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Series Title		
Chapter Title	Lipoperoxyl Radical Scavenging and Antioxidative Effects of Red Beet Pigments	
Chapter SubTitle		
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Abstract	Aerobic life is characterized by a steady formation of reactive oxygen species and free radicals, which is almost entirely counteracted by endogenous primary and secondary antioxidant systems. Maintenance of these systems is then imperative to ensure a continuous defense to cells and to avoid conditions known as oxidative stress. Apart from antioxidant vitamins, many compounds from the plant kingdom are now considered very helpful to maintain a proper cell redox balance. Among them, betalain pigments have received recent attention. Betanin (betanidin-5-*O*-β glucoside) is the main betacyanin from red beet. Redox potential, ability to interact with lipid structures and bioavailability in humans make this molecule a potential natural antioxidant with protective effects in vivo. This review summarizes the peroxyl radical-scavenging activity of the molecule and of its aglycone betanidin, as observed in a few chemical or biological models.

Chapter 6
Lipoperoxyl Radical Scavenging and Antioxidative Effects of Red Beet Pigments

Maria A. Livrea and Luisa Tesoriere

Abstract Aerobic life is characterized by a steady formation of reactive oxygen species and free radicals, which is almost entirely counteracted by endogenous primary and secondary antioxidant systems. Maintenance of these systems is then imperative to ensure a continuous defense to cells and to avoid conditions known as oxidative stress. Apart from antioxidant vitamins, many compounds from the plant kingdom are now considered very helpful to maintain a proper cell redox balance. Among them, betalain pigments have received recent attention. Betanin (betanidin-5-*O*-β glucoside) is the main betacyanin from red beet. Redox potential, ability to interact with lipid structures and bioavailability in humans make this molecule a potential natural antioxidant with protective effects in vivo. This review summarizes the peroxyl radical-scavenging activity of the molecule and of its aglycone betanidin, as observed in a few chemical or biological models.

6.1 Introduction

It is now acknowledged that cell and tissue wellbeing relies on an appropriate cell redox status. Indeed, a million years of evolution led aerobic organisms to produce free radicals and oxidants (reactive oxygen species [ROS]), as well as to exploit an effective antioxidant machinery to control redox-sensitive signaling pathways responsible for a variety of processes including, among others, cell differentiation and proliferation, inflammation, apoptosis and aging itself (Hancock 2009; Dröge 2002; Matsuzawa and Ichijo 2008; Giles 2006; Wu et al. 2006; Giorgio et al. 2007; Valko et al. 2007; Lee and Griendling 2008; Pan et al. 2009).

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B. Neelwarne (ed.), *Red Beet Biotechnology: Food and Pharmaceutical Applications*,
DOI 10.1007/978-1-4614-3458-0_6, Springer Science+Business Media New York 2012

While ROS production in a finely controlled fashion is required to maintain the natural oxidative homeostasis, uncontrolled generation and/or aggression by environmental oxidants, toxicants and heavy metals can modify the balance between pro- and antioxidative processes, resulting in the condition known as "oxidative stress", initiating biochemical events resulting in pathological conditions (Ma 2010; Martin and Barrett 2002).

Cells are endowed with primary antioxidant defenses, i.e. enzymes such as superoxide dismutase, catalase and glutathione peroxidase, that remove ROS before they may attack cell components, and various repair systems needed to cope with damaged molecules, including low molecular weight antioxidants such as glutathione and vitamins E, C, A and carotenoids. By these means, cells protect all compartments, thus preventing damage to nucleic acids, proteins and membrane lipids.

Because endogenous antioxidants are continuously consumed, the organism should be helped to keep their optimal level to avoid oxidative damage. This can be accomplished by introducing new reducing molecules to replace the consumed ones. Numerous epidemiological studies (Willett et al. 1995; Kushi et al. 1995) point out the importance of diets based on herbs, fruits, grains, and vegetables in reducing the incidence of chronic and degenerative diseases such as cancer and cardiovascular disease, the etio-pathogenesis of which is strongly supported by oxidative stress (Lin 1995; Cao et al. 1997). Indeed plants are the main source of dietary antioxidants. Apart from the antioxidant vitamins, a vast array of phytochemicals, from bioflavonoids to phytosterols and terpenoids, with potential antioxidative activity and/or ability to modulate redox-sensitive signaling pathways, have been isolated. Recently, the radical-scavenging activity and antioxidant capacity of betalains have been the object of research in our as well as in other laboratories (Kanner et al. 2001; Escribano et al. 1998; Butera et al. 2002; Livrea and Tesoriere 2004; Gliszczynska-Swiglo et al. 2006; Czapski et al. 2009).

Betalain pigments, secondary metabolites of plants of the *Caryophyllales* order, share the chemical structure of betalamic acid and include two classes of compounds, i.e. the yellow betaxanthins and red betacyanins, according to the structure bound to betalamic acid. When the latter is conjugated with amino acids or corresponding amines (including dopamine), betaxanthins arise. Betacyanins are derivatives of betanidin, the conjugate of betalamic acid with *cyclo*-DOPA, with additional substitutions through varying glycosylation and acylation patterns at C5 or C6 positions. Betanin (5-*O*-glucose betanidin) and vulgaxanthin I (glutamine–betaxanthin) are the main pigments found in raw red beet (Fig. 6.1). On the other hand, in accordance with studies showing that vulgaxanthin I is poorly stable under a number of physical and chemical conditions (Herbach et al. 2006), vulgaxanthin did not appear detectable in the steamed red beet, nor in other beet preparations such as juice and jam (Tesoriere et al. 2008).

When treating with the potential health-promoting effects of dietary compounds, it is important to consider their bioavailability, i.e. how much of the active molecule is absorbed, its eventual transformation at the level of the digestive tract and, finally, the distribution to tissues and cells. Factors such as the chemistry of the molecule, the nature of co-ingested compounds as well as the complexity of the food matrix

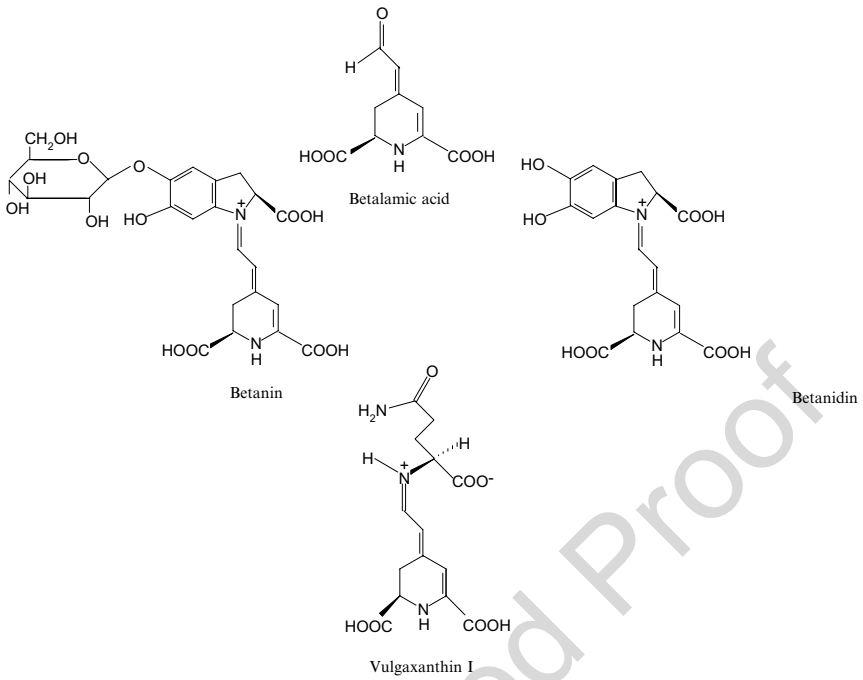

Fig. 6.1 Chemical structure of betalamic acid, vulgaxanthin I and main betacyanin derivatives

may largely affect bioavailability. Studies in humans reporting kinetics of absorption and extent of plasma concentration and urinary excretion (Kanner et al. 2001; Tesoriere et al. 2004a; Frank et al. 2005) provided evidence that discrete amounts of betanin can reach the circulation and distribute in low-density lipoproteins (LDL) (Tesoriere et al. 2004a) and red blood cells (Tesoriere et al. 2005), where the molecule presumably was involved in antioxidant protection. On this basis, investigating the activity of betanin as a lipid antioxidant and providing kinetic parameters of the activity has been a stimulating challenge for our group (Tesoriere et al. 2009). To this purpose chemical lipid systems such as methanolic solutions of methyl linoleate and soybean phosphatidylcholine liposomes have been used. In other studies, the antioxidant activity of betanin has been evaluated in more complex biological lipid matrixes such as LDL (Tesoriere et al. 2003; Allegra et al. 2007).

6.2 Oxidation of Lipids

Oxidation of membrane unsaturated lipids is believed to contribute to human ageing and disease by disrupting the structure and the packaging of the lipid components and, ultimately, by preventing membrane function. Beside causing local disruption, this process may also affect intracellular signaling, since reactive end-products of

lipid peroxidation such as unsaturated aldehydes may easily migrate from membranes, causing intracellular injury and remarkable modifications of the oxidative homeostatic signaling (Uchida 2007; Echtay et al. 2003). Due to the importance of maintaining membrane integrity, numerous bioactive substances present in foods have been explored as potential lipid antioxidants.

Peroxidation of polyunsaturated lipids (PUFA) is characterized by radical chain reactions, where a single initiating free radical (R·) may cause the peroxidation of a large number of lipids (LH). In the presence of appropriate initiators, the process takes place according to a mechanism exemplified in

Initiation

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$
$$ROO^{\bullet} + LH \rightarrow ROO^{\bullet} + L^{\bullet}$$

Propagation

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$$
$$LOO^{\bullet} + LH \xrightarrow{kp} LOOH + L^{\bullet}$$

Termination

$$2\ LOO^{\bullet} \xrightarrow{kt} \text{non - radical products}$$

where L·, LOO·, and LOOH are the alkyl and alkylperoxyl radicals and hydroperoxide generated, and kp and kt are the rate constants for propagation and termination of the radical chain, respectively. Classical chain-breaking antioxidants, such as vitamin E, inhibit the peroxidation process by scavenging the chain-carrying lipoperoxyl radicals, thus preventing the radical attack of other lipids and production of hydroperoxides. The effectiveness of these antioxidants is determined by the rate at which they actually scavenge lipoperoxyl radicals, comparable with the rate at which the radicals are produced, as well as by the number of radicals scavenged per mole of antioxidant. In the presence of a chain-breaking antioxidant, lipid peroxidation is stopped as long as the antioxidant is totally consumed, a time interval known as the inhibition period or lag time. Due to the primary importance of vitamin E (α-tocopherol) in protecting membrane lipids (Fukuzawa 2008), the comparison between kinetic parameters measured for natural antioxidants and those of vitamin E may provide an indication of the compound's effectiveness.

The oxidation of methyl linoleate (LAME) under controlled conditions is the simplest way to study the oxidation of polyunsaturated lipids, and it has widely been adopted to carry out kinetic studies with antioxidants. Since the linoleic acid has two double bonds, peroxidation occurs at the bis-allylic hydrogens and generates stoichiometric amounts of conjugated dienes (CD) lipid hydroperoxides that can be measured spectrophotometrically (Pryor and Castle 1984). Methanolic LAME solutions

are oxidized by radicals thermally generated from a lipophilic azo-initiator such as AMVN (2,2′-azobis (2,4-dimethylvaleronitrile)) (Niki 1990) to ensure a linear production of lipoperoxides propagating chain reactions. Analysis of the peroxidation curve generated by monitoring the formation of CD hydroperoxides at time intervals permits the calculation of kinetic parameters for the reaction of lipoperoxyl radicals with antioxidants. The propagation rate, R_p, is 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, is measured by the inhibition period (t_{inh}) produced by a known amount of α-tocopherol, following the equation

$$R_i = n[IH] / t_{inh} \quad (6.1)$$

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 (Burton and Ingold 1981).

In the curve of peroxidation in the presence of antioxidant, the inhibition period, t_{inh}, is 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. When inhibition periods are measured, the inhibition rate constant, K_{inh}, in solution of peroxidizing LAME is calculated as

$$K_{inh} = K_p[LH] / R_{inh} t_{inh}, \quad (6.2)$$

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} (Yamamoto et al. 1982). The inhibition rate, R_{inh}, that is the rate of production of lipid hydroperoxides during the inhibition period, is calculated by the coordinates of the intercept of the extrapolations of the parts of the curve representing the inhibition and propagation phases.

Soybean phosphatidylcholine (PC) unilamellar liposomes are a suitable membrane-mimetic system to obtain quantitative data of the peroxyl radical-scavenging activity of antioxidants, due to the peculiar composition in unsaturated fatty acids, 95% of which consist of linoleic acid. The use of a hydrophilic azo-initiator such as AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) (Niki 1990) causes a linear hydroperoxide formation, thereby R_i can be evaluated by the classic inhibitor method according to Eq. 6.1.

6.3 Antioxidant Activity of Betacyanins

Betacyanins are heterocyclic tyrosine-derived pigments. The phenol moiety and/or the cyclic amine group have been considered to confer reducing properties to this class of compounds (Kanner et al. 2001; Gliszczynska-Swiglo et al. 2006; Gandia-Herrero et al. 2010). In addition, because of their chemistry, including

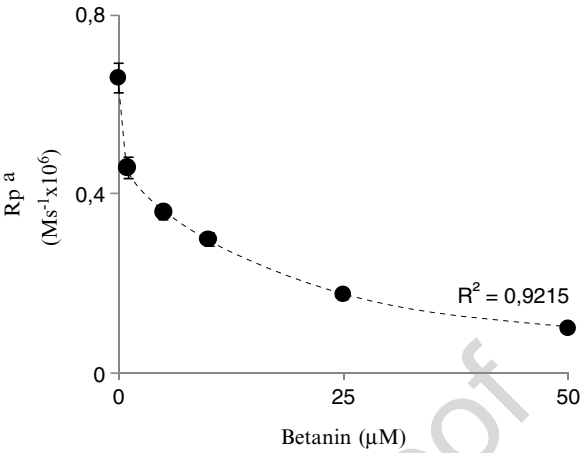

Fig. 6.2 Relationships between the propagation rate (Rp) and betanin concentrations in AMVN-induced oxidation of methyl linoleate in methanol. [a]CD-hydroperoxide formation per second

charged portions and ionizable groups as well as lipophilic moieties, these molecules may behave as amphiphilic-like compounds at physiological pH. Kinetic measurements of the peroxyl radical-scavenging activity of betanin and of its aglycone, betanidin, in organic solution and liposomes, and the identification of oxidized products, have recently provided mechanistic insights on the antioxidant properties of these compounds, consistent with the activity of the glucose-substituted monophenol and *ortho*-diphenol moieties, respectively (Tesoriere et al. 2009). Though both pigments appear to be peroxyl radical scavengers, betanidin exhibits an effectiveness higher than betanin.

(a) *Peroxyl radical-scavenging activity of betanin and betanidin in methanol*
Betanin does not cause any delay of the oxidation of LAME in methanol solution, but only a decrease of the peroxidation rate that depends exponentially on the betanin amount (Fig. 6.2). This is typical of antioxidants known as retarders. These may react so slowly with chain-carrying lipoperoxyl radicals that termination also occurs by the bimolecular self-reaction of peroxyl radicals, which finally does not result in a well-defined inhibition period. The redox potential of betanin (0.4 V) (Butera et al. 2002), would make the molecule an efficient reductant for lipid-derived peroxyl radicals (Buettner 1993). Nevertheless, kinetic solvent effects (Avila et al. 1995; Valgimigli et al. 1995), in particular polarity and hydrogen bond-accepting ability (HBA) of the solvent, may strongly affect the capacity of phenol antioxidants to transfer the hydroxylic H-atoms to radicals, because of preferential formation of a H-bonded complex between the reducing phenol-OH and a molecule of solvent (Barclay et al. 1999). Since methanol has a high HBA (Kamlet and Taft 1976), a strong interference could account for the very modest antioxidant effects of betanin in this solvent. In the absence of defined inhibition periods, Eq. 6.2 cannot be applied, then the K_{inh} for the reaction of betanin with peroxyl radicals in methanol cannot be determined. On the other hand, the hydrophilic nature of the pigment makes more apolar solvents inapplicable (Livrea and Tesoriere, unpublished data).

The interference of protic solvents on the H-atom-donating ability of *ortho*-diphenols is lower than monophenolic compounds (Foti and Ruberto 2001). Indeed, LAME autoxidation is very effectively inhibited by the betanin aglycone (betanidin) that acts as a classic chain-breaking antioxidant, with well-defined concentration-dependent inhibition periods, and total consumption at the end of the inhibition phase. According to a chain-breaking mechanism, the length of the inhibition period is determined by the number of radicals scavenged per each molecule of antioxidant (Niki 1996). Equations 6.1 and 6.2 can be then applied to calculate the stoichiometric factor n and K_{inh} of betanidin. The kinetic parameters characterizing the lipoperoxyl radical-scavenging activity of betanidin in methanol are reported in Table 6.1. Interestingly, K_{inh} of α-tocopherol (n = 2) was measured 6.4×10^5 $M^{-1} s^{-1}$ under comparable conditions (Tesoriere et al. 2009). Therefore, K_{inh} and stoichiometric factor of the reaction between betanidin and peroxyl radicals are of the same order as those of α-tocopherol.

The 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 (Barclay 1993; Ingold 1969; Barclay et al. 1990). According to spectrophotometric and parallel high-performance liquid chromatography (HPLC) analysis, betanidin ~~quinine~~ to an extent consistent with the consumed betanidin, was the only product generated during LAME peroxidation in methanol (Tesoriere et al. 2009). The stoichiometry of the reaction between betanidin and peroxyl radicals suggests that, after H-atom transfer from the *ortho*-diphenol moiety, the intermediate radical undergoes termination reactions with lipoperoxyl radicals leading to the stable betanidin quinone (Fig. 6.3, pathway A).

Other studies reported on the antioxidant activity of betanin and betanidin against peroxidation of linoleic acid in buffered detergent solution (Kanner et al. 2001). In those experiments linoleate peroxidation was induced by cyt c, metmyoglobin or lipoxygenase. Betanin acted slightly better than betanidin when cyt c or lipoxygenase were the oxidizing agents, and exhibited almost the same effect when metmyoglobin was the oxidant. Then, in aqueous micellar dispersions, the molecules were allowed to act in a nearly comparable manner. This appears to be in substantial agreement with recent observations, discussed below.

(b) *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 the hydrophilic azo-initiator AAPH can be followed by the time-course of formation of lipid hydroperoxides either in the absence or in the presence of antioxidants (Niki 1990). Both betanin and betanidin exhibit a net chain-breaking antioxidant activity in the etherogeneous aqueous-soybean phosphatidylcholine vesicular system (Fig. 6.4). The stoichiometric factors reported in Table 6.1 are calculated from the length of the relevant inhibition periods in accordance to Eq. 6.1.

Table 6.1 Kinetic parameters of the antioxidant activity of betacyanins in in vitro oxidation models

Model	Betacyanin	$10^8 \times Rp^a$ (Ms^{-1})	$10^9 \times Ri^b$ (Ms^{-1})	$10^9 \times R_{inh}$ (Ms^{-1})	kcl	t_{inh} (s)	n[c]	$10^{-5} \times K_{inh}$[d] ($M^{-1}s^{-1}$)	K_{inh}(betacyanin)/ K_{inh}(α-toc)[e]
LAME[f]	None	66	22		30[g]				
	10 μM betanidin			204	9.2[h]	900	1.98	3.75	
Liposomes[i]	None	8.6	2.77		31[g]				
	5.0 μM betanin			12.25	4.4	1841	1.02		0.53
	5.0 μM betanidin			3.90	1.4	3574	1.98		0.84

[a]Rates are expressed for total solution

[b]Measured by the duration of inhibition of 10 μM a-tocopherol

[c]Calculated by Eq. 6.1

[d]Calculated by Eq. 6.2

[e]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)

[f]AMVN (2 mM)-induced oxidation of 300 mM methyl linoleate (LAME) in methanol (Tesoriere et al. 2009)

[g]*kcl* kinetic chain length in the absence of antioxidant (Rp/Ri)

[h]kcl_{inh} kinetic chain length during the inhibition period (R_{inh}/Ri)

[i]Unilamellar soybean PC liposomes (10 mM lipid concentration) were oxidized by AAPH (2 mM) (Tesoriere et al. 2009)

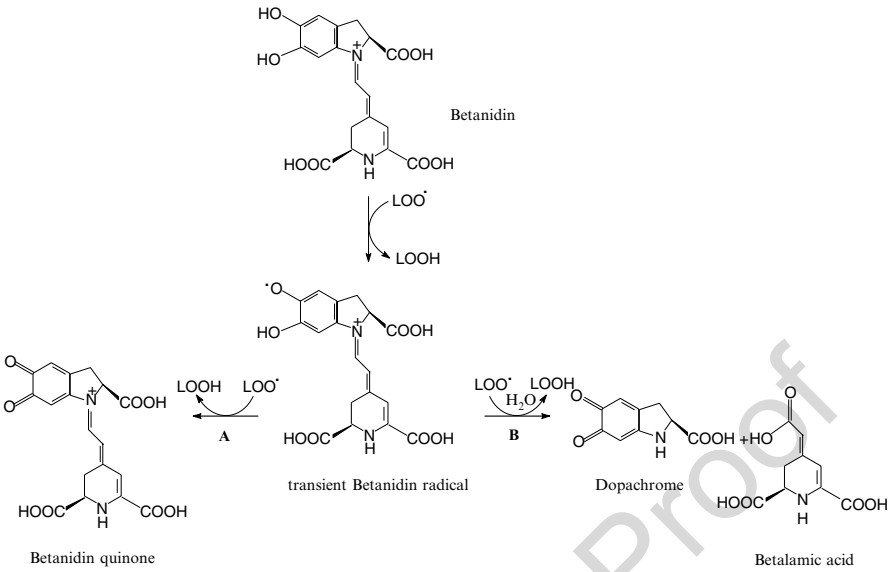

Fig. 6.3 The oxidation pathway of betanidin by lipoperoxyl radicals ($LOO^{\cdot}$) from LAME in methanol (*pathway A*), or from soybean PC in an etherogenous aqueous/vesicular system (*pathway B*)

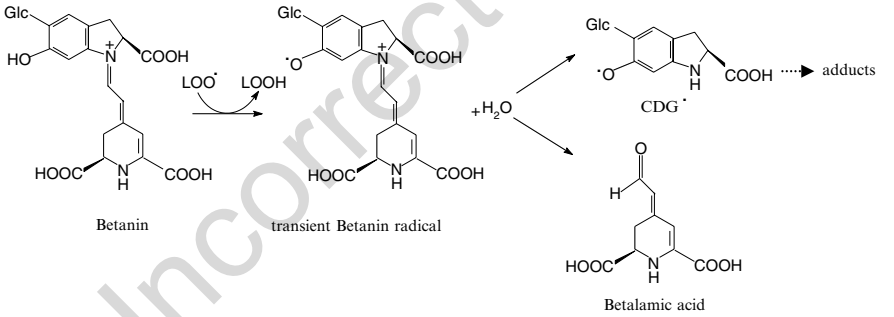

Fig. 6.4 The oxidation pathway of betanin by lipoperoxyl radicals ($LOO^{\cdot}$) in an aqueous/vesicular system. CDG *cyclo*-DOPA 5-*O*-b-D-glucoside radical

With respect to the organic solution, an increase of the antioxidant effectiveness of betanin in the aqueous/lipid system may be expected for a number of reasons. Since the reaction medium is buffered at pH 7.4, the molecule is in a deprotonated state favoring hydrogen atom and/or electron donation (Gliszczynska-Swiglo et al. 2006; Gandia-Herrero et al. 2010). In addition, the HBA of water is lower than methanol (Kamlet and Taft 1976), thus the influence of the solvent on the H-atom-donating activity is less pronounced. Furthermore, partition between the water and

lipid phase is to be considered a major factor determining the activity of antioxidant phytochemicals in membranes and lipid bilayers, with compounds partitioned more in the water phase showing less effectiveness (Rice-Evans et al. 1996; Shirai et al. 2001; Zou et al. 2005). According to other findings, betanin can partition in the lipid core of dipalmitoyl-phosphatidylcholine vesicles (Turco-Liveri et al. 2007). All these observations suggest that, despite the hydrophilic sugar substituent, location of the aromatic *cyclo*-DOPA in the membrane would allow its reducing phenol hydroxyl to easily interact with lipoperoxyl radicals floating from the membrane interior.

Partition and location of betanidin in liposomal phospholipids are not known. In comparison with betanin, the absence of the hydrophilic sugar substituent might finally 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 could account for the effectiveness of betanidin in the liposomal model.

K_p, the rate constant for the propagation of the radical chain of phosphatidylcholine, is not known, which prevents application of Eq. 6.2 to evaluate the absolute inhibition constant of betanin and betanidin in the lipid bilayer. However, an estimate of the antioxidant activity of the pigments 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. Taking into account Eqs. 6.1 and 6.2, R_{inh} can be expressed by

$$R_{inh} = K_p[LH]\, R_i / n\, K_{inh}[IH] \tag{6.3}$$

Therefore, when comparable amounts of antioxidant and α-tocopherol are used, the ratio $R_{inh[betacyanin]}/R_{inh[\alpha\text{-tocopherol}]}$ will represent $nK_{inh[\alpha\text{-tocopherol}]}/nK_{inh[betacyanin]}$. Then, the effectiveness of betanin and betanidin can be calculated, which were 53% and 84%, respectively, of the effectiveness of α-tocopherol. The kinetic parameters of the inhibition of AAPH-induced peroxidation of unilamellar liposomes are summarized in Table 6.1.

In the liposomal system, the oxidation of betanidin resulted in stoichiometric amounts of dopachrome, as the oxidation product of the *cyclo*-DOPA moiety, and the chromophore betalamic acid, the yield of which was lower than the parent compound, which was explained by molecular degradation (Tesoriere et al. 2009). Then, in the heterogeneous water/lipid vesicular system, the betanidin radical generated after H-atom abstraction by lipoperoxyl radicals undergoes nucleophilic attack of water to the C adjacent to the indolic nitrogen, before being oxidized by a second lipoperoxyl radical, with final release of dopachrome and betalamic acid (Fig. 6.3, pathway B).

Betalamic acid, again to an extent not consistent with the amount of the parent compound, was found as a product from betanin during liposomal oxidation (Tesoriere et al. 2009), indicating that, similarly to betanidin, the intermediate betanin radical generated after reaction of its phenol moiety undergoes solvolytic splitting of the aldimine bond (Fig. 6.5). On the basis of spectrophotometric evidence, unidentified product(s) from the reaction has/have been considered as derivatives of the *cyclo*-DOPA 5-*O*-β-D-glucoside radical (CGD·), possibly highly conjugated structures of adducts from self-termination reactions (Tesoriere et al. 2009).

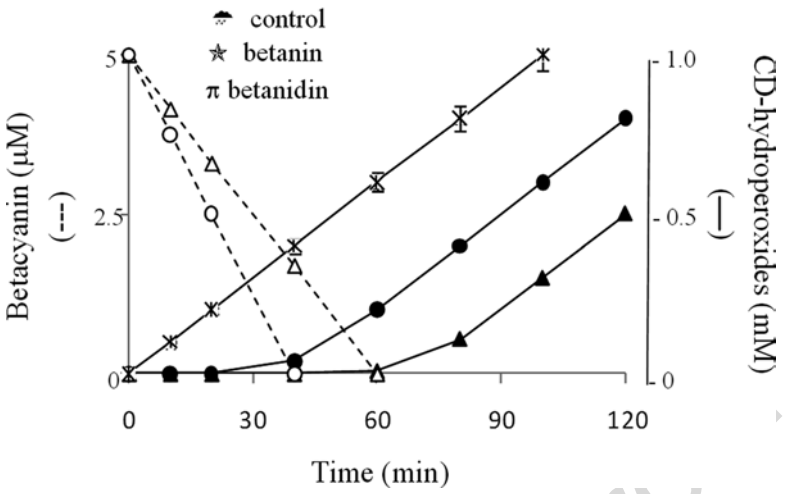

Fig. 6.5 Time course of CD-hydroperoxides formation (*filled symbols*) during the AAPH-induced soybean PC liposome oxidation in the absence (*control*) or in the presence of betacyanins and consumption of the pigments (*open symbols*) (Tesoriere et al. 2009)

The investigations reported above, showing that betanidin is a lipoperoxyl radical-scavenger better than betanin both in solution and lipid bilayers, confirm the importance of peculiar structural features conferring antiradical capacity to betalains. A recent systematic study assessed the reducing activity, as Trolox equivalence antioxidant capacity (TEAC) of 15 betalains with increasingly complex chemistry, from 1-ethylamine betaxanthin to betanin (Gandia-Herrero et al. 2010). The data support the existence of a strong "intrinsic" antiradical activity, possibly linked to the electron resonance system supported by both nitrogen atoms, which is common to all betalains. The presence of a mono/diphenol moiety in resonance with the betalamic acid moiety, plus a second cycle fused in an indoline manner, as in betacyanins, implies a significant enhancement of the radical-scavenging capacity (Gandia-Herrero et al. 2010). The formation of betanidin quinone or dopachrome from the oxidation of betanidin in methanol or liposomes, respectively (Tesoriere et al. 2009), while confirming the importance of the phenol hydroxyls, may rule out that the cyclic nitrogen is involved in the antioxidant mechanism of the molecule in the model systems considered.

6.4 Inhibition of Low-Density Lipoprotein Oxidation by Betanin

Free radical-induced oxidation of low-density lipoproteins (LDL) proceeds by a chain mechanism generating phosphatidylcholine hydroperoxides and cholesteryl ester hydroperoxides as the major primary products (Esterbauer et al. 1992).

These reactions, and the consequent internalization of oxidized LDL (ox-LDL) in macrophages, are considered key events in the progression and eventual development of atherosclerosis (Steinbrecher et al. 1987; Steinberg et al. 1989; Heinecke 1998). LDL are endowed with several lipophilic antioxidants, the most abundant being α-tocopherol (Esterbauer et al. 1992); however oxidants from endogenous and/or exogenous sources can reduce the defense, which makes the particle prone to oxidize, thus becoming an agent of damage. Under these circumstances, dietary bioavailable antioxidants that may interact with and/or partition in LDL and be involved in LDL protection ~~has~~ have continuously been explored.

The oxidation of human LDL by transition metal ions such as iron or copper has been a model for generating knowledge of the kinetics of LDL oxidation (Esterbauer et al. 1992), and has widely been considered for assessing intrinsic activity of natural antioxidants. The biological relevance of such a model has been questioned, however. In more recent studies, oxidation of LDL in vivo has been suggested to depend on the activity of myeloperoxidase (MPO) (Daugherty et al. 1994; Heller et al. 2000), a heme-enzyme that utilizes hydrogen peroxide and a variety of co-substrates to generate reactive enzyme intermediates, namely compound I and compound II (Heinecke 1998; Daugherty et al. 1994; Klebanoff 1980). MPO activity also depends on the metabolism of nitric oxide (NO) forming nitrite, the final oxidation product of NO metabolism, a substrate for the enzyme (Burner et al. 2000; Eiserich et al. 1998; van der Vliet et al. 1997; Sampson et al. 1998). Nitrogen dioxide radical $\left(NO_2^{\bullet}\right)$, the one-electron oxidation product of nitrite by MPO compound I, has been proposed as the reactive species to start massive oxidation of the LDL lipids (Byun et al. 1999; Kostyuk et al. 2003). Both these models have been used to assess whether the sensitivity of human LDL to oxidation could be altered by betanin (Tesoriere et al. 2003; Allegra et al. 2007).

The production of lipid hydroperoxides in LDL exposed to oxidative challenge does not start before all LDL antioxidants are consumed in the sequence from the most active (α-tocopherol) to the least active (β-carotene) (lag phase). After the lag period, peroxidation begins to accelerate and formation of CD hydroperoxides can be measured (propagation phase), until all lipid is oxidized. Betanin can incorporate in human LDL in vivo and in vitro (Tesoriere et al. 2004a, 2003). In ex vivo experiments, betanin-enriched LDL were isolated after spiking human plasma with pure betanin, then the resistance of these particles to copper-induced oxidation was measured in comparison with LDL obtained from the same plasma that did not undergo the spiking procedure (Tesoriere et al. 2003). Betanin-enriched LDL showed a significant elongation of the time preceding lipid oxidation, during which betanin was totally consumed (Fig. 6.6). Behaving as a lipoperoxyl radical scavenger, betanin affects the chain process of the copper-induced LDL lipid oxidation. In this system, vitamin E consumption is unaltered in the presence of betanin, whereas consumption of β-carotene is delayed. Betanin starts declining only after vitamin E depletion, and is totally consumed before β-carotene. While indicating the higher effectiveness of vitamin E in protecting LDL lipids and all LDL antioxidants, these findings show that betanin acts as a lipoperoxyl radical-scavenger better than β-carotene in the copper-oxidized LDL model.

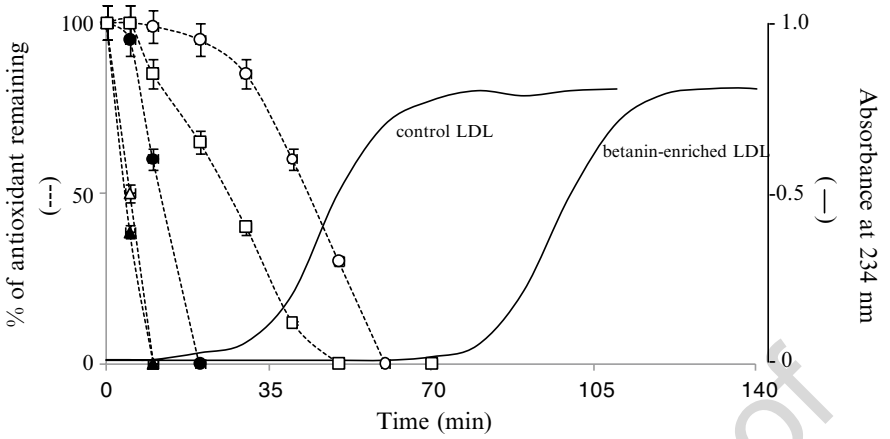

Fig. 6.6 Time course of the consumption of vitamin E (*triangle*), β-carotene (*circle*) and betanin (*square*) during the copper-induced oxidation of control (*filled symbols*) or betanin-enriched (*open symbols*) LDL. LDL oxidation is followed by the formation of CD hydroperoxides at 234 nm (Tesoriere et al. 2003)

As for the other model, betanin effectively inhibited the production of lipid hydroperoxides in human LDL submitted to a MPO/nitrite-induced oxidation (Allegra et al. 2007). In this system, the time-course of lipid oxidation follows the same phases as the copper-oxidised LDL, followed by the formation of lipid hydroperoxides. It was imperative from a number of kinetic measurements that the betalain can block the process at various levels, that betanin not only acts as a scavenger of the initiator radical nitrogen dioxide, but can also act as a lipoperoxyl radical scavenger. In addition, unidentified products from the oxidation of betanin by MPO/nitrite further inhibit LDL oxidation as effectively as the parent compound (Allegra et al. 2007), thus extending the antioxidative protection of LDL beyond the time in which betanin is consumed. It should be mentioned that other studies showed that betanin is a reducing substrate for the intermediates—compound I/II of the peroxidative MPO cycle (Allegra et al. 2005), an action potentially pro-oxidant in this LDL model. This however appears to be counteracted by the activity of betanin and possibly by its oxidized products through scavenging of NO_2. Figure 6.7 depicts the catalytic cycle of MPO/nitrite and suggests sites of action of betanin.

6.5 Interactions of Betanin and Betanidin with Vitamin E

In living organisms, antioxidants do not function individually, rather, they function cooperatively or even in synergism with each other. Since α-tocopherol is the main lipid antioxidant in membranes, exploring interactions between dietary antioxidants and α-tocopherol is considered important to envisage eventual effects and possibly mechanism of action of these molecules in vivo. For instance, either synergistic or

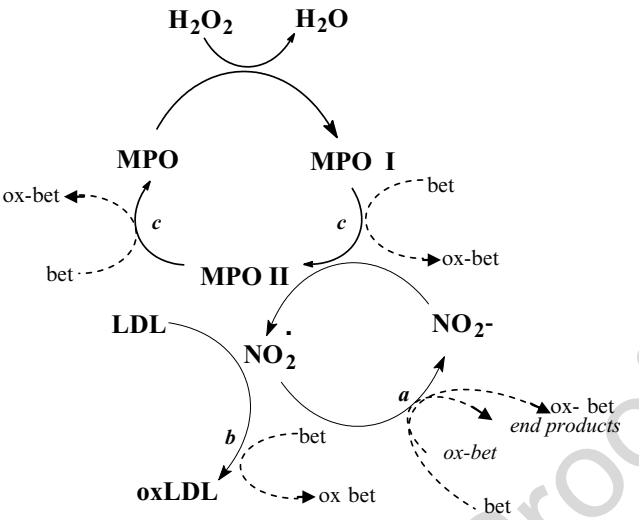

Fig. 6.7 Proposed mechanisms of antioxidant activity of betanin on myeloperoxidase-induced LDL oxidation. *a* scavenger of $NO_2^{\cdot}$, *b* lipoperoxyl radical scavenger, *c* reductant for compound I and II of MPO

additive effects or co-antioxidant action have been reported between polyphenol phytochemicals and α-tocopherol (Zou et al. 2005; Jia et al. 1998; Pedrielli and Skibsted 2002). In soybean PC liposomes, at a 1:1 betacyanin:α-tocopherol ratio, either betanin or betanidin cannot extend the inhibition period beyond the sum of the individual inhibition periods, providing evidence of merely additive effects (Tesoriere et al. 2009). On the other hand, even in a model of copper-oxidized LDL, the time-course of vitamin E consumption, either in the absence or in the presence of betanin, suggests an independent antioxidant activity of the two molecules (Tesoriere et al. 2003). The redox potential of betanin is lower than α-tocopherol (0.5 mV) (Buettner 1993), which would allow reduction of the α-tocopheroxyl radical at the membrane surface (Fukuzawa 2008), provided favorable site-specific interactions (Barclay 1993). The absence of cooperative effects may be the expression of the partition of betanin in either the lipid bilayer or LDL and of its activity in scavenging lipoperoxyl radicals.

6.6 Peroxyl Radical-Scavenging Activity of Vulgaxanthin I

Antioxidative effects of vulgaxanthin I were evaluated in an oxidation model of LAME in the presence of AMVN (Tesoriere et al. 2008). The amount of lipid hydroperoxides formed after a 30-min incubation was taken as a reference end-point, and the inhibition by vulgaxanthin I was expressed in terms of IC_{50}, that is, the amount of pigment required for a 50% inhibition. Under these conditions, vulgaxanthin I

showed an IC_{50} of 0.75 μM, of the same order as betanin and α-tocopherol taken as a comparison, 1 μM and 0.56 μM, respectively.

6.7 Conclusions

The unanimously recognized dual and complex role of radical species and oxidants in the cell functioning and in pathology points to the necessity to get better knowledge of what the so-called antioxidant compounds may really do, since scavenging of reactive species and interactions with cell constituents involved in maintaining the redox homeostasis may significantly interfere with cell signal transduction. These new concepts have recently led to consider the role of antioxidant vitamins even as modulators of redox-regulated cell signaling, and must be used to investigate and interpret effects, including eventual adverse effects, of phytochemicals with redox properties at a molecular level (Leonarduzzi et al. 2010).

Phenolic hydroxyls have been repeatedly proven as efficient reducers of prooxidant/oxygen radicals under a wide range of conditions (Valgimigli et al. 1995; Barclay et al. 1999). In accordance, the higher the number of hydroxyl groups, the higher the antioxidant activity of polyphenol phytochemicals such as flavonoids, has been shown (Rice-Evans et al. 1996). The betacyanin pigments, betanin and betanidin, exhibit an antioxidant effectiveness linked to the presence of the glucose-substituted phenol moiety of betanin and to the *ortho*-diphenol moiety of its aglycone, the latter being a much more efficient reductant in both organic solvent and liposomal lipid bilayers. These findings may be of an even greater interest since the calculated constants characterizing the activity in solution and in liposomes have appeared of the same order as those of α-tocopherol (Tesoriere et al. 2009), the major lipid antioxidant in our body (Niki 1996). More importantly, betanin also shows antioxidant activity in a biologically relevant LDL oxidation model (Allegra et al. 2007).

Information on chemistry, reactivity in as many as possible different systems, particularly biological environments, and interactions with physiological antioxidants, are first steps to characterize dietary antioxidants. Activity in cell cultures and investigation of cell redox changes and specific signaling may further enhance our knowledge and allow hypotheses on potential health effects. However none of these studies make sense until it is proven that the compound of interest can really reach body sites and the observed in vitro actions may be accomplished in vivo. Studies in this direction have shown that betanidin, being a highly unstable molecule (Gandia-Herrero et al. 2007; Stintzing and Carle 2004), was not found after a simulated digestion of betanin-containing foods, including beet root, though the aglycone could have been generated by pancreatic amylase (Tesoriere et al. 2008). These observations make its eventual systemic activity in vivo hard to determine. Beneficial effects could be considered at the gastrointestinal level, however (Halliwell et al. 2005). Betanin, instead, has been shown to be bioavailable in humans, after ingestion of either cactus pear fruits or red beet (Kanner et al. 2001; Tesoriere et al. 2004a,

2005; Frank et al. 2005), reaching plasma concentrations sufficient to promote its incorporation in LDL and red blood cells (Tesoriere et al. 2004a, 2005). It is in light of these findings that the chemistry of the peroxyl radical-scavenging activity of betanin, and relevant parameters, deserve to be considered. It is suggested that betanin, and foods rich in betanin, such as beetroot and the fruits of the *Opuntia* cactus, may be of nutraceutical interest and contribute to maintain the natural redox homeostasis and possibly prevent disease states. With focus on the latter point, a small clinical trial carried out with eight healthy volunteers who consumed cactus pear fruit pulp for 15 days demonstrated a remarkable positive effect on the body's redox status that was reasonably attributed to betalains, and not to the fruit vitamin C (Tesoriere et al. 2004b). As a final note, current studies in the authors' laboratory show that betanin is transported through human CaCo-2 cell monolayers with an apparent permeability coefficient ~~that rules out paracellular transport and suggests~~ that dietary betanin can be absorbed quite effectively during its intestinal transit (data to be published). While these data appear to confirm the observations in humans (Kanner et al. 2001; Tesoriere et al. 2004a, 2005; Frank et al. 2005), the actual amounts recovered in vivo, quite lower than suggested by in vitro experiments, would indicate metabolism and/or bacterial degradation of the molecule in gut, which should be investigated.

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[AU1]

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