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Betacyanins as phenol antioxidants. Chemistry and mechanistic aspects of the lipoperoxyl radical-scavenging activity in solution and liposomes

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Abstract
Reaction kinetics of betanin and its aglycone betanidin towards peroxyl radicals generated from the azo-initiated oxidation of methyl linoleate in methanol and of a heterogeneous aqueous/soybean phosphatidylcholine liposomal system were studied by monitoring formation of linoleic acid hydroperoxides and consumption of the pigments. Betanin was a weak retarder in methanol and an effective chain breaking antioxidant in the liposomal model, indicating that kinetic solvent effects and partition in lipid bilayers may affect its activity. Betanidin behaved as a chain terminating antioxidant in both models. Kinetic parameters characterizing peroxyl radical-scavenging activity showed that betanidin was more effective than betanin, in terms of both radical-scavenging rate constant and stoichiometric factor, with effectiveness of the same order as vitamin E under comparable conditions. Products identified by spectrophotometric and HPLC techniques indicated reaction of the glucose-substituted monophenol and *ortho*-diphenol moieties of betanin and betanidin, respectively, and suggested mechanisms of the antioxidant activity. Either betanin or betanidin incorporated in liposomes with α-tocopherol had additive effects, supporting partition of the pigments in the bilayer and lipoperoxyl radical reduction.

Keywords: *Betacyanins, betanin, betanidin, lipid peroxides, liposomes, antioxidant phytochemicals*

Introduction

Lipid peroxidation, a degradative process that concerns unsaturated membrane lipids under conditions of oxidative stress [1], is believed to contribute to human ageing and disease by disrupting the structure, the packing of lipid components and, ultimately, preventing the membrane function. Humans have a rather complex network of defense barriers that protect cell membranes against oxidation by eliminating reactive oxygen species. The dietary intake of antioxidants plays a major role in this network. Vitamins C and E and carotenoids and flavonoids are the most known and widely studied antioxidants, but other bioactive substances are continuously being explored in foods.

Betalains are nitrogen-containing pigments occurring in the Caryophyllales order of plants, including beetroot and cactus pear, and in some fungal genera [2]. They include two main groups; betaxanthins, that may be considered as immonium derivatives of betalamic acid with either amino acids or amines, and betacyanins, condensation products of betalamic acid with a *cyclo*-DOPA structure with additional substitutions through varying glycosylation and acylation patterns at C-5 or C-6. Betalains have been shown to be bioavailable from various food sources [3–5], may reach peak plasma concentrations of the micromolar order [4] and can distribute in low density lipoproteins (LDL [4]) and cells [6]. Betanin, betanidin 5-O-β glucoside, is the major dietary betacyanin and its aglycone betanidin may be formed during digestion (Figure 1) [7].

A number of data have been published on the antioxidative and protective effects of betalains in

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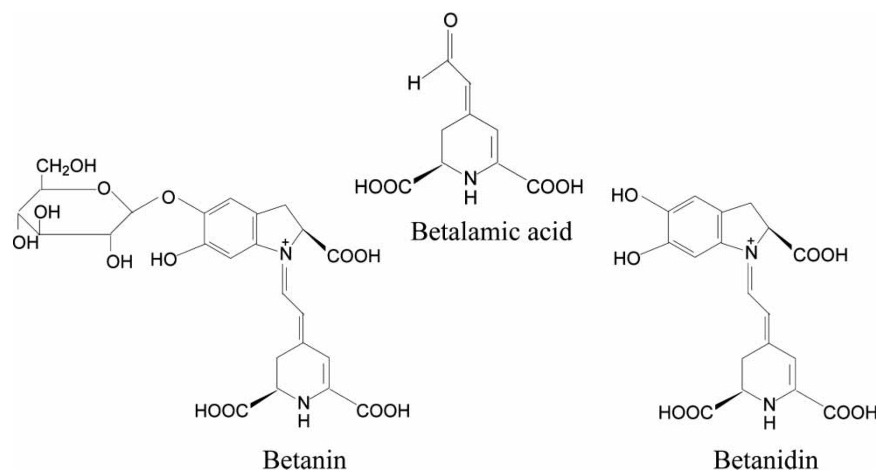

Figure 1. Molecular structure of betalamic acid and betacyanins.

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various biological environments from LDL [8], to either healthy [6] or pathological [9] red blood cells, as well as cell cultures [10]. Investigating the chemistry of the reaction between the pigments and lipid-derived radicals can contribute to elucidate the molecular basis of their antioxidant effects. Whereas kinetic parameters of the lipoperoxyl-radical scavenging activity of betaxanthins (as indicaxanthin) have recently been obtained [11], antioxidant efficiency values of betacyanins are not available. Recent studies showing that betacyanins can bind to microsomes [3] and partition within the lipid core of membrane mimetic phosphatidylcholine vesicles [12] may add further interest to such investigation.

In this work we evaluated the reducing activity of betanin and betanidin vs (lipo)peroxyl radicals generated in methanol from methyl linoleate by the activity of the lipid-soluble 2,2′-azobis (2,4-dimethyl-valeronitrile) (AMVN) and in an heterogeneous aqueous/soybean phosphatidylcholine membrane model, whose oxidation was induced by the hydrophilic 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH). Mechanisms of the reaction of the betacyanins with peroxyl radicals are proposed on the basis of the stoichiometry of reduction and of the products revealed by the joint use of spectrophotometric and HPLC techniques.

Methods

Chemicals

Almond β-glucosidase, linoleic acid methyl ester (LAME), soybean phosphatidylcholine (PC), α-tocopherol, 13(S)-hydroperoxy-(9Z,11E)-octadecadienoic acid, L-Dopa, Chelex-100 ion-exchange resin and Sephadex G-25 were from the Sigma Chemical Co. (St. Louis, MO). LAME was purified on a Florisil column (Floridin, New York). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) and 2, 2′-azobis(2-amidinopropane)hydrochloride (AAPH) were from Polyscience, Inc. (Warrington, PA). All other reagents and chemicals were of analytical or HPLC degree. Buffers used throughout this study were chromatographed over Chelex-100, to minimize the effects of adventitious metals.

Preparation of betalain substrates and reference compounds

Betanin was isolated from cactus pear (*Opuntia ficus-indica* L. *Mill*) fruits (red cultivar). The phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25 [13]. Fractions containing the pigment were submitted to cryo-dessiccation. The dessiccated material was re-suspended in 1% acetic acid in water and submitted to semi-preparative HPLC using a Varian Pursuit C-18 column (250×10 mm i.d.; 5 μm; Varian, Palo Alto, CA), eluted with a 20 min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile), with a flow of 3 ml/min [11]. Spectrophotometric revelation was at 536 nm. The elution volumes relevant to betanin were collected. Samples after cryo-dessiccation were re-suspended in methanol or 0.9% NaCl in 5 mM phosphate buffer, pH 7.4 (PBS), at a suitable concentration and used immediately or stored at -80°C. Concentration of the samples was evaluated spectrophotometrically in a DU-640 Beckman spectrophotometer by using a molar coefficient at 536 nm of 65 000 $M^{-1}cm^{-1}$ [3].

Almond β-glycosidase (14 U/mL) was used to obtain betanidin from 4.0 μM purified betanin, in 50 mM acetate buffer, 37°C, pH 5.0 [14]. The enzyme was removed by ultrafiltration through YM-10 membranes (Sigma). Transformation was complete in 10 min according to spectrophotometric analysis, taking an extinction coefficient of 54 000 $M^{-1}cm^{-1}$ at 536 nm [15]. The pigment was submitted to cryo-dessication, re-suspended in suitable solvent at opportune concentrations and immediately used.

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Indicaxanthin was purified from cactus pear fruits (yellow cultivar) as described [11]. Betalamic acid was prepared by alkaline hydrolysis of indicaxanthin and the amount evaluated spectrophotometrically at 420 nm using an extinction coefficient 30 000 M^{-1} cm^{-1} [16].

Betanidine quinone was obtained from betanidin by oxidation with equimolar amounts of Na-periodate, at 25°C, in 100 mM phosphate buffer, pH 3.5 [17]. A spectrophotometric analysis, taking an extinction coefficient of 18 600 $M^{-1}cm^{-1}$, at 400 nm [14], showed that the transformation was complete within 5 min.

Dopachrome was obtained by oxidation of L-DOPA with $NaIO_4$ in 0.01 M acetate buffer, pH 4.8 [18], with a 4-fold excess of oxidant to allow total conversion of substrate. The product was quantified by the absorption band peaking at 485 nm [19], using an extinction coefficient 4770 M^{-1} cm^{-1} [20].

Peroxyl radical-scavenging assay in solution

Peroxidation of methyl linoleate, either in the absence or in the presence of antioxidants, was performed by incubating 300 mM LAME and 2.0 mM AMVN, in a final methanol volume of 0.25 mL, in a water bath at 50°C, under air. Portions of the mixture (10 μl) were taken at intervals and dissolved in 500 volumes of methanol. Aliquots (100 μl) were injected onto a Supelco Supelcosil^{TM} (Bellafonte, PA) LC-18 column (250 × 4.6 mm i.d., 5 μm), equilibrated and then eluted with methanol at a flow rate of 1.0 ml/min. Quantitation was by reference to a standard curve constructed with known amounts of linoleic acid hydroperoxide (R_t 4.2). Methanolic solution of antioxidants were added to the solution of methyl linoleate, then the azo-initiator was added and the incubation was carried out as above.

Peroxyl radical-scavenging assay in liposomal suspensions

Liposomes were prepared by adding chloroform solution of soybean PC and betacyanins and/or α-tocopherol in methanol when required, in a round-bottom tube kept in an ice bath. Solvent was removed, under a nitrogen stream and the thin film obtained was re-suspended with PBS to a final 10 mM lipid concentration and vortexed in an ice bath for 10 min. The resulting multilamellar dispersion was then transferred into an Avestin Liposofast (Avestin, Inc., Ottawa, Canada) small-volume extrusion device provided with a polycarbonate membrane of 100 nm pore size, designed to obtain a homogeneous population of large unilamellar liposomes.

Liposomes were incubated in a water bath, at 37°C, under air. Oxidation was induced by 5 mM AAPH, added to the suspensions in a small PBS volume. Aliquots of liposomes (20 μl) were taken at intervals and dissolved in 50 vol of absolute ethanol. Spectra were then recorded in the range 200–300 nm and the conjugated diene (CD) lipid hydroperoxide production was evaluated by the increase in absorbance at 234 nm, using an extinction coefficient 27 000 M^{-1} cm^{-1} [21]. All oxidations were carried out under dim red light to avoid possible photo-oxidation of fatty acid by low-energy quanta of visible light and to preserve light-sensitive α-tocopherol and pigments.

Assays for betanin/betanidin oxidation and analysis of reaction products

The consumption of betanin and betanidin, as well as formation of products, were monitored by both HPLC and spectrophotometric measurements. Suitable aliquots of the incubation mixture were taken at time intervals, diluted in 1% acetic acid in water and analysed by HPLC using a RP-18e Performance column (100 × 4.6 mm; Merck, Darmstadt, Germany), equipped with RP-18e Chromolith guard cartridge (5 × 4.6 mm, Merck) and eluted with a 20-min linear gradient elution from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile), at a flow rate of 1 mL/min (solvent system 1). Spectrophotometric detection was at 536 nm for either betanin (R_t 12.5 min) or betanidin (R_t 16.5 min). Formation of betalamic acid was investigated under the conditions described above (R_t 8.2 min), with spectrophotometric revelation at 425 nm. Automatic wavelength change allowed evaluation of betalamic acid and betacyanins in the same run. Formation of betanidin quinone from betanidin was assessed using the chromatographic column as above with a linear gradient in 12 min, from 10% to 60% methanol in 10 mM sodium acetate buffer, pH 5.0 (solvent system 2), at 1.0 mL/min and detection at 540 nm [14]. Under these conditions betanidin quinone eluted with R_t 3.7 min and betanidin with R_t 6.2 min. Dopachrome was analysed by a LiChrosphere^{R} 100RP-8 column (250 × 4.0 mm i.d., 5 μm, Merck, USA), equilibrated and then eluted with 5% acetonitrile in 10 mM aqueous ammonium acetate (corrected to pH 2.0 with HCl, solvent system 3) at 1.0 mL/min and detection at 485 nm [22]. Quantitation was by reference to standard curves constructed with 5–100 ng of the relevant purified compounds and by relating the amount of the compound under analysis to the peak area. In parallel, aliquots of the incubation mixtures were diluted with methanol and spectra between 300–700 nm were recorded.

Kinetic theory

The oxidation of methyl linoleate under controlled conditions is the simplest way for studying the oxidation of polyunsatured lipids and it has widely

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been adopted to carry out kinetic studies with antioxidants. Since the linoleic acid has two double bonds, peroxidation occurs at the bisallylic hydrogens and generates stoichiometric amounts of CD lipid hydroperoxides. LAME solutions were oxidized by radicals thermally generated from lipophilic azo-initiator to ensure a linear production of lipoperoxides propagating chain-reactions. The experimental data were treated according to the classical lipid oxidation theory with a computer-assisted analysis (TableCurve 2D, Jandel, CA) of the peroxidation curves to calculate kinetic parameters for the reaction of lipoperoxyl radicals with antioxidants. The propagation rate, R_p, was measured as the amount of CD lipid hydroperoxides formed per second, either in the absence (control) or in the presence of antioxidant. The rate of chain initiation, R_i, was measured by the inhibition period (t_{inh}) produced by a known amount of α-tocopherol following equation (1)

$$R_i = n[IH]/t_{inh} \tag{1}$$

where IH is the concentration of α-tocopherol and n, the stoichiometric factor that represents the peroxyl radicals scavenged by each molecule of antioxidant, is assumed to be 2 [23]. The inhibition period, t_{inh}, in the curve of peroxidation in the presence of antioxidant was measured as the time interval between the addition of free radical initiator and the point of intersection of the tangents to the tracts of the curve representing the inhibition and propagation phases.

The inhibition rate constant, k_{inh}, was calculated in solution of peroxidizing LAME following equation (2)

$$k_{inh} = k_p[LH]/R_{inh}t_{inh} \tag{2}$$

where $[LH]$ is the concentration of the lipid and k_p, the absolute rate constant for the oxidation of LAME at 50°C is to be assumed 230 $M^{-1}s^{-1}$ [24]. The inhibition rate, R_{inh}, that is the rate of production of lipid hydroperoxides during the inhibition period, was calculated by the coordinates of the intercept of the extrapolations of the parts of the curve representing the inhibition and propagation phases.

Soybean phosphatydilcholine liposomes are a suitable system to obtain quantitative data of the peroxyl radical-scavenging activity of antioxidants in membrane-mimetic vesicles, due to the peculiar composition in unsatured fatty acids, 95% of which consist of linoleic acid. The use of hydrophilic azo-initiator causes a linear hydroperoxide formation, thereby R_i can be evaluated by the classical inhibitor method reported above.

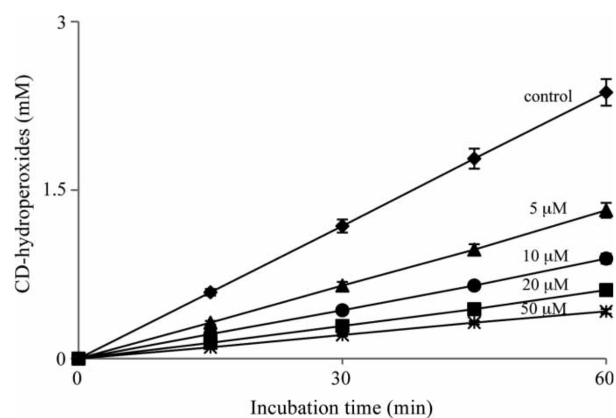

Figure 2. AMVN-induced oxidation of methyl linoleate in methanol in the absence (control) or in the presence of 5–50 μM betanin. Oxidation conditions are reported in methods. Each point represents the mean ± SD of four-to-six determinations carried out with different incubation mixtures.

Results

Peroxyl radical-scavenging activity of betanin and betanidin in methanol

Methanolic LAME solutions were oxidized by AMVN, in the absence and in the presence of variable amounts of either betanin or betanidin. Betanin was unable to delay LAME peroxidation, but caused only a decrease of the peroxidation rate, that depended exponentially ($r^2 = 0.93$) on the betanin amount, a behaviour characteristic of antioxidants known as retarders [25] (Figure 2). On the contrary, betanidin showed a classical chain-breaking antioxidant activity. Clear inhibition periods, the length of which was linearly correlated to the concentration of the antioxidant ($r^2 = 0.99$, $p < 0.01$), preceded the active hydroperoxide formation, while the rate of peroxidation during the active phase of lipid oxidation was comparable with that of the non-inhibited reaction (Figure 3). In addition betanidin was totally

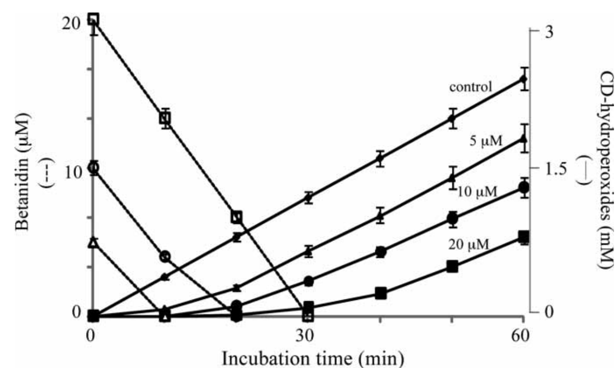

Figure 3. AMVN-induced oxidation of methyl linoleate in methanol in the absence (control) or in the presence of 5–20 μM betanidin and consumption of the antioxidant. Oxidation conditions and HPLC analysis of betanidin are reported in methods (solvent system 1). Each point represents the mean ± SD of five-to-seven determinations carried out with different incubation mixtures.

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consumed within the inhibition period, at a constant rate (Figure 3). The length of the inhibition period of chain-breaking antioxidants is determined by the number of radicals scavenged for each molecule of antioxidant [26], then the stoichiometric factor was calculated according to equation (1). In accordance with equation (2), k_{inh} of betanidin was calculated from the rate of the lipid hydroperoxide formation during the inhibition period. Table I reports the kinetic parameters characterizing the lipoperoxyl radical-scavenging activity of betanidin in methanol. For a comparative purpose, k_{inh} of α-tocopherol calculated under the same experimental conditions was $6.4 \times 10^5\ M^{-1}s^{-1}$.

Oxidized products from betanidin were researched. Spectra of the incubation mixture containing peroxidizing methyl linoleate in the presence of 20 μM betanidin were recorded at time-intervals within the inhibition period. A decay of the absorbance at 541 nm with a progressive shift to 550 nm and appearance of a large band centred at 400 nm characteristic of *ortho*-quinone structure, were observed (Figure 4). The product was assigned to betanidin quinone by comparison with the spectrum of the authentic compound obtained by reacting betanidin with excess $NaIO_4$ (Figure 4A) and by parallel HPLC analysis of the mixture, showing a product eluting with the R_t of the reference betanidin quinone (Figure 4B). Measurements of the peak areas, provided evidence that the betanidin quinone formed was consistent with the consumed betanidin, indicating that the product was stable, at least within the experimental time.

Table I. Oxidation of methyl linoleate in methanol[a] and inhibition by 10 μM betanidin.

R_p	0.66×10^{-6} (Ms^{-1})[b]
R_i	2.20×10^{-8} (Ms^{-1})[c]
R_{inh}	2.04×10^{-7} (Ms^{-1})
t_{inh}	900 (s)
kcl	30[d]
kcl_{inh}	9.2[e]
n	1.98[f]
k_{inh}	3.75×10^5 ($M^{-1}s^{-1}$)[g]

[a] Methyl linoleate and AMVN concentrations were 300 mM and 2.0 mM, respectively.
[b] Rates are expressed for total solution.
[c] Measured by the duration of inhibition of 10 μM α-tocopherol.
[d] kcl, kinetic chain length in the absence of antioxidant (R_p/R_i).
[e] kcl_{inh}, kinetic chain length during the inhibition period (R_{inh}/R_i).
[f] calculated by equation (1).
[g] calculated by equation (2).

Peroxyl radical-scavenging activity of betanin and betanidin in liposomes

Liposomes are convenient biomimetic models to study the activity of natural antioxidants. The oxidation kinetics of water-dispersed unilamellar soybean PC liposomes exposed to AAPH were studied either in the absence or in the presence of 2.0–10.0 μM of either betanin or betanidin. The formation of lipid hydroperoxides was inhibited in the presence of the phytochemicals, with inhibition periods concentration-related ($r^2 = 0.98$ and $r^2 = 0.97$, for betanin and betanidin, respectively, Figure 5). Both pigments were consumed during lipid oxidation, at a constant rate of 2.7×10^{-9} Ms^{-1} (betanin) and 1.39×10^{-9} Ms^{-1} (betanidin), with total depletion within the relevant inhibition periods. In the absence of

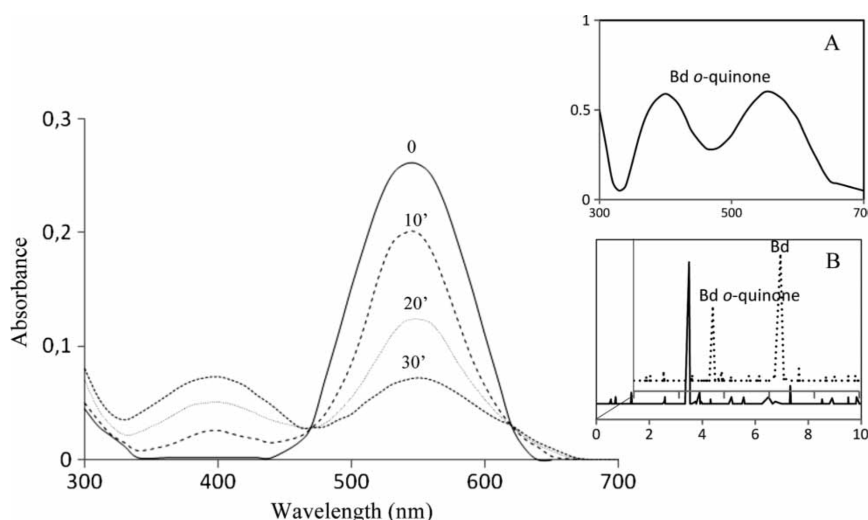

Figure 4. Scan spectra of the incubation mixture during the AMVN-induced methyl linoleate oxidation in methanol in the presence of 20 μM betanidin. Oxidation conditions are reported in methods. Aliquots were drawn before (0) and at the indicated time intervals after the azoinitiator addition, diluted 1:4 (v:v) with methanol and submitted to spectral analysis. (A) Spectrum of betanidin *o*-quinone (Bd *o*-quinone) generated by oxidation of betanidin with sodium periodate as reported in methods. (B) HPLC chromatograms of the oxidation mixture after 30 min incubation (full line) and of betanidin (Bd) and Bd *o*-quinone (dashed line). HPLC was as reported in methods following solvent system 2.

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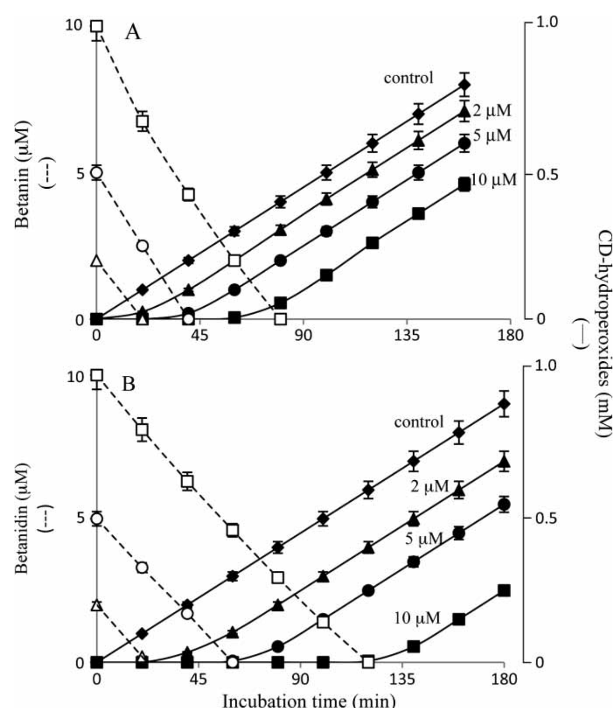

Figure 5. AAPH-induced oxidation of unilamellar soybean PC liposomes in the absence (control) or in the presence of 2–10 μM betanin (A) or betanidin (B) and consumption of the antioxidants. Oxidation conditions and HPLC analysis of betanin or betanidin are reported in methods (solvent system 1). Each point represents the mean ± SD of six-to-seven determinations carried out with different incubation mixtures.

liposomes, betanin and betanidin were oxidized by peroxyl radicals derived from AAPH at a rate of 1.9×10^{-10} Ms^{-1} and 0.9×10^{-10} Ms^{-1}, respectively. When the R_i of the system was measured from the inhibition period caused by known amounts of α-tocopherol, the stoichiometric factors 1.02 and 1.98, for betanin and betanidin, respectively, were calculated from the length of the relevant inhibition periods in accordance with equation (1).

Since k_p, the rate constant for the propagation of the radical chain of phosphatidylcholine, is not known, equation (3) cannot be applied and the absolute inhibition constant of betanin and betanidin in the lipid bilayer cannot be evaluated with the method used here. However, an estimate of the activity of the pigments as antioxidants in liposomes can be obtained by relating the value of R_{inh} measured in the presence of either betanin or betanidin and of α-tocopherol. According to equations (1) and (2), R_{inh} can be expressed by

$$R_{inh} = k_p[LH]R_i / n\, k_{inh}[IH] \quad (3)$$

Therefore, when comparable amounts of antioxidant and α-tocopherol are used, the ratio $R_{inh[betacyanin]}/R_{inh[\alpha\text{-tocopherol}]}$ will represent $nk_{inh[\alpha\text{-tocopherol}]}/nk_{inh[betacyanin]}$. From this calculation the effectiveness of betanin and betanidin appeared 53% and 84% of that of α-tocopherol, respectively. The kinetic parameters evaluated for the inhibition of AAPH-induced peroxidation of unilamellar liposomes are summarized in Table II.

Products from the oxidation of 10 μM of either betanin or betanidin were investigated by the spectral changes of the reaction media, monitored at time-intervals within the inhibition period, with parallel HPLC analysis. Spectral analysis showed a progressive decrease of the betanin absorbance at 536 nm, with a shift to 531 nm, accompanied by the increase of a single band peaking at 420 nm (Figure 6). The latter was assigned to the chromophore betalamic acid by comparing with the spectra of authentic compound (Figure 6A) and confirmed by analysis of the HPLC profile of the reaction mixture showing a peak whose R_t matched that of the authentic compound (Figure 6B). Evaluation of the peak areas showed that the accumulated betalamic acid was not consistent with betanin consumed. The nature of the product(s) with absorbance at 531 nm, accumulated after consumption of betanin, was not investigated.

Spectral analysis of the reaction media containing betanidin clearly showed a time-dependent decay of the betanidin absorbance at 541 nm, while three new absorbance bands were evident (Figure 7). The band peaking at 420 nm was assigned to betalamic acid in

Table II. Parameters of the antioxidant activity of betanin and betanidin in unilamellar soybean PC liposomes[a] as compared to α-tocopherol.

Antioxidant	$10^8 \times R_p^b$ (Ms^{-1})	$10^9 \times R_i$ (Ms^{-1})	$10^9 \times R_{inh}$ (Ms^{-1})	kcl	t_{inh} (s)	n	k_{inh} (betacyanin)/ k_{inh} (α-toc)[c]
None	8.6	2.77		31[d]			
5.0 μM α-toc			3.33	1.20[e]	3610	2[f]	
5.0 μM betanin			12.25	4.42	1841	1.02	0.53
5.0 μM betanidin			3.90	1.40	3574	1.98	0.84

[a]Unilamellar liposomes were prepared and assayed as described in Methods.
[b]Rates are expressed for total solution.
[c]The relative antioxidant activity of betacyanins is evaluated with respect to α-tocopherol (α-toc) by the ratio R_{inh} (betacyanin)/R_{inh} (α-toc) = nk_{inh} (α-toc)/nk_{inh} (betacyanin) (see text).
[d]kcl, kinetic chain length in the absence of antioxidant (R_p/R_i).
[e]kcl_{inh}, kinetic chain length during the inhibition period (R_{inh}/R_i).
[f]Assumed.

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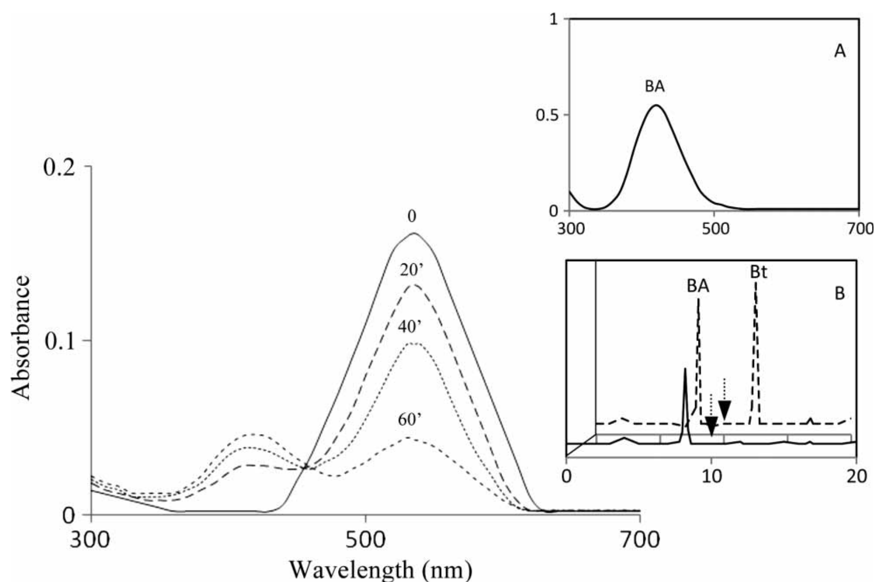

Figure 6. Scan spectra of the incubation mixture during the AAPH-induced unilamellar soybean PC liposome peroxidation in the presence of 10 μM betanin. Oxidation conditions are reported in methods. Aliquots were drawn before (0) and at the indicated time intervals after the azoinitiator addition, diluted 1:4 (v:v) with methanol and submitted to spectral analysis. (A) Spectrum of betalamic acid (BA) generated by alkaline hydrolysis of indicaxanthin as reported in methods. (B) HPLC chromatograms of the oxidation mixture after 60 min incubation (full line) and of BA and betanin (Bt) (dashed line). HPLC was as reported in methods (solvent system 1). Arrow indicates automatic wavelength change from 420 to 536 nm.

accordance with the HPLC profile of the incubation mixture and of the authentic compound (Figure 7A). The absorbance profile with bands peaking at 305 nm and 485 nm was assigned to dopachrome, as the oxidation product of the *cyclo*-Dopa moiety of the pigment, by comparison with spectra of authentic compound obtained by reacting Dopa with excess $NaIO_4$ (Figure 7B) and by matching the HPLC profile of the reaction mixture with that of dopachrome (Figure 7C). Quantitation of the products at

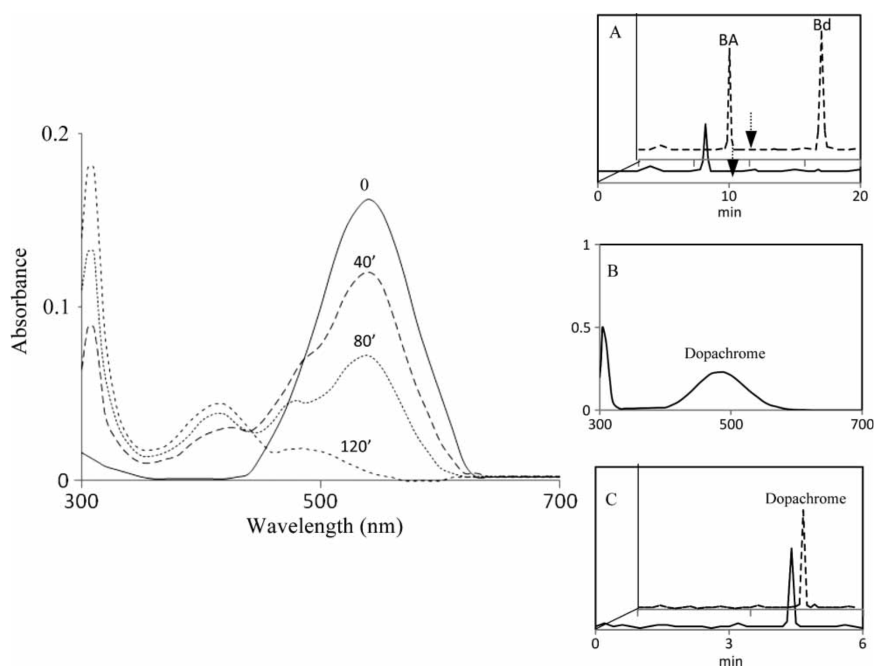

Figure 7. Scan spectra of the incubation mixture during the AAPH-induced unilamellar soybean liposome peroxidation in the presence of 10 μM betanidin. Oxidation conditions are reported in methods. Aliquots were drawn before (0) and at the indicated time intervals after the azoinitiator addition, diluted 1:4 (v:v) with methanol and submitted to spectral analysis. (A) HPLC chromatograms of the oxidation mixture after 120 min incubation (full line) and of betalamic acid (BA) and betanidin (Bd) (dashed line). HPLC was as reported in methods (solvent system 1). Arrow indicates automatic wavelength change from 420 to 536 nm. (B) Spectrum of dopachrome generated by oxidation of L-Dopa with sodium periodate as reported in methods. (C) HPLC chromatograms of the oxidation mixture after 120 min incubation (full line) and of dopachrome (dashed line). HPLC was as reported in methods (solvent system 3).

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time-intervals provided evidence that betanidin yielded equimolar amounts of dopachrome. The amounts of betalamic acid, instead, were not consistent with the native compound.

Interactions between either betanin or betanidin and vitamin E in suppressing the AAPH-induced soybean PC liposome oxidation were explored by measuring the inhibition periods induced by the individual antioxidants or their combination with α-tocopherol. Co-incubation of 2 μM of either betanin or betanidin with equimolar amounts of α-tocopherol resulted in inhibition periods the length of which was the sum of the inhibition periods caused by the individual antioxidants, which ruled out synergistic interactions (Figure 8).

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Discussion

Betacyanins are heterocyclic tyrosine-derived dietary pigments with antioxidant and anti-radical activity [3,7,13,27]. The phenol moiety and/or the cyclic amine group characterizing these molecules have been considered to confer this class of compounds their reducing properties [3,28]. In the present work kinetic measurements of the peroxyl radical-scavenging activity of betanin and betanidin in solution and liposomes and identification of oxidized products provide mechanistic insights on the antioxidant properties of both compounds, consistent with the activity of their glucose-substituted monophenol and *ortho*-diphenol moieties, respectively. Although both pigments appeared to be peroxyl radical scavengers, betanidin exhibited an effectiveness quite higher than betanin in both solution and lipid bilayers.

When dispersed in methanolic solution of peroxidizing LAME, betanin behaved as a weak retarder. This kind of antioxidants may react so slowly with chain-carrying lipoperoxyl radicals that chain termination also occurs by the bimolecular self-reaction of peroxyl radicals, which finally does not result in a well-defined induction period, but only a decrease of the peroxidation rate [25]. Alternatively, or in addition, such an effect may be the expression of a moderate trapping of initiating peroxyl radicals from the decomposition of the azo-initiator, resulting in a decrease of the R_i of the system. Voltammetric measurements showed that betanin has a redox potential of 0.4 V [13], which would make the molecule an efficient reductant for lipid-derived peroxyl radicals [29], as also observed in LDL [7]. Kinetic solvent effects [30,31] may be considered to rationalize the present findings. Polarity and hydrogen bond accepting ability (HBA) of the solvent are known to strongly affect the capacity of phenol antioxidants to transfer the hydroxylic H-atoms to radicals, because of preferential formation of an H-bonded complex between the reducing phenol-OH and a molecule of solvent [32]. Because of high HBA [33], a strong interference of methanol on the reducing activity of betanin could account for the very modest antioxidant effects observed in this solvent.

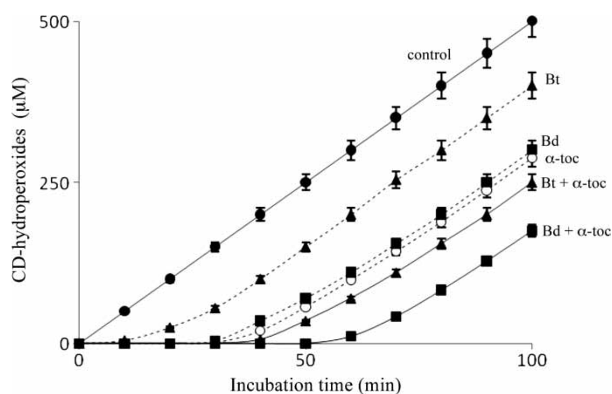

Figure 8. AAPH-induced oxidation of unilamellar soybean PC liposomes incorporating 2 μM of either betanin (Bt, ▲) or betanidin (Bd, ■) or α-tocopherol (α-toc, ○), separately (dashed line) or in combination (full line). Oxidation of liposomes in the absence (control) or in the presence of antioxidants is reported in methods. Each point represents the mean $\pm$SD of four determinations carried out with different incubation mixtures.

The interference of protic solvents on the H-atom donating ability of *ortho*-diphenols is lower than monophenolic compounds [34]. This may have contributed to the activity of betanidin in methanol, where LAME auto-oxidation was inhibited very effectively according to a net chain-breaking antioxidant mechanism, with well-defined concentration-dependent inhibition periods and total consumption of antioxidant at the end of the inhibition phase. This behaviour allowed the calculation of the k_{inh} and n, the stoichiometric factor of the reaction between betanidin and peroxyl radicals, that appeared of the same order as those measured for α-tocopherol under comparable conditions. In contrast, in the absence of defined inhibition periods, equation (2), based on the assumption that the peroxyl radicals are all quenched by the antioxidant, does not hold anymore, which is why the k_{inh} for the reaction of betanin with peroxyl radicals in methanol could not be determined by the kinetic approach used in this study.

The antioxidant effectiveness of betanin showed a dramatic increase upon changing to the etherogeneous aqueous/soybean phosphatidylcholine vesicular system, where a net chain-breaking antioxidant activity was observed. A less pronounced influence of the solvent on the reactivity of betanin may be expected, since HBA of water is lower than methanol [33]. However, partition between the water and lipid phase has been shown to be a major factor determining the antioxidant activity of a number of phytochemicals in membranes and lipid bilayers, with compounds partitioned more in the water phase showing less effectiveness [35–37]. Recent findings from a chemico-physical spectrophotometric study revealed that

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betanin interacts with dipalmitoyl-phosphatidylcholine vesicles, partitioning in the lipid portion of the bilayer [12]. Then, despite the sugar substituent adding hydrophilic character to the molecule, the aromatic *cyclo*Dopa could locate in the membrane, placing the reducing phenol hydroxyl at a polar interface, to possibly promote interactions with lipoperoxyl radicals floating from the membrane interior. Eventually the amphiphilic nature of the antioxidant can allow a facile diffusion from one vesicle to another, which has been shown to be rate-limiting for hydrophobic phenolic inhibitors such as α-tocopherol [38,39].

Affinity for microsomal membranes has been shown [3], however location of betanidin in a phospholipid bilayer is not known. In comparison with betanin, the absence of the hydrophilic sugar substituent might enhance partition in lipid bilayers. Then, in addition to the antioxidant chemistry of its *ortho*-diphenol moiety, accessibility of lipoperoxyl radicals to the reducing hydroxyl groups may be an important factor to account for the effectiveness of betanidin in the liposomal model. Finally, the observation that the rate of consumption of both betanin and betanidin by peroxyl radicals derived from the azo-initiator in the absence of liposomal lipids was one order of magnitude lower than in their presence, supporting that lipoperoxyl radicals may have a major role in the oxidation of both compounds.

Interactions between a number of polyphenol phytochemicals and α-tocopherol have been explored to get knowledge of eventual effects and mechanism of action of these dietary antioxidants in living organisms. Either synergistic or additive effects or co-antioxidant action have been reported [37,40,41]. In soybean PC liposomes, at a 1:1 betacyanin: α-tocopherol ratio, neither betanin nor betanidin did extend the inhibition period beyond the sum of the individual inhibition periods, providing evidence that only additive effects had occurred. The redox potential of betanin is lower than α-tocopherol (0.5 mV) [29] which would allow reduction of the α-tocopheroxyl radical at the membrane surface [42], provided favourable site-specific interactions [39]. The absence of synergism under the applied conditions may be the expression of the partition of betacyanins in the phospholipid bilayer and of their activity in scavenging lipoperoxyl-radicals. Other studies are required to assess or rule out that cooperative interactions between these antioxidants may be promoted in chemical environments or biological systems.

Oxidation of phenol antioxidants by peroxyl radicals proceeds through H-atom abstraction and formation of the transient resonance-stabilized aryloxyl radical that can either undergo reactions of fast termination leading to formation of adducts or quinones or even self-termination reactions forming dimers or other products [39,43,44]. Stoichiometry of the reaction between betanidin and peroxyl radicals in both methanol and liposomes and identification of products suggest that, after H-atom transfer from the *ortho*-diphenol moiety, the destiny of the intermediate radical is governed by the reaction medium. In methanol, this undergoes termination reactions with (lipo)peroxyl radicals leading to the stable betanidin quinone (Figure 9, reaction A). On the other hand, in the heterogeneous water/lipid vesicular system, the intermediate betanidin radical appears to undergo nucleophilic attack of water to the C adjacent to the indolic nitrogen, before being

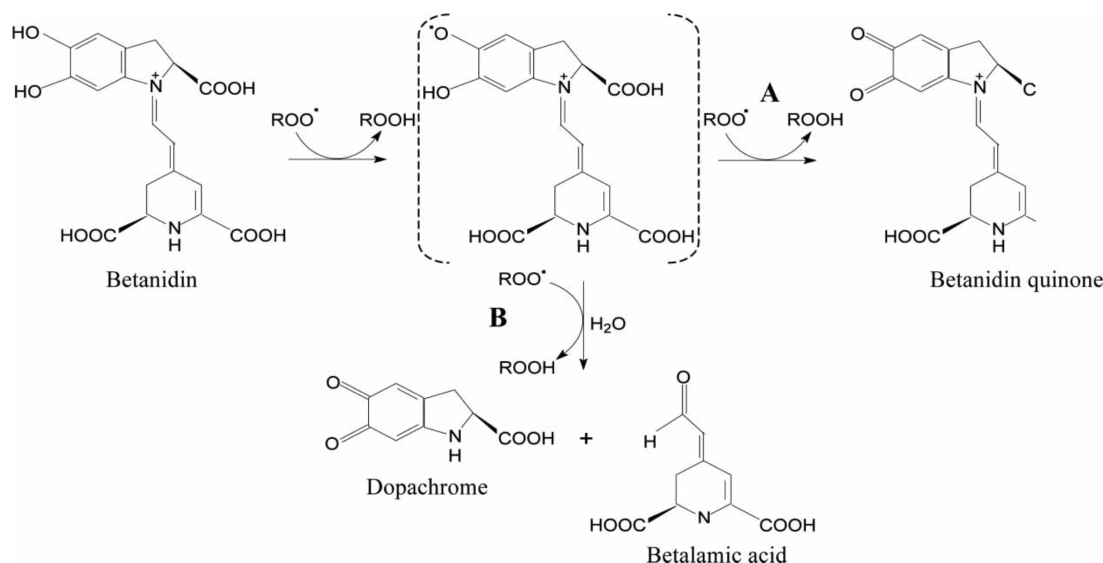

Figure 9. Proposed reaction mechanism of betanidin with (lipo)peroxyl radicals in methanol (A) or in aqueous/lipid vesicular system (B). In brackets, the transient betanidin radical.

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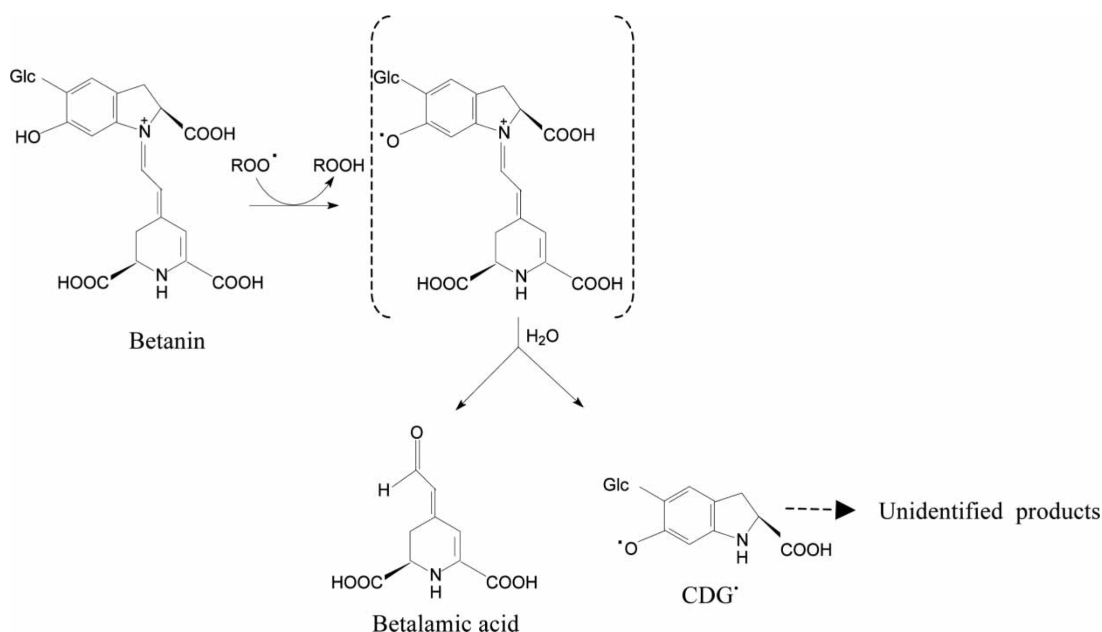

Figure 10. Proposed reaction mechanism of betanin with (lipo)peroxyl radicals in aqueous/lipid vesicular system. In brackets, the transient betanin radical. CDG·, cyclo DOPA 5-*O*-b-D-glucoside radical.

oxidized by a second (lipo)peroxyl radical, with final relase of dopachrome and betalamic acid (Figure 9, reaction 2). Whereas the solvolytic splitting of the betanidin radical generated equimolar amounts of dopachrome, betalamic acid accumulated to a quite lower extent, suggesting molecular degradation. Although betalamic acid has been reported to be a reducing compound [27], its consumption did not appear to contribute to the betacyanin antioxidant effectiveness. Because of its polar nature betalamic acid may slip out the vesicular lipids and be consumed by azoinitiator-derived peroxyl radicals, in a process quite ineffective to inhibit lipid oxidation.

Release of betalamic acid from betanin during liposomal peroxidation indicates that, similarly to betanidin, the intermediate betanin radical, generated after reaction of its phenol moiety, undergoes solvolytic splitting of the aldimine bond (Figure 10). The final product(s) from the *cyclo*Dopa 5-O-β-D-glucoside radical (CDG·) has (have) not been identified, however the peak of absorbance in the yellow-green spectrum, detected after depletion of betanin, would indicate the presence of compounds with highly conjugated structures, possibly adducts from self-termination reactions. On the other hand, our kinetic measurements rule out that CDG· is involved in pro-oxidant reactions.

It may be interesting to mention that release of betalamic acid during oxidation of both betanin and betanidin may rule out that the indolic nitrogen is involved in the antioxidant mechanism, at least under the conditions applied.

In conclusion the antioxidant ability of two betalain pigments, betanin and betanidin, has been evaluated by determining the kinetics of the H-atom transfer reaction to peroxyl radicals in a hydrogen-bond accepting organic solvent and in an heterogeneous aqueous/liposomal system. Our data show that the antioxidant effectiveness of the glucose-substituted phenol moiety of betanin is highly influenced by the nature of the microenvironment where the auto-oxidation takes place, with the partition in the vesicular bilayer being a major factor to potentiate its activity. On the other hand the *ortho*-diphenol moiety of the aglycone betanidin appeared a much more efficient reductant in both organic solvent and lipid bilayer, with an effectiveness of the same order as vitamin E. Then, as for other phytochemicals such as flavonols, the glycosylated form is less active than the parent aglycone [37,40,45]. While being of help to advance our understanding of mechanisms involved in the antioxidant effects of betalains, these findings suggest that these dietary bioavailable compounds may contribute to the antioxidant human defense.

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