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Peroxyl radical trapping activity of anthocyanins and generation of free radical intermediates

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Abstract
The inhibition by anthocyanins of the free radical-mediated peroxidation of linoleic acid in a SDS micelle system was studied at pH 7.4 and at 37°C, by oxygraphic and ESR tecniques. The number of peroxyl radicals trapped by anthocyanins and the efficiency of these molecules in the trapping reaction, which are two fundamental aspects of the antioxidant action, were measured and discussed in the light of the molecular structure. In particular the contribution of the substituents to the efficiency is —OH > —OCH_3 > —H. By ESR we found that the free radicals of anthocyanins are generated in the inhibition of the peroxidation of linoleic acid. The life time of these radical intermediates, the concentration of which ranges from 7 to 59 nM under our experimental conditions, is strictly correlated with the anthocyanin efficiency and with the heat of formation of the radical, as calculated by a semiempirical molecular orbital approach.

Keywords: *Antioxidant, electron spin resonance, free radicals, lipid peroxidation, peroxyl radical, polyphenols*

Introduction

In the recent years the antioxidant role of anthocyanins, the pigmented compounds responsible of the colors of most fruits, vegetables and flowers, has been assessed and many papers deal with this subject [1–3]. The antioxidant activity of these polyphenolic pigments derives from their structure characterized by the presence of three rings differing for the nature of the substituents in the positions 3′ and 5′ of B ring, see Figure 1 [4].

Increasing attention has been focused on their possible health effects, and recently on their interactions at the blood–brain barrier [5–8], however, the benefits of anthocyanins as dietary supplements are still only potential [9] and many aspects of their antioxidant action must be clarified. In fact the comparison of the results obtained in several studies on the antioxidant activity of these compounds, shows a different order of activity of these antioxidants reflecting the oxidant agent, end-point and quantification systems used in activity tests. Most of the data reported have been obtained from inhibition measurements carried out with a single or few antioxidant concentrations, in experiments involving H abstraction by long lived DPPH radicals, chemiluminescence emission by H_2O_2–acetaldehyde and ORAC assay [1,3,10]. Furthermore in aqueous solutions anthocyanins are present in various forms: flavylium cation, carbinol pseudobase, quinoidal bases and chalcone, the relative amount and reactivity of which vary with pH [11,12].

In spite of the fact that peroxidation of polyunsaturated fatty acids of membranes and LDL may lead

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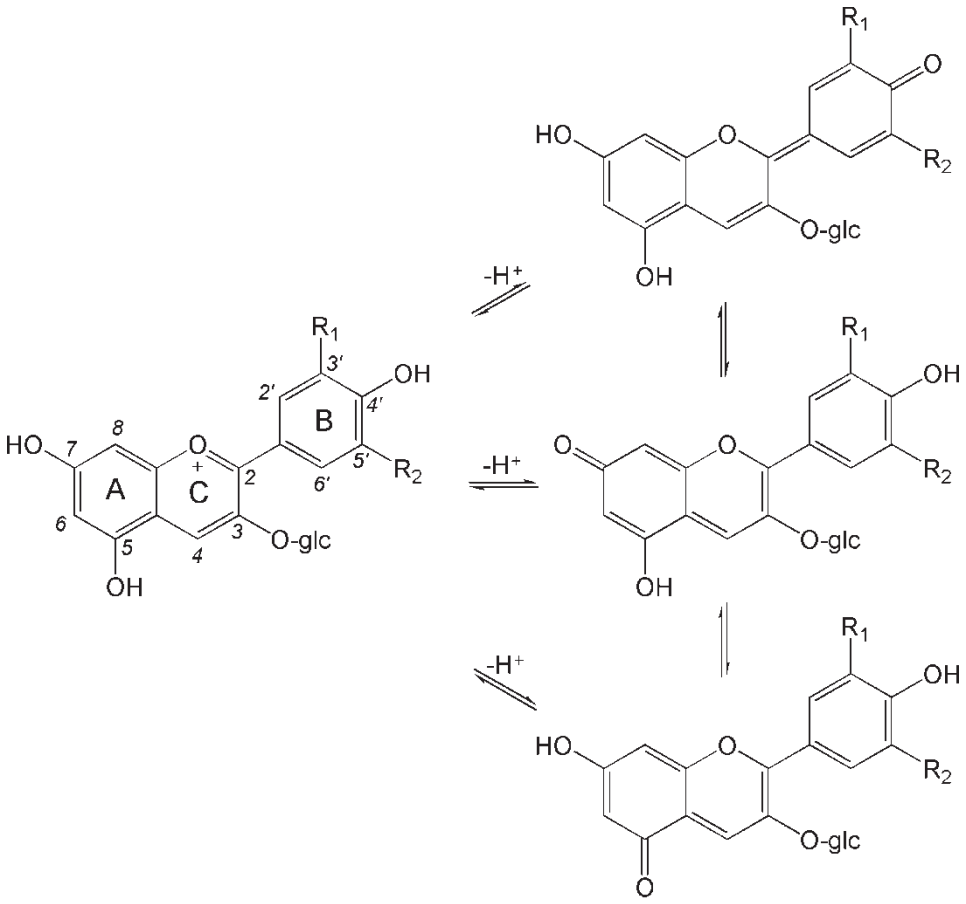

	R_1	R_2
Delphinidin 3-glucoside	OH	OH
Petunidin 3-glucoside	OH	OCH_3
Malvidin 3-glucoside	OCH_3	OCH_3
Cyanidin 3-glucoside	OH	H
Peonidin 3-glucoside	OCH_3	H
Pelargonidin 3-glucoside	H	H

Figure 1. Acid–base equilibria and tautomerism of quinoidal base of anthocyanins.

to many diseases, none of the above reported studies deals with the capacity and efficiency of anthocyanins in trapping peroxyl radicals [13,14]. At physiological pH values some important parameters of this reaction are: (i) the capacity, that is the number *n* of peroxyl radicals scavenged by a single anthocyanin molecule; (ii) the efficiency, that is the ability of the anthocyanin molecule to compete with other molecules for peroxyl radicals; (iii) the reactivity of the anthocyanin free radicals, and the presence of anthocyanin quinoidal bases which are formed when the flavylium pigment is brought to pH 7 [15].

In this paper we would like to report about the ability of 3-O-β-glucoside of the six anthocyanins to trap peroxyl free radicals both in term of capacity and efficiency at physiological pH values. Furthermore, by ESR spectroscopy, we detected anthocyanin free radicals as reaction intermediates, the steady-state concentrations of which correlated very well with their heat of formation and with their efficiency in scavenging peroxyl radicals.

Materials and methods

Reagents

The reagents were of the highest available quality (HPLC or standard for GC). 2,2′-azobis[2-(2-imidazolin-2-yl)propane] (ABIP) was a kind gift of Wako Chemicals (Neuss, Germany). All the aqueous solutions were prepared with bi-distilled water and, when necessary, were passed throughout a column of

Chelex-100 (Biorad, Richmond, CA), to minimize the concentration of heavy metal ions.

Delphinidin 3-O-β-glucoside (delphinidin 3-glucoside), petunidin 3-O-β-glucoside (petunidin 3-glucoside), peonidin 3-O-β-glucoside (peonidin 3-glucoside), malvidin 3-O-β-glucoside (malvidin 3-glucoside), cyanidin-3-O-β-glucoside (cyanidin 3-glucoside) and pelargonidin 3-O-β-glucoside (pelargonidin 3-glucoside) were obtained from Extrasyntese (France) and were of HPLC grade.

Antioxidant activity test

The antioxidant activity of the anthocyanins was measured by the method of Wayner et al. [16] as modified by Rigo et al. [17,18]. This method reproduces *in vitro* the free radical-induced peroxidation of polyunsaturated fatty acid, which was monitored by electrochemical detection of oxygen concentration. The standard assay solution of the experiments reported in this paper contained a micelle system formed by 2.5 mM linoleic acid, 50 mM SDS in 50 mM phosphate at pH 7.4, equilibrated with atmospheric oxygen at the test temperature (37°C). After the addition of 8 mM ABIP to this solution a constant flux of free radical was generated by thermal decomposition of the azo compound, $k_d = 2.44 \cdot 10^{-4}\,\text{min}^{-1}$ at 37°C [17]. Under these conditions the oxygen consumption rate (α_0) in the assay solution, due to the not inhibited chain peroxidation of LH catalyzed by the radicals generated by thermal decomposition of ABIP, is constant with time until the disappearance of O_2. The addition to the test solution containing ABIP of an anthocyanin at micromolar concentration strongly decreases the oxygen uptake rate (α_1). This rate appears almost constant for some minutes (lag time (LT)) after which the oxygen consumption rate slowly increases to the α_0 value, due to the disappearance of the anthocyanin. The decrease of the oxygen consumption rate or better the ratio α_1/α_0, is fitted by the equation:

$$\alpha_1/\alpha_0 = A + B \cdot e(-\ln 2 \cdot C/\text{IC}_{50}) \qquad (1)$$

where C is the concentration of the anthocyanin injected in the testing solution, α_1 is the slope of oxygen traces just after the injection of the antioxidant, and IC_{50} is the antioxidant concentration that halves the rate of oxygen consumption due to peroxidation of LH while A and B are constants from the fitting procedure [19]. The constant A, the average value of which was found to be 0.26 ± 0.02 under our experimental conditions, represents the value of α_1/α_0 ratio when LH peroxidation is fully inhibited by the antioxidant. The constant B is the value of $(\alpha_1/\alpha_0 - A)$ extrapolated at antioxidant concentration equal to 0, and usually $B = 1 - A$ was found.

The number n of highly reactive peroxyl radicals scavenged by a single molecule of antioxidant was calculated from the LT by the equation:

$$n = R_0 \cdot \text{LT} \cdot \eta / C \qquad (2)$$

where R_0 is the rate of generation of peroxyl radicals and η is the fraction of LOO° radical scavenged by the antioxidant during the lag time. The rate R_0 was calculated as $R_0 = 2e \cdot k_d \cdot [\text{ABIP}]$, where e is the efficiency of the radical generation process ($e = 0.43$ under our experimental conditions). In first approximation, taking into accounts the steady state concentration of peroxyl radicals in the presence and in the absence of the antioxidant, η was calculated according to the equation:

$$\eta = 1 - \left[\frac{(\alpha_1 - R_0)}{(\alpha_0 - R_0)}\right]^2 \qquad (3)$$

ESR measurements

A Bruker X band (9.8 GHz) D200 spectrometer, equipped with a TM110 standard cavity, was employed. The antioxidant activity test solution was injected in a flat cell inserted in the ESR cavity. All the ESR measurements were performed at 37°C.

Typical instrument settings were as follows: modulation frequency 100 KHz and power 20 mW. For the evaluation of the radical concentrations, all the spectra were acquired with modulation amplitude 4 G, scan rate 0.4 G/s and time constant 5 s. In particular the radical concentrations were calculated as second integral of the derivative curve using manganese oxide as internal standard. For this purpose a small sealed vial containing MnO doped CaO was fixed on the exterior surface of the quartz flat cell. The signal of the external manganese was calibrated by injecting in the flat cell a copper solution obtained by oxidation of high purity copper shots by 10% HNO_3.

To detect hyperfine interactions the spectra were acquired also with modulation amplitude 0.8 G, scan rate 0.05 G/s, time constant 0.5 s.

Quantum chemical calculations

The semiempirical quantum chemical method AM1 [20], as provided in MOPAC 2002 (Fujitsu Limited, Tokyo), was used for all the calculations. In order to simplify the calculations, the glucosides bound to the C3 atom of the anthocyanins were replaced with methoxy groups. To form the radicals, one H atom was removed from each hydroxyl group of the various forms of the anthocyanin quinoidal bases present in aqueous solution at pH 7. The spin restricted Hamiltonian was used for both the closed and open-shell species. Stable geometries were fully optimized

from approximate starting geometries using the eigenvector following routine, until the energy gradient norm dropped below 0.05 kcal/mol. The absolute energy minimum was calculated exploring the conformational space as a function of the torsional angles defined by the relative position of the C and B rings, and by the orientation of the hydroxy and methoxy groups. For every stable conformation of the radicals, the heat of formation of the possible free radical (ΔH_f^0) was calculated.

Results and discussion

The efficiency values were obtained from the plots of the ratio α_1/α_o versus the injected anthocyanin concentration according to Equation (1). At least eight concentrations for each anthocyanin were tested, obtaining a fitting parameter $r > 0.98$. In Table I the anthocyanin efficiency values are reported both as IC_{50}^{-1} and as ratio $(IC_{50})^{-1}_{anthocyanin}/(IC_{50})^{-1}_{trolox}$. From Table I it clearly appears that the contribution of the B ring substituents to the efficiency is —OH > —OCH_3 >>—H, being the delphinidin 3-glucoside ($R_1 = R_2 =$ —OH) eight times more efficient than pelargonidin 3-glucoside ($R_1 = R_2 =$ —H).

The number n of peroxyl radicals trapped by each molecule of antioxidant was calculated according to Equation (2), taking into accounts that only a fraction η of LOO° radicals is scavenged by the antioxidant during the lag time, see Equation (3). On the basis of the n value, the anthocyanins may be grouped in two classes: the first one containing —OH and/or —OCH_3 in position 3′ and 5′ of the B ring with an average n value of 3.5 and the second one with at least one H atom in these positions with a n value of about 1.5. The —OH group in 3′ or 5′ can be directly involved in hydrogen donor antioxidant mechanism, while —OCH_3 group could contribute to the same mechanism with intramolecular electron donor effect. n values higher than 2, found in the case of delphinidin 3-glucoside, petunidin 3-glucoside and malvidin 3-glucoside, indicate that the antioxidant mechanism of anthocyanins involves also —OH groups in A ring. This is possible because at neutral pH anthocyanins are present as quinoidal-base isomers involving ring A, B and C in wide conjugated structures (Figure 1).

The standard assay solution containing 8 mM ABIP in the absence of anthocyanins did not show any ESR resonance, indicating that the steady-state concentration of the free radicals generated by ABIP or those derived by LH peroxidation (LOO°) were below the detection limit, under our experimental conditions. The addition of anthocyanins to this solution gave rise to an ESR signal which was associated to the presence of a free radical intermediate generated by anthocyanins itself in the inhibition of the peroxidation process. In fact no ESR signal was detected following the addition of anthocyanins in the assay solution in the absence of ABIP.

The spectra of the radicals generated by the various anthocyanins in trapping LOO° radicals are shown in Figure 2. The spectra of column A, recorded with high modulation amplitude and high integration constant to increase the sensitivity [21], are characterized by a variable intensity and by a g value close to that of DPPH. It should be noted that the ESR spectrum of trolox, which intensity is comparable to those of anthocyanins, shows a not well resolved multiplet (Figure 2, spectrum g). Under the above reported conditions the area is directly proportional to the modulation amplitude regardless of the lineshape [21]. The ESR spectra of anthocyanin radicals were also acquired under high resolution conditions, see Figure 2, column B. Hyperfine interactions were observed only in the case of delphinidin-3 glucoside spectrum, which is a doublet with an a value of 1.1 gauss (Figure 2, spectrum a′). In the case of petunidin 3-glucoside and malvidin 3-glucoside no hyperfine interaction was observed, while for all the others anthocyanins no detectable ESR signal was obtained under the high resolution conditions. The lack of hyperfine structure of the signal or its absence could be due to a decrease of the ESR signal and/or to the presence of a variety of radical species [22] leading to a wider splitting.

In Table I, column 5, the steady-state concentration of anthocyanin free radicals, generated in the presence of 15 μM anthocyanins, is reported. Under these

Table I. Peroxyl radical trapping efficiency and capacity of anthocyanins and characteristics of the intermediate anthocyanin radicals.

Compound	IC_{50}^{-1} $(\mu M)^{-1\star}$	$(IC_{50})^{-1}_{anthocyanin}/(IC_{50})^{-1}_{trolox}$	$n^{\dagger}$	$[R^0]$ (nM)‡	ΔH_f^0 (kcal/mol)
Delphinidin-3-*O*-glucoside	1.25 ± 0.07	1.39 ± 0.08	3.6 ± 0.4	59 ± 8	−157.2
Petunidin-3-*O*-glucoside	1.00 ± 0.06	1.11 ± 0.08	3.7 ± 0.3	50 ± 7	−148.9
Malvidin-3-*O*-glucoside	0.91 ± 0.06	1.01 ± 0.09	3.4 ± 0.3	45 ± 9	−138.9
Cyanidin-3-*O*-glucoside	0.50 ± 0.05	0.56 ± 0.11	1.8 ± 0.2	22 ± 4	−112.5
Peonidin-3-*O*-glucoside	0.17 ± 0.01	0.19 ± 0.08	1.2 ± 0.1	13 ± 2	−104.3
Pelargonidin-3-*O*-glucoside	0.16 ± 0.01	0.18 ± 0.08	nd	7 ± 2	−66.9
Trolox	0.90 ± 0.05	1	2.0 ± 0.1	28 ± 5	

⋆ Standard error obtained fitting the data to Equation (1) (number of experiments = 8); † Standard deviation (number of experiments = 8); ‡ Standard deviation (number of experiments = 3).

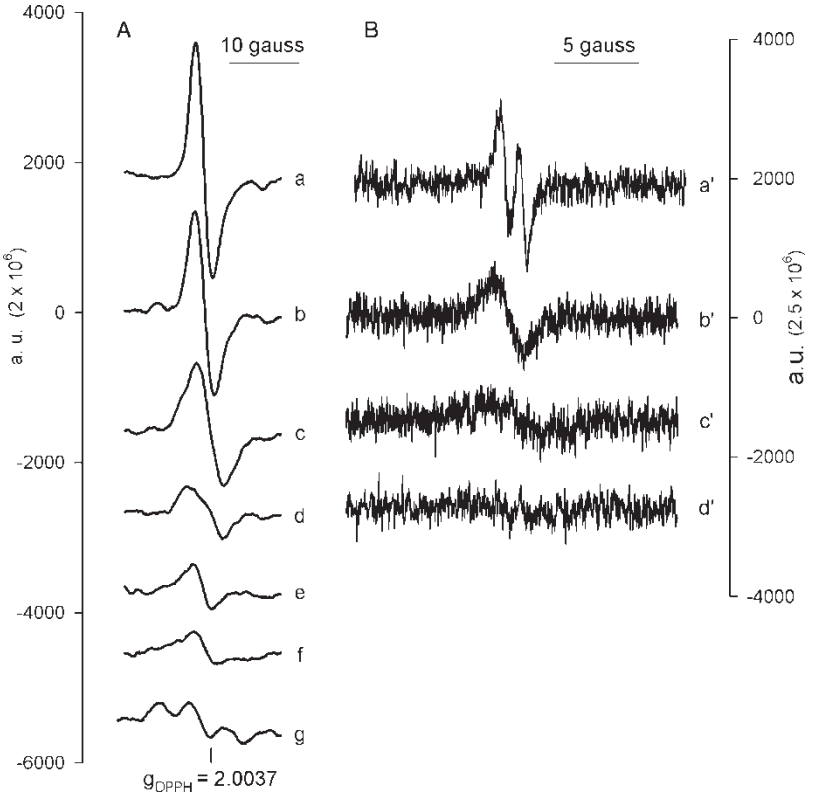

Figure 2. ESR spectra of anthocyanin radicals generated by lipid peroxidation. Peroxidation was carried out in the SDS-LH-ABIP system at 37°C in phosphate buffer, pH 7.4, in the presence of 15 μM antioxidant. (a) and (a′) delphinidin 3-glucoside; (b) and (b′) petunidin 3-glucoside; (c) and (c′) malvidin 3-glucoside; (d) and (d′) cyanidin 3-glucoside; (e) peonidin 3-glucoside; (f) pelargonidin 3-glucoside; (g) trolox. Column A modulation amplitude 4 G, scan rate 0.4 G/s, time constant 5 s. Column B modulation amplitude 0.8 G, scan rate 0.05 G/s, time constant 0.5 s.

conditions the α_1/α_0 ratio is about 0.26, which means that all the peroxyl radicals produced by ABIP react with the anthocyanins, the radicals of which are therefore, generated with a constant rate. As a consequence the concentration of the free radicals of anthocyanins should be controlled by their stability, which in turn appears to be controlled by the substituents present in the B ring.

In Table I, last column, the lowest value of heat of formation (ΔH_f^0) among those calculated for every stable conformation of radicals of the various quinoidal bases according the semiempirical quantum chemical method AM1, are reported. A highly significant inverse linear correlation between the logarithm of concentration of the anthocyanin free radicals and ΔH_f^0 was found ($r < -0.99$, Figure 3). This correlation indicates that, under the above reported experimental conditions, the life time of anthocyanin radicals is governed by their thermodynamic stability. Moreover, also the efficiency IC_{50}^{-1} appears quite well correlated with the concentration of anthocyanin free radical ($r > 0.99$, Figure 4). A similar correlation was found between the concentration of stable free radicals present in red wines and their peroxyl trapping efficiency in scavenging peroxyl radicals [23].

The resonance stabilization energy theory, according to which electron-donor groups such as —OH and —OCH_3 stabilize the radicals, so facilitating their formation, may be the basis of this behavior [24]. This stabilization of the radicals facilitates also their formation by hydrogen abstraction reactions and may explain the correlation between

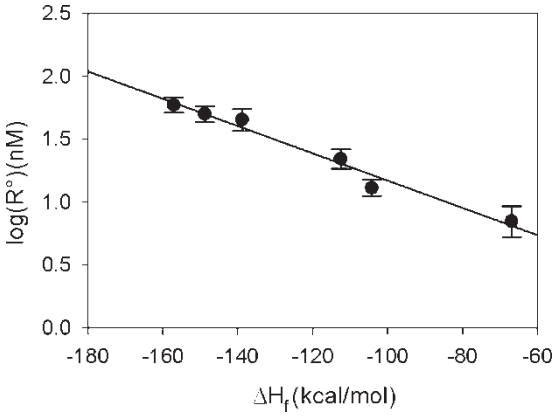

Figure 3. Inhibition of lipid peroxidation: correlation between the logarithm of steady-state concentration of anthocyanin free radicals (nM) and their heat of formation (ΔH_f^0).

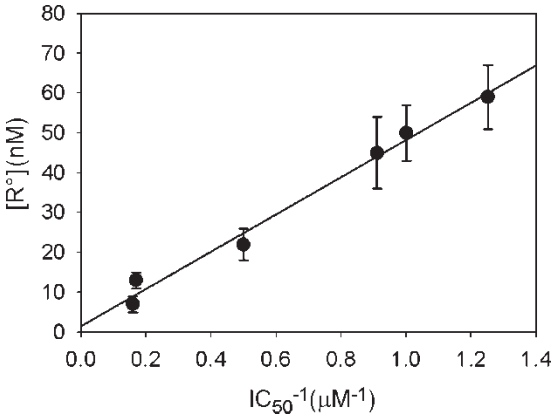

Figure 4. Inhibition of lipid peroxidation: correlation between steady-state free radical concentration and antioxidant efficiency $(IC_{50})^{-1}$ of anthocyanins. The uncertainties of $(IC_{50})^{-1}$ are reported in Table I.

the efficiency values and the concentration of free radicals we have found.

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