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Characterization of the Activity of Tyrosinase on Betanidin

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Betalains are an important class of water-soluble pigments, with radical scavenging capacity, which is characteristic of the order Caryophyllales. The structural unit of the violet betacyanins, betanidin is reported as a substrate for the enzyme tyrosinase (EC 1.14.18.1), which plays a key role in the betalains biosynthetic scheme. The compound was identified in *Lampranthus productus* violet flowers, from which it was extracted and purified. The tyrosinase-mediated oxidation of betanidin was characterized in depth and followed by high-performance liquid chromatography and spectrophotometry. The addition of ascorbic acid reversed the reaction product, betanidin-quinone, to the original pigment. Kinetic analysis revealed a $K_m = 0.66$ mM. Betanidin degradation kinetics was also studied in the absence of the enzyme and demonstrated that pH values over 6.0 and high ionic strength reduce the pigment stability.

KEYWORDS: Tyrosinase; metabolic pathway; betalains; betacyanins; betanidin; caryophyllales

INTRODUCTION

Betalains are water-soluble nitrogen-containing pigments which are present in plants belonging to the order Caryophyllales and in some fungal genera (*1*), and they include the red-violet betacyanins and yellow/orange betaxanthins. They are considered to be immonium derivatives of betalamic acid with 5,6-dihydroxyindoline-2-carboxylic acid (*cyclo*-DOPA) and amino acids or amines. All betacyanins are composed of aglycone (betanidin) and glycosyl moieties, and some betacyanins are modified with acyl groups.

Although the betalain metabolic pathway is still to be fully clarified, the involvement of the enzyme tyrosinase or polyphenol oxidase (monophenol, *o*-diphenol: oxygen oxidoreductase; EC 1.14.18.1) is generally considered as probable (*1*−*3*). Tyrosinase is a copper-containing enzyme that catalyzes two different reactions using molecular oxygen: the hydroxylation of monophenols (monophenolase activity) and the oxidation of *o*-diphenols (diphenolase activity) (*4*). The enzyme tyrosinase has been purified from betalain-containing extracts of *Amanita muscaria* (*5*), *Portulaca grandiflora* (*6*), and *Beta vulgaris* (*7*).

Physical−chemical properties of betalains have been characterized (*8*, *9*), and their stability has attracted the attention of researchers, since they are used as food colorants (*10*) and exhibit antioxidant capacity (*11*−*13*).

The first substantial evidence for the existence of enzymatic activity on betacyanins came from studies with protein fractions from red beet (*14*). These studies were continued (*15*), and the implication of the enzyme peroxidase in the degradation of betanin was established. However, it was not until the studies of Martínez-Parra and Muñoz (*16*, *17*) that the mechanism of action of peroxidase on betacyanins was established and the reaction products were identified.

Decolorizing enzyme activities are not restricted to red beets and have also been detected in Amaranthus species (*18*, *19*) and in pokeweed leaves (*Phytolacca americana*) (*20*).

Despite the relevance of tyrosinase in the metabolism of betalains, its activity toward betacyanins has never been characterized. In this work, the structural unit of betacyanins is extracted and purified from a natural source and used for kinetic studies with tyrosinase. The aim of the work described here was to study previously unconsidered reactions occurring once this labile molecule, betanidin, is constituted and to propose new steps that are integrated into the final steps of the biosynthetic scheme of betalains. The stability of betanidin under different conditions is also investigated.

MATERIALS AND METHODS

Chemicals. Mushroom tyrosinase (2590 units mg^{-1} solid, lot 092K70491), β-glucosidase (almonds, Sigma G4511), ascorbate oxidase (cucurbita sp.), chemicals, and reagents were purchased from Sigma (St. Louis, MO). Solvents were from Merck Chemicals Ltd. (Dorset, UK). HPLC-grade acetonitrile and methanol were purchased from Labscan Ltd. (Dublin, Ireland). Distilled water was purified using a Milli-Q system (Millipore, Bedford, MA).

Plant Material and Sample Preparation. *Lampranthus productus* (L. Bol.) N.E.Br. plants with violet flowers were grown by the authors in Murcia (Spain). Flower samples were carefully collected, and the petals were removed and washed. Pigments were extracted in 10 mM sodium acetate buffer pH 5.0, containing 10 mM ascorbic acid (AA) in a Polytron homogenizer (Kinematica AG, 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

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filtered through Centriplus YM-10 membranes (Millipore) to remove proteins, and the filtrate was used for pigment analysis or further purification. The whole process was carried out at 4 °C. For analytical purposes, flower extracts were also carried out in pure MeOH and filtered through 0.45 μm PVDF filters (Millipore) before analysis.

FPLC Purification. Anionic exchange chromatography of betanidin from *Lampranthus* was performed in an Äkta purifier apparatus (Amersham Biosciences, Uppsala, Sweden). The equipment was completely operated via a PC using Unikorn software version 3.00. Elutions were followed at 280 and 536 nm.

The solvents used were sodium acetate buffer 10 mM, pH 5.0 (solvent A), and sodium acetate buffer 10 mM, pH 5.0, 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 was used. After sample injection, the elution process was as follows: 0% B from beginning to 7 mL; 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 re-equilibration (7 mL, 100% A) steps were performed between each sample injection. Injection volume was 1 mL, and the flow rate was 0.5 mL min^{-1}. A total of 100 μL was injected for analytical purposes and comparison with standards.

Desalting on SPE Cartridges. For desalting betanidin, 1 mL C-18 cartridges (Waters, Milford, MA) were conditioned with 5 mL of methanol followed by 10 mL of purified water. Aqueous solutions of betanidin were applied and bound to the minicolumn. Salts and buffers were washed off by rinsing the column with purified water. Betanidin was eluted with acetone, and the resulting fraction was evaporated to dryness under reduced pressure at room temperature. Samples were then redissolved in sodium acetate buffer 10 mM, pH 5.0, at 4 °C or in purified water (for pH studies).

Standard Betanin and Betanidin Obtainment. Standard betanin for HPLC identification analysis and calibration was obtained from betanin purified 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) 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. (*11*).

Standard betanidin was obtained enzymatically from purified betanin through β-glucosidase (EC 3.2.1.21, β-D-glucoside glucohydrolase) treatment. A 4 μM betanin solution was incubated for 30 min with 14 units mL^{-1} of β-glucosidase in 50 mM sodium acetate buffer, pH 5.0, at 25 °C. The enzyme was removed by ultrafiltration through YM-10 membranes. Transformation was complete according to HPLC analysis.

HPLC Analysis. *HPLC-PDA.* A Shimadzu LC-10A apparatus equipped with a SPD-M10A photodiode array detector (Shimadzu, Kyoto, Japan) 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 system 1*: Solvent A was water acidified with 0.05% trifluoroacetic acid, and solvent B was composed of acetonitrile with 0.05% trifluoroacetic acid. Linear gradient was performed in 20 min from 0% B to 28% B. *Solvent system 2*: Solvent A was sodium acetate buffer 10 mM, pH 5.0, and solvent B was pure methanol. Linear gradient was performed in 11 min from 10% B to 60% B. In both cases, the flow rate was 1 mL min^{-1}, operated at 25 °C. Injection volume was 20 μL.

HPLC-MS. For HPLC-ESI-MS analyses, an Agilent VL 1100 apparatus with LC/MSD Trap (Agilent Technologies, Palo Alto, CA) was used. Elution conditions were as described above for solvent system 1, using a Zorbax SB-C18 (30 × 2.1 mm, 3.5 μm) column (Agilent Technologies) with a flow rate of 0.3 mL min^{-1}. 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.

Absorbance Spectroscopy. For absorbance spectroscopy, a Uvikon 940 spectrophotometer (Kontron Instruments, Zurich, Switzerland) was used. Consecutive wavelength scans were performed in a HP 8452A diode array UV−visible spectrophotometer (Hewlett-Packard, Waldbronn, Germany).

Quantification of Betacyanins. Betanin and betanidin concentration were evaluated through absorbance, taking a molar extinction coefficient of $\epsilon = 65\,000\ M^{-1}\ cm^{-1}$ and $\epsilon = 54\,000\ M^{-1}\ cm^{-1}$, respectively, at 536 nm (*21*). Measurements were made in water at 25 °C.

Tyrosinase Assays. The commercial mushroom tyrosinase was used as an enzyme source since it is used extensively as a model for studies at molecular and kinetic levels (*22*).

Unless otherwise stated, the reaction medium (1.0 mL) contained 20 mM sodium acetate buffer, pH 5.0, and 50 μg mL^{-1} of the enzyme at 25 °C. Quinone formation rates were followed at 400 nm. Other conditions and reagents are detailed in the text and in the figure legends. All experiments were performed in triplicate. Cuvettes of 0.5 and 1 cm of light path were used. Chemical oxidation of betanidin was negligible during the assay time.

The molar extinction coefficient corresponding to betanidin-quinone at 400 nm was determined by an end-point method, through a set of experiments at initial concentrations from 8.0 to 20.0 μM, and at sufficiently high enzyme concentration and incubation time. The reaction was allowed to proceed until the substrate had been completely converted. The data thus obtained were fitted by least-squares linear regression. Absorption spectra were also recorded with the above instrument. 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.).

Experiments were performed in triplicate, and the mean and standard deviation were plotted.

Stability of Betanidin. The effect of pH on betanidin stability was measured in 20 mM sodium acetate buffer (pH 3.0−5.5) or 20 mM sodium phosphate buffer (pH 5.5−8.0). For the study of the effect of ionic strength, a solution 6.4 μM betanidin, in the presence or absence of H_2O_2, was prepared in sodium acetate buffer (pH 5.0) 10−180 mM at 25 °C.

Betanidin degradation rate was followed spectrophotometrically at 536 nm.

Experiments were performed in triplicate, and the mean and standard deviation were plotted.

Ascorbate Oxidase Treatment. *L. productus* extract was carried out in 10 mM sodium acetate buffer pH 5.0 at 4 °C, under the same conditions as described above but without the addition of AA. Endogenous AA was depleted from the extract enzymatically through treatment with the enzyme ascorbate oxidase (EC 1.10.3.3, L-ascorbate: O_2 oxidoreductase). Fresh extract aliquots were diluted to give final absorbance at 265 nm around 1 unit and incubated with 0.07 units mL^{-1} of the enzyme in 10 mM sodium acetate buffer, pH 5.0, at 25 °C. The process was followed by UV−visible spectrophotometry until it was completed. The concentration of oxidized AA was calculated from the decrease in absorbance at 265 nm using the molar extinction coefficient of $16\,500\ M^{-1}\ cm^{-1}$ for AA (*24*). The experiment was performed in triplicate with different extracts.

RESULTS AND DISCUSSION

Extraction and Purification of Betanidin. A major problem limiting the number of studies on betanidin has been the low availability of the compound. In addition, it is known to be a labile molecule (*25*), and it degrades quickly under the working and storage conditions. Betanidin has been obtained from betanin by acid hydrolysis or deglycosilation (*17*, *26*).

The first aim of the present work was to find a natural source from which to obtain natural betanidin, and to purify it. The pigment content of violet flowers of different plants belonging to the Caryophyllales was analyzed by HPLC, solvent system 1 (results not shown). Among them, *L. productus* deep-violet flowers were selected because they revealed a high content of betanidin (0.56 mg/g fresh flower) which was only accompanied by its 5-*O*-β-glucoside, betanin (0.10 mg/g) (**Figure 1**, inset). Spectra and retention times were compared with those of standard purified betanin, extracted from beet root, and standard betanidin, obtained by deglycosilation of the former. Masses

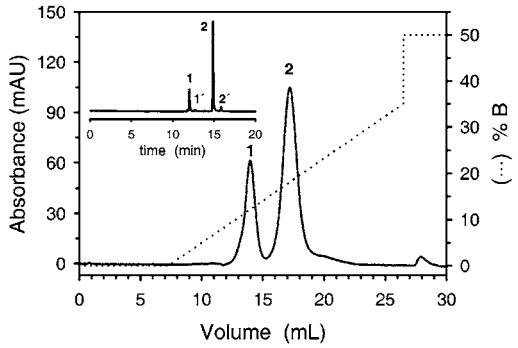

Figure 1. Chromatographic profile for the purification of betanidin (peak 2) from extracts of *L. productus* violet flowers. Peak 1 corresponds to betanin. Elution was followed at 536 nm (—), and conditions are described in the Materials and Methods section. A total of 100 μL of an extract containing betanidin 29 μM was injected. Inset: HPLC elution profile of the extract showing betanin (peak 1), betanidin (peak 2), and their corresponding (2*S*/*R*)-isomers (peaks 1′ and 2′). Analysis was performed with solvent system 1 and followed at 536 nm. Full scale is $A_{536} = 0.15$ absorbance units. A total of 20 μL of extract containing betanidin 24 μM was injected.

were determined by HPLC-ESI-MS and corresponded to the expected protonated molecular ions $[M + H]^+$ of the pigments: *m*/*z* 551 (betanin) and *m*/*z* 389 (betanidin). No betalamic acid was detected. *Lampranthus* is a genus of plants that belongs to the Aizoaceae family and which is native to South Africa. The existence of betalains in the genus *Lampranthus* has been known since Piattelli and Impellizzeri (*27*) described the presence of betacyanins in violet flowers. They demonstrated the existence of feruloyl-*p*-coumaroyl and diferuloyl-*p*-coumaroyl derivatives of betanin, but the presence of betanidin was not reported. Betanidin lability may have prevented proper identification in previous studies.

Pigments were extracted from the petals in buffer at pH 5.0 with 10 mM (AA). AA was added in order to avoid any possible oxidation of the pigment by the extract. The analysis of the extract was also carried out in pure methanol, with the same results being obtained. Betalains are highly soluble in water, and pH 5.0 favored the stability of betanidin as preliminary studies demonstrated (results not shown). Once proteins were removed by ultrafiltration, the extract was submitted to purification in an automated system based on ionic exchange chromatography. The elution profile obtained for the extracted pigments is shown in **Figure 1**. Fractions containing betanidin were pooled, and pure betanidin was obtained according to HPLC analysis. A total of 99% of the starting betanidin was recovered and submitted to C-18 cartridges in order to remove the NaCl content used during elution. The yield for the last process was 95%, and the final sample was evaporated to dryness and used in further experiments.

Calibration parameters have never been reported for betanidin in the literature. In this study, calibration graphs suitable for betanidin quantification in plant extracts were made by applying the described HPLC protocol (solvent system 1). Linear regression analysis was followed by plotting concentration (μM) against peak area at $\lambda = 536$ nm. Five points were used, and samples were injected in triplicate. The regression factor calculated was $r = 0.9999$ (peak area $= 49595 \pm 303 \times$ [betanidin] $- 13943 \pm 4597$). The detection limit (LOD $= 0.66$ μM) was calculated on the basis of 3σ (σ being the residual SD around the regression line) and the quantitation limit (LOQ $= 1.53$ μM) on the basis of 10σ. Data correspond to the calibration of the main isomer (2*S*/*S*), whose individual content was determined as a percentage of peak area.

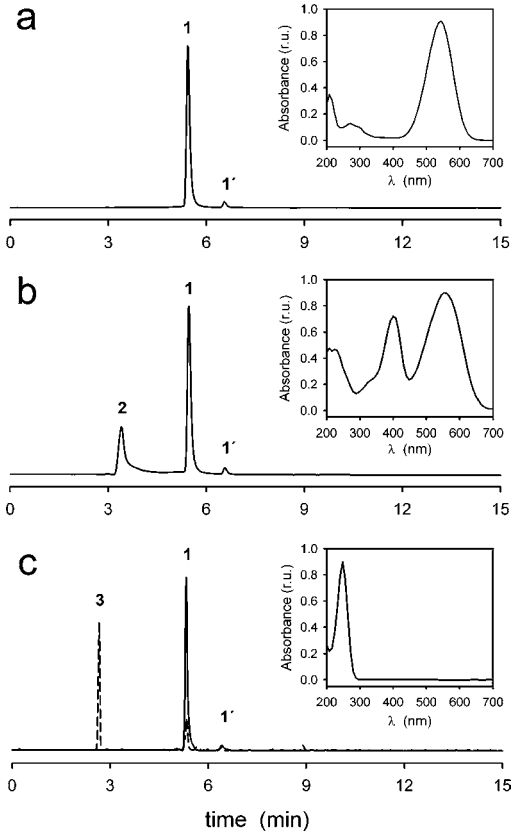

Figure 2. HPLC elution profiles for a solution of 37 μM betanidin in 20 mM sodium acetate buffer pH 5.0, before (**a**) and after (**b**) the addition of 85 μg/mL tyrosinase. Betanidin (peak 1) and the (2*S*/*R*) diastereoisomeric form (peak 1′) shared the same spectrum (**a**, inset). In **b**, the reaction was stopped after 15 min. The spectrum of the product obtained by the action of tyrosinase on betanidin (peak 2) is shown in **b**, inset. Panel **c** shows the chromatogram after the addition of 0.2 mM AA to the previous one, recovering the original pigment. Dashed line in **c** represents absorbance at 240 nm (full scale $A_{240} = 0.4$), with 3 being the peak corresponding to AA (spectrum in inset). Full scale is $A_{536} = 0.4$ absorbance units in **a** and **c**; $A_{536} = 0.125$ in **b**. Solvent system 2 was used.

Enzymatic Oxidation of Betanidin by Tyrosinase. *HPLC Analysis.* Purified betanidin was analyzed by HPLC (solvent system 2) and showed the existence of the isomers (2*S*/*S*) (major peak) and (2*S*/*R*) (minor peak), as can be seen in **Figure 2a**. The solution was stable under pH 5.0 at 25 °C during the assay time. However, when the enzyme tyrosinase was added (85 μg/mL), the pigment content evolved and a new peak appeared, concomitant with the reduction of the peak corresponding to the original betanidin (**Figure 2b**). The peak derived from the activity of tyrosinase on betanidin was assigned to betanidin-quinone by following the well-established mechanism of action of the enzyme (*4*) and by spectral comparison with the betanidin-quinone obtained by peroxidase activity (*17*). The contribution of the minor diasteroisomer (2*S*/*R*) to the reaction could not be separated from the major one (2*S*/*S*), and a single peak for betanidin-quinone was obtained. Tyrosinase may preferably oxidize betanidin compared to isobetanidin in the same way as tyrosinase showed stereospecificity in its affinity toward betaxanthins (*28*).

Spectroscopic Analysis of the Oxidation of Betanidin by Tyrosinase. Pure betanidin was obtained in sufficient amounts to follow the tyrosinase-mediated oxidation of the compound by observing changes in the UV−visible spectrum with time at pH 5.0 (**Figure 3a**). Maximum spectral changes were observed

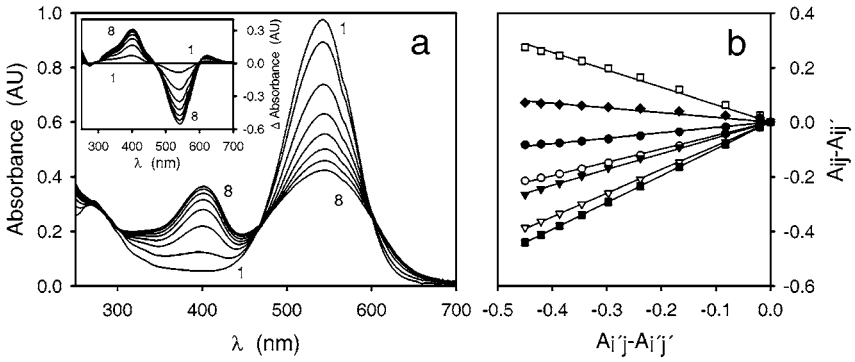

Figure 3. (**a**) Consecutive scan spectra of betanidin oxidation by tyrosinase. The assay medium contained 18 μM betanidin in 20 mM sodium acetate buffer, pH 5.0. The reaction was started by the addition of the enzyme (50 μg/mL). Scans were performed at 5 min intervals. Inset: differential spectra derived from the previous scans on 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 $i' = 540$ nm, $i = \blacklozenge$ (320 nm), $i = \square$ (400 nm), $i = \bullet$ (482 nm), $i = \circ$ (500 nm), $i = \triangledown$ (522 nm), $i = \blacksquare$ (534 nm), $i = \blacktriangledown$ (570 nm), and j' = first tracing.

at 542 nm (decrease in absorbance), and an isosbestic point was formed in the visible range of the electromagnetic spectrum at $\lambda = 466$ nm, during the first 40 min of reaction. Another maximum was detected at 400 nm (increase in absorbance). These changes were not observed in the absence of the enzyme and thus were considered results of tyrosinase activity. The spectroscopic evolution was as expected, considering the spectrum of betanidin-quinone obtained by HPLC-PDA (**Figure 2b**, inset).

Coleman graphic analysis (*29*) was applied to the oxidation of betanidin by tyrosinase. The convergence of straight lines at the origin of the coordinate axis (**Figure 3b**) also supported the existence of only two related absorbing species evidenced by HPLC. To ascertain that the product of the reaction was betanidin-quinone, AA (final concentration 0.2 mM) was added to the medium after the action of tyrosinase and once the enzyme had been removed by ultrafiltration. Immediately after the addition of AA, the original color was recovered and betanidin was formed, as HPLC analysis indicated (**Figure 2c**). AA is a reducing agent able to reverse *o*-quinones to the diphenols (*30*). All the data collected in the present work indicated that a single quinone was the product of the oxidation of betanidin mediated by tyrosinase.

Kinetic Characterization. Betanidin oxidation rate by tyrosinase was dependent on substrate concentration in the reaction medium (**Figure 4**). When the concentration of betanidin was varied and the resulting steady-state rates were fitted to the Michaelis−Menten equation, the kinetic parameters for tyrosinase were evaluated as $V_m = 41.1 \pm 0.6$ μM/min, $K_m = 0.662 \pm 0.012$ mM.

The affinity that tyrosinase exhibits for this substrate is in line with the data reported for tyrosinases from different sources acting on plant pigments. A $K_m = 0.646$ mM was obtained for the oxidation of the flavonoid quercitin catalyzed by broad bean tyrosinase (*31*), and a $K_m = 0.20$ mM was found for the oxidation of catechin by the enzyme from peach (*32*). A lower value was calculated for the oxidation by mushroom tyrosinase of the related compound dopaxanthin ($K_m = 84.3$ μM) (*2*). The tyrosinase-homologue aureusidin synthase presented a high specificity in the catalysis of chalcones oxidation, and a $K_m = 15.7$ μM was determined for the diphenolic pigment PHC (2′,3,4,4′,6′-pentahydroxychalcone) (*33*).

For the first time, kinetic parameters are reported for tyrosinase acting on a betacyanin. To determine the reaction rates of betanidin oxidation it was necessary to calculate the molar extinction coefficient of the product. An $\epsilon = 18\,600$ M^{-1} cm^{-1} was obtained at 400 nm for betanidin-quinone.

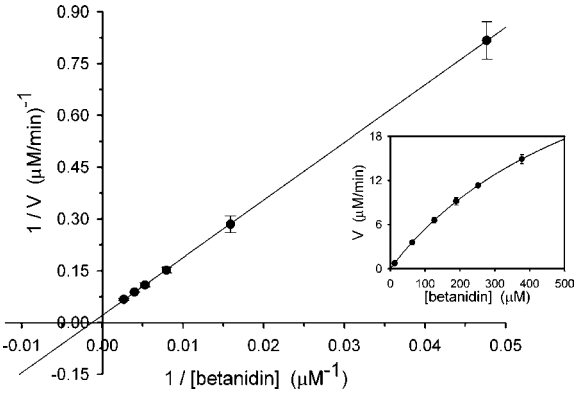

Figure 4. Lineweaver–Burk plot for the kinetic analysis of the action of tyrosinase on betanidin. Inset: dependence of betanidin oxidation on its concentration. The reaction medium contained different substrate concentrations in 20 mM sodium acetate buffer, pH 5.0, at 25 °C with 50 μg mL^{-1} of tyrosinase.

Betanidin Stability. Studies on the chemical stability of betacyanins have mainly been focused on betanin. Huang and von Elbe (*34*) evaluated the influence of pH on the stability of this compound, although data have been reported for other pigments, e.g., amaranthin (*35*). Whereas the betanin degradation generates betalamic acid and *cyclo*-DOPA−5-O-β-D-glucoside (*13*), the chemical degradation of betanidin leads to the formation of betanidin-quinone that evolves to polymers (*17*). To our knowledge, kinetic studies on the degradation of betanidin have not been carried out.

Figure 5A shows the effect of pH 3.0−8.0 on the degradation rate of betanidin at 25 °C in the absence of any enzyme. The data for each pH could be adjusted to a first-order degradation kinetics, and the corresponding degradation constants, k_d, were calculated. The results (**Figure 5A**, inset) indicated that betanidin was stable in solution in the pH range 3.0−6.0. However, at higher pH values, betanidin degrades quickly and a color loss is observed. The vacuolar location of betalains (*36*) may favor betanidin stability since pH is lower than in cytosol.

The influence of ionic strength on the stability of betanidin solutions was also studied (**Figure 5B**). Buffer concentrations of 20, 60, and 100 mM (pH 5.0) were assayed in the presence of increasing H_2O_2 concentrations, which chemically conducted to the formation of betanidin-quinone. The data clearly showed that the stability of betanidin is negatively affected by ionic strength and decreases with increasing concentrations of peroxide. Even in the absence of peroxide an increase in the ionic

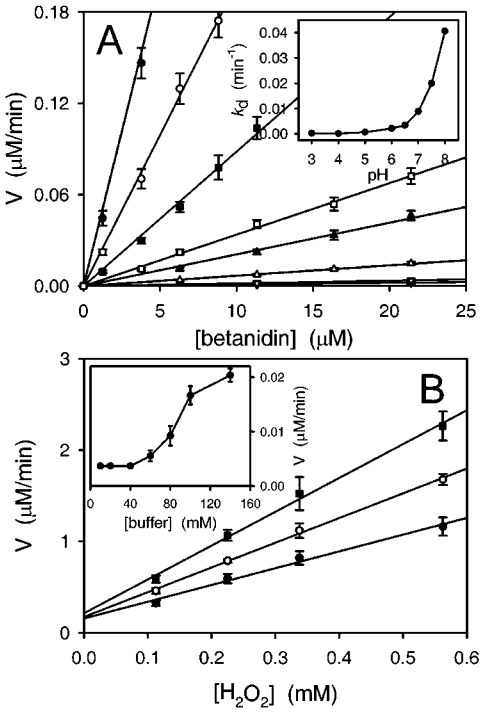

Figure 5. Degradation kinetics of betanidin, in the absence of tyrosinase, under different conditions of pH (**A**) and ionic strength (**B**). In **A**, the effect of betanidin concentration on the initial degradation rate was tested at the following pH values: 3.0 (▽), 4.0 (▼), 5.0 (△), 6.0 (▲), 6.5 (□), 7.0 (■), 7.5 (○), and 8.0 (●), thus allowing the determination of the corresponding first-order degradation constants, k_d (inset). The medium contained 20 mM sodium acetate (pH 3.0–5.0) or sodium phosphate buffer (pH 6.0–8.0) at 25 °C. In **B**, the effect of the ionic strength was tested in the presence of increasing concentration of H_2O_2, which magnified the effect obtained in its absence (inset). The medium contained betanidin 6.4 μM and sodium acetate buffer (pH 5.0) 20 mM (●), 60 mM (○), or 100 mM (■) at 25 °C.

strength is enough to promote betanidin degradation (**Figure 5B**, inset). Thus, not only the presence of oxidases like tyrosinase or peroxidase but also the oxidative stress in structures exposed to direct sunlight may lead to the formation of betanidin-quinone under unspecific conditions (*37*, *38*).

Endogenous Ascorbic Acid Prevents Betanidin Oxidation. Among the reducing compounds available in plant cells, AA (vitamin C) is considered to be the most abundant (*39*), and it may be one of the possible systems present in the petal cell of betanidin-containing flowers to avoid the pigment's oxidation. The effect of AA on the tyrosinase-mediated catalysis is the reduction of the *o*-quinone product of the enzymatic reaction back to the corresponding *o*-diphenol with the concomitant oxidation of AA to dehydroascorbic acid (DAA) (*30*). It has been proposed that AA and DAA neither activate nor inhibit the enzyme (*40*). Due to the relevance of the presence of a reducing agent for the stability of betanidin, and hence in the biosynthetic scheme of betacyanins, the occurrence of endogenous AA was evaluated for the first time in violet flowers of a plant belonging to the Caryophyllales.

Endogenous AA was determined in extracts of violet flowers of *L. productus* using a spectrophotometric method (**Figure 6**). AA was depleted by the enzyme ascorbate oxidase which catalyzes the oxidation of L-AA to L-DAA in the presence of molecular oxygen. The evolution of the depletion of AA (absorbance decrease at 265 nm) is better shown when differential spectra are represented, and it can be seen that betanidin was also affected by the enzyme activity (absorbance decrease

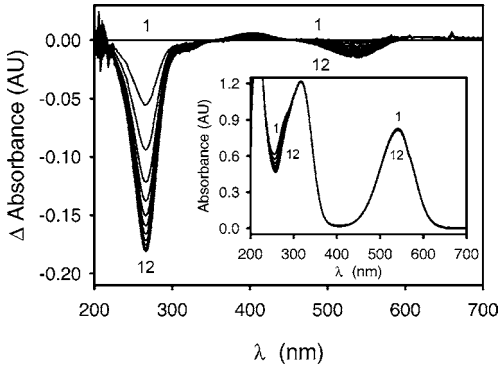

Figure 6. Endogenous AA depleted from *L. productus* violet flowers extract after the addition of ascorbate oxidase (0.07 unit mL^{-1}). Differential spectra show negative recordings of the characteristic absorbance spectrum of AA (decrease at 265 nm) derived from the spectra shown in the inset. Scans were performed at 1 min intervals.

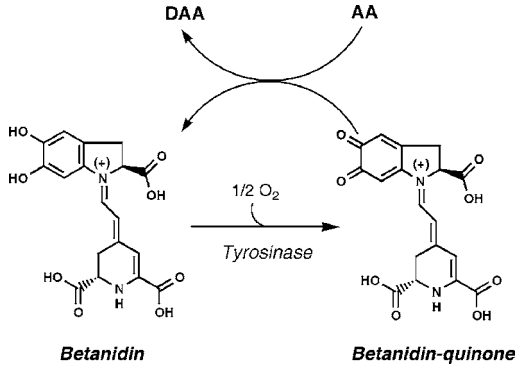

Figure 7. Oxidation of betanidin by tyrosinase (step 1) and the involvement of AA on betanidin regeneration (step 2).

at 542 nm). The level of AA determined was 0.19 mg g^{-1} petal fresh weight. This value is comparable to those reported for betalain containing beet roots (0.15 mg g^{-1}) (*41*) and cactus pears (0.18−0.23 mg g^{-1}) (*42*) and lower than the level obtained for dopaxanthin-containing yellow flowers of *L. productus* (0.8 mg g^{-1}) (*2*).

When tyrosinase (25 μg/mL) was added to the extracts used for the evaluation of endogenous AA, oxidation of betanidin was not observed until all AA was depleted from the medium (results not shown). Betanidin-quinone was being formed by tyrosinase and continuously being transformed back to betanidin by AA, evidencing the protection by the latter of the original pigment. **Figure 7** shows the oxidation of betanidin by tyrosinase where the formation of betanidin-quinone by tyrosinase (step 1) and the protecting effect of AA (step 2), demonstrated in the present work, are considered.

In conclusion, the pivotal molecule in the biosynthesis of plant pigments betacyanins, betanidin, can be extracted and purified from violet flowers of *L. productus*. The activity of the enzyme tyrosinase on this secondary metabolite is characterized for the first time, and the formation of betanidin-quinone, as a single compound, is demonstrated.

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