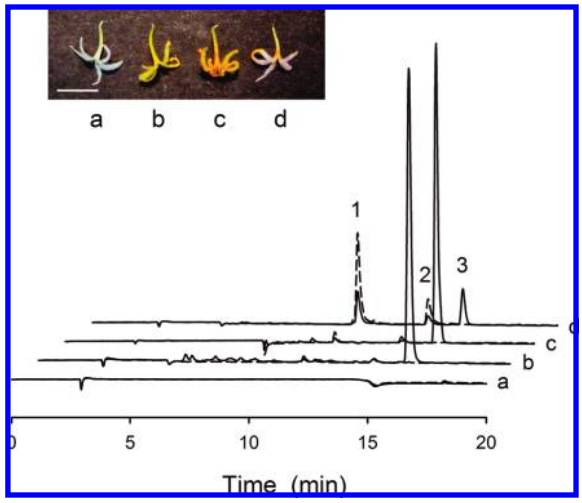

Figure 6. HPLC profiles for pigment analysis of pistils removed from white (a), yellow (b), red (c), and pink (d) flowers, at 480 nm (—) and 536 nm (- - -). Peaks identified correspond to betanin (1), betanidin (2), and dopamine-betaxanthin (3). Full scale is $A = 0.1$ absorbance unit, sample c being diluted 1/3.3 for comparison purposes. Inset scale bar is 5 mm.

The study of *P. oleracea* pigment content extends the knowledge of betalain pigment distribution, and reveals yellow flowers as a possible source of dopamine-betaxanthin. The fluorescent properties of the pigment make petal cell visualization possible. Tyrosinase catalyzed oxidation of a betaxanthin is monitored for the first time through fluorescence, allowing the characterization of the phenomenon, and the development of a new continuous detection procedure.

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