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Kinetics of the lipoperoxyl radical-scavenging activity of indicaxanthin in solution and unilamellar liposomes

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Abstract
The reaction of the phytochemical indicaxanthin with lipoperoxyl radicals generated in methyl linoleate methanol solution by 2,2′-azobis(2,4-dimethylvaleronitrile), and in aqueous soybean phosphatidylcholine unilamellar liposomes by 2,2′-azobis(2-amidinopropane)hydrochloride, was studied. The molecule acts as a chain-terminating lipoperoxyl radical scavenger in solution, with a calculated inhibition constant of $3.63 \times 10^5\,M^{-1}s^{-1}$, and a stoichiometric factor approaching 2. Indicaxanthin incorporated in liposomes prevented lipid oxidation, inducing clear-cut lag periods and decrease of the propagation rate. Both effects were concentration-dependent, but not linearly related to the phytochemical concentration. The consumption of indicaxanthin during liposome oxidation was remarkably delayed, the lower the concentration the longer the time-interval during which it remained in its native state. Indicaxanthin and α-tocopherol, simultaneously incorporated in liposomes, exhibited cooperative antioxidant effects and reciprocal protective interactions. The extent of synergism decreased at the increase of the ratio (indicaxanthin)/(α-tocopherol). A potential antioxidant mechanism of indicaxanthin is discussed in the context of the chemistry of the molecule, and of the possible reactivity of a short-lived intermediate.

Keywords: *Indicaxanthin, phytochemical, lipid oxidation, nutritional value*

Introduction

ROS-mediated damage to biological lipid environments, including membranes and lipoproteins, is involved in the initiation and/or progression of a number of pathological conditions, from atherosclerosis and coronary heart disease to cell senescence and cancer. The epidemiological evidence that clinical manifestations of such conditions are reduced by dietary intake of antioxidant vitamins and of a number of other non-nutrient phytochemicals [1–4], brought about considerable work to inspect new natural compounds for potential bioactivity.

Pigments such as anthocyanins, carotenoids and curcuminoids have been shown to possess antioxidant and anti-inflammatory activity [5,6]. Betalains, which replace anthocyanins in almost all plants of the order of Cariophyllalae and occur in some superior fungi [7], have recently been investigated as novel antioxidants [8–12]. These molecules are derivatives of betalamic acid, and encompass 2 classes of compounds [13]: betacyanins and betaxanthins. Betalamic acid may condense either with cyclo-DOPA, with additional substitutions through varying glycosylation and acylation patterns at C-5 or C-6, to produce betacyanins, or with various amino acids or amine derivatives to produce betaxanthins (Figure 1). Betalains are cationized compounds with a positive nitrogen in a polyene system. Their cyclic amine has been considered as the reactive group conferring to this class of molecules their reducing activity [14,15]. Indicaxanthin, the yellow pigment characterizing the edible fruit of the cactus *Opuntia ficus indica*, is the adduct of betalamic acid with proline (Figure 1). The redox potential and radical scavenging activity of this compound have recently been measured [12], and a number of data have been

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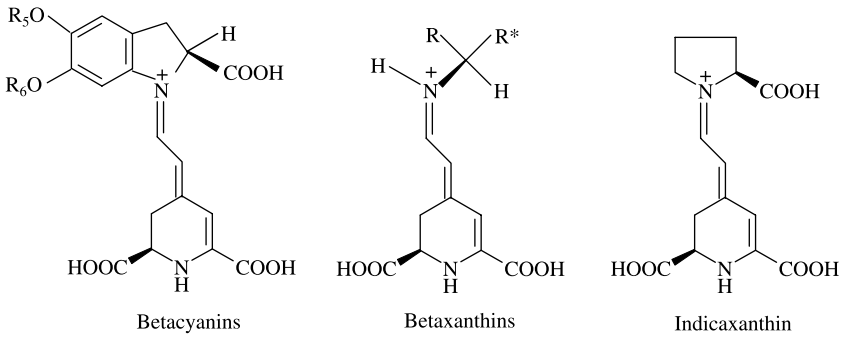

Figure 1. Basic structure of betacyanins and betaxanthins and molecular structure of indicaxanthin.

published on the antioxidant and protective effects of indicaxanthin in various biological environments from low density lipoproteins (LDL) [16], to either healthy [17] or pathological [18] red blood cells, as well as cell cultures [19]. These findings led us to study the interaction between indicaxanthin and lipoperoxyl radicals produced under controlled conditions in either methyl linoleate (LAME) solution, or in liposomal bilayers of soybean phosphatidylcholine (PC), in order to provide chemical parameters characterizing the antioxidant properties of this compound. In the light of our recent finding of cooperative effects of vitamin E and indicaxanthin in LDL [16], we were also motivated to study the interactions of indicaxanthin and α-tocopherol in soybean PC liposomes.

Methods

Chemicals

Linoleic acid methyl ester (LAME), soybean or dipalmitoyl (DP) phosphatidylcholine (PC), α-tocopherol, 13(S)-hydroperoxy-(9Z,11E)-octadecadienoic acid, Chelex-100 ion-exchange resin, Sephadex G-25, and dialysis tubing cellulose membrane (23 mm flat) were from the Sigma Chemical Co. (St Louis, MO). LAME was purified on a Florisil column (Floridin, New York, NY) (Terao, J., Nagao, 20). 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 to adventitious metals.

Isolation of indicaxanthin

Indicaxanthin was isolated from cactus pear (*Opuntia ficus-indica*) fruits (yellow cultivar). The phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25 [12]. Fractions containing the pigment were submitted to cryo-dessiccation, and purified according to Stintzing et al. [20]. Briefly, the dessiccated material was re-suspended in 1% acetic acid in water and submitted to semi-preparative HPLC using a Varian Pursuit C18 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. Spectrophotometric revelation was at 482 nm. The elution volumes relevant to indicaxanthin were collected. Samples after cryo-dessiccation were re-suspended in methanol 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 482 nm of 42,800 [21].

Peroxyl radical-scavenging assay in solution

Peroxidation of methyl linoleate was performed by incubating 300 mM LAME and 2.0 mM AMVN, in a final methanol volume of 1.0 ml, in a water bath at 37°C, under air. Portions of the mixture (10 μl) were taken at intervals and injected onto a Supelco Supelcosil™ (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. When required, suitable amounts of antioxidant in methanol were added to the methanol solution of methyl linoleate and allowed to equilibrate at 37°C for 60 s. The azoinitiator was added, and the incubation was carried out as above.

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 indicaxanthin. The propagation rate, R_p, was measured as the amount of conjugated diene (CD) lipid hydroperoxides formed per second in the absence of antioxidant or after the inhibition period. The initiation rate, R_i, was measured with the induction period method [22], according to Equation 1

$$R_i = n\,[IH]/t_{inh} \quad (1)$$

where [IH] is the concentration of the antioxidant, n is the reaction stoichiometric factor and t_{inh} the inhibition period. α-Tocopherol was used as a reference assuming that two peroxyl radicals are scavenged per molecule of antioxidant [23]. The inhibition period, t_{inh}, in the curve of peroxidation in the presence of antioxidant corresponds to 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, R_{inh}, is calculated by the coordinates of the intercept of the extrapolations of the parts of the curve representing the inhibition and propagation phases. The inhibition rate constant, k_{inh}, was calculated from the duration of the inhibition period and the inhibition rate, following Equation 2

$$k_{inh} = k_p[LH]/R_{inh}t_{inh} \quad (2)$$

where [LH] is the concentration of the lipid and k_p the absolute rate constant for the oxidation of LAME in methanol which is to be assumed $100\,M^{-1}\,s^{-1}$ [24].

Peroxyl radical-scavenging assay in liposomal suspensions

Liposomes were prepared by adding, in this order, chloroform solution of either soybean PC or DPPC and antioxidant in methanol, when required, in a round-bottom tube kept in an ice bath. Solvent was removed, after each addition, under a nitrogem stream, and the thin film obtained was vortexed in an ice bath, for 10 min with 0.9% NaCl in 5 mM phosphate buffer, pH 7.4 (PBS). 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 2 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 CD lipid hydroperoxide production was evaluated by the increase in absorbance at 234 nm, using a molar absorption coefficient of 27,000 [25]. All oxidations were carried out under dim red light to avoid possible photooxidation of fatty acid by low-energy quanta of visible light [26,27] and to preserve light-sensitive α-tocopherol.

Indicaxanthin and α-tocopherol analysis

The consumption of indicaxanthin and α-tocopherol during liposomal oxidation was assessed by extracting aliquots of the incubation mixture, diluted 1:10 (v:v) with PBS, at suitable time intervals, followed by HPLC quantitation. Indicaxanthin was extracted by mixing 1 volume of the diluted sample with 3 volumes of chloroform/methanol (2:1, v:v). The methanol phase was dried under nitrogen, re-suspended in 1% acetic acid in water and analysed on a Supelco Supelcosil™ column as above, eluted with the same gradient, with a flow rate of 1.5 ml/min. α-Tocopherol was extracted by mixing 1 volume of the diluted sample with 2 volumes of absolute ethanol and 8 volumes of petroleum ether. The organic extract was gathered, dried under nitrogen, re-suspended in several microliters of methanol, and analysed by HPLC using the same column reported above. The eluent was methanol with a flow rate of $1.0\,ml\,min^{-1}$. Fluorometric detection was with excitation at 290 nm and emission at 335 nm. Quantitation was by reference to standard curves constructed with 5–100 ng of the relevant purified compound, and by relating the amount of the compound under analysis to the peak area.

Suitable aliquots of the incubation mixture, without preliminary extraction, were injected on top of the HPLC column and analysed as described above, to measure the consumption of indicaxanthin during peroxidation of LAME.

Dialysis

Indicaxanthin, free or incorporated in 10 mM either soybean- or DP–PC liposomes, was inserted in a dialysis tube containing 5 ml of PBS, to obtain a final concentration of 10 μM, and dialysed against 10 ml of the same buffer, at 37°C. At suitable time intervals indicaxanthin outside the dialysis tube was determined by HPLC as reported above.

Results

Lipoperoxyl radical-scavenging activity of indicaxanthin in solution

LAME methanol solutions were oxidized by AMVN, to ensure a linear lipoperoxide production. The kinetic curves of the hydroperoxide formation either in the absence or in the presence of indicaxanthin are shown in Figure 1. The phytochemical caused inhibition periods preceding the active hydroperoxide formation (lag phase), the length of which was correlated to the concentration of antioxidant ($r^2 = 0.99$, $P < 0.01$), while the peroxidation rate (R_p) following the lag phase was comparable to that of the non-inhibited reaction. Measurements of indicaxanthin during LAME oxidation provided evidence that the molecule disappeared steadily, and was totally depleted within the lag phase (Figure 2). The consumption rate of indicaxanthin during LAME oxidation was calculated as $2.7 \pm 0.3 \times 10^{-9}\,M\,s^{-1}$ from the data presented in Figure 2. On the other hand, the consumption was two orders of magnitude lower ($7.7 \pm 0.82 \times 10^{-11}\,M\,s^{-1}$, $n = 4$), when indicaxanthin

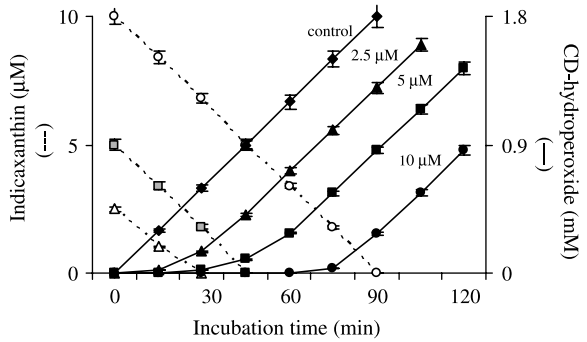

Figure 2. AMVN-induced oxidation of methyl linoleate in methanol in the absence (control) or in the presence of 2.5–10 μM indicaxanthin and consumption of the antioxidant. Oxidation conditions and HPLC analysis of indicaxanthin are reported in methods. Each point represents the mean ± SD of six to eight determinations carried out with different incubation mixtures.

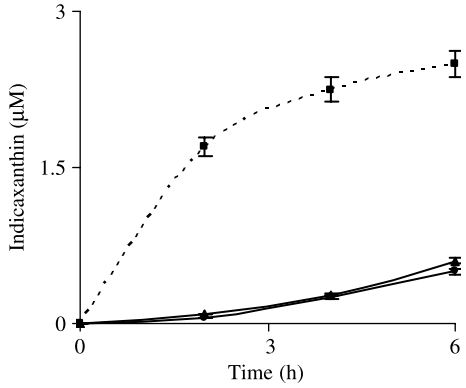

Figure 3. Diffusion of indicaxanthin (10 μM) through a dialyzing tube in the absence (dashed line) or in the presence (full line) of 10 mM soybean (triangle) or dipalmitoyl (circle) phosphatidylcholine unilamellar liposomes.

was exposed to AMVN in the absence of lipid. Table I reports the kinetic parameters characterizing the uninhibited, as well as the inhibited reaction, as calculated from the experimental data.

Peroxyl radical-scavenging activity of indicaxanthin in liposomes

The affinity of indicaxanthin to liposomal bilayers is shown in Figure 3. The diffusion of the phytochemical from the dialysis tube in the presence of either soybean or DPPC liposomes, was significantly lower than that exhibited in their absence, and was comparable in both systems, indicating similar partition in liposomes from the two phosphatidylcholines.

The kinetics of lipid oxidation in water-dispersed unilamellar soybean PC liposomes exposed to AAPH were studied either in the absence or in the presence of 2.5–20 μM indicaxanthin. The phytochemical caused both clear inhibition periods and decrease of the kinetic chain length during the propagation phase (Figure 4). Neither the length of the lag phase, nor the calculated R_p appeared linearly dependent on the indicaxanthin concentration (Figure 4, insets, and Table II).

The changes of indicaxanthin concentration during the reaction were monitored. Though actively prevented the production of peroxides, indicaxanthin did not appear to be consumed for a certain time period after starting of lipid oxidation, the lower the concentration, the longer the time-interval in which remained in its native state (Figure 5A). By contrast, when indicaxanthin in the range 2.5–10 μM was incorporated in 10 mM unilamellar DPPC liposomes, its consumption started soon after the addition of 2 mM AAPH (Figure 5B).

Cooperative effects of indicaxanthin and alpha-tocopherol in liposomal bilayers

Interactions between indicaxanthin and vitamin E in suppressing the AAPH-induced soybean PC liposome oxidation were investigated by measuring the inhibition periods (τ) induced either by the individual antioxidants or by a variety of combinations of indicaxanthin and

Table I. Oxidation of methyl linoleate in methanol* and inhibition by 5 μM indicaxanthin.

R_p	0.32×10^{-6} $(Ms^{-1})^{\dagger}$
R_i	0.40×10^{-8} $(Ms^{-1})^{\ddagger}$
R_{inh}	3.33×10^{-8} (Ms^{-1})
t_{inh}	2480 (s)
kcl[¶]	80
kcl_{inh}[§]	9.6
n[‖]	1.98
K_{inh}[#]	3.63×10^{5} $(M^{-1}s^{-1})$

* Methyl linoleate and AMVN concentrations were 300 and 2.0 mM, respectively; † Peroxidation rate (R_p) is expressed for total solution; ‡ Initiation rate (R_i) is measured by the duration of inhibition of 10 μM α-tocopherol; ¶ kcl, kinetic chain length in the absence of antioxidant (R_p/R_i); § kcl_{inh}, kinetic chain length during the inhibition period (R_p/R_{inh}); ‖ calculated by Equation 1; # calculated by Equation 2.

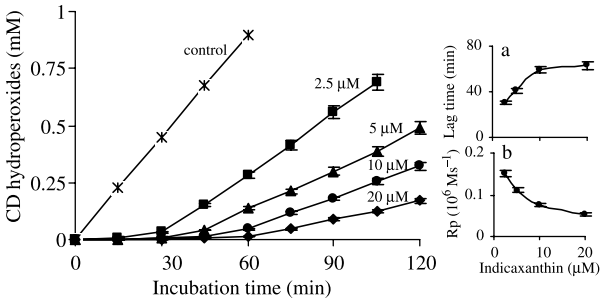

Figure 4. AAPH-induced oxidation of unilamellar soybean PC liposomes in the absence (control) or in the presence of 2.5–20 μM indicaxanthin. Oxidation conditions are reported in methods. Each point represents the mean ± SD of six to nine determinations carried out with different liposomal preparations. Insets: Relationships between either length of lag time (a) or Rp (b) and indicaxanthin concentrations.

Table II. Kinetic parameters measured during AAPH-induced oxidation of soybean PC liposomes* in the presence of indicaxanthin.

Antioxidant concentration μM	Lag time min	R_p 10^6 Ms^{-1}	*n*
0		0.25 ± 0.022	6
2.5	30 ± 2.1	0.15 ± 0.020	9
5	41 ± 3	0.11 ± 0.019	9
10	59 ± 5	0.075 ± 0.009	8
20	63 ± 7	0.052 ± 0.006	7

*Unilamellar PC liposomes and AAPH concentration were 10 and 2.0 mM, respectively. The values are the mean ± SD of *n* experiments carried out with different liposomal preparations, as represented in Figure 4.

α-tocopherol, as well as their consumption, when alone or combined. Co-incubation of indicaxanthin and α-tocopherol, both at 5 μM, resulted in an inhibition period the length of which was higher than the sum of the inhibition periods caused by the individual antioxidants, providing evidence that the two molecules acted cooperatively (Figure 6). The decay kinetics of indicaxanthin and α-tocopherol showed that, while producing synergistic interactions, the combination of

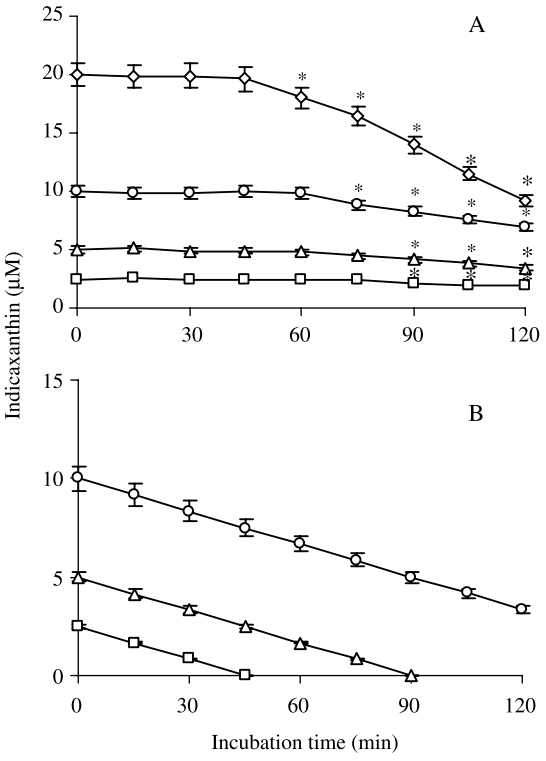

Figure 5. Consumption of indicaxanthin incorporated in either unilamellar soybean PC (panel A) or unilamellar DPPC liposomes (panel B) exposed to AAPH. Oxidation conditions, extraction and HPLC analyis of indicaxanthin are reported in methods. Panel A: each point represents the mean ± SD of six to nine determinations carried out with different liposomal preparations. *With respect to the relevant value at time 0, values were significant with $P < 0.01$ (Student's *t*-test). Panel B: each point represents the mean ± SD of three determinations carried out with different liposomal preparations.

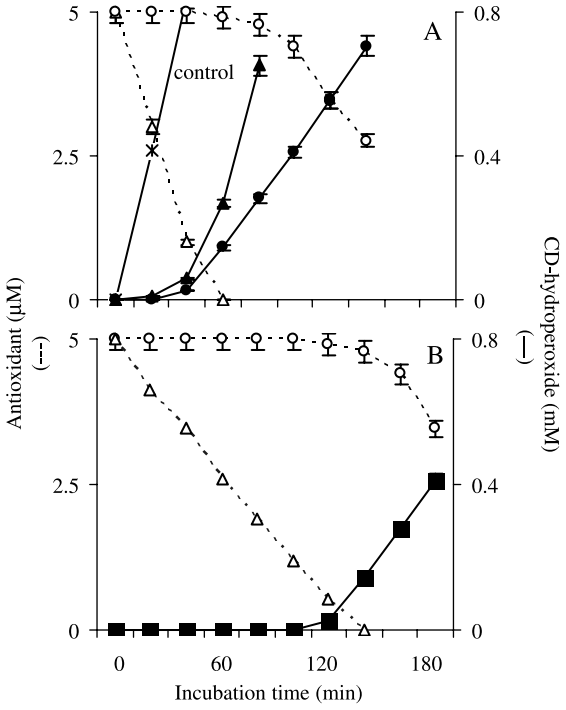

Figure 6. AAPH-induced oxidation of unilamellar soybean PC liposomes incorporating indicaxanthin and α-tocopherol separately (A) or in combination (B), and time course of the consumption of the antioxidants. Oxidation of liposomes (closed symbols), containing no antioxidant (control), or 5 μM indicaxanthin (circle), or 5 μM α-tocopherol (triangle), or both (square), and consumption (open symbols) of indicaxanthin (circle), or α-tocopherol (triangle), are reported in methods. Each point represents the mean ± SD of six determinations carried out with different liposomal preparations.

the molecules resulted in a reciprocal protective effect. Indeed, the decay of α-tocopherol appeared slowered down, and consumption of indicaxanthin was remarkably delayed (Figure 6).

The extent of synergism, as expressed by $\tau_{(E+I)} - (\tau_E + \tau_I)$ increased according to a non-linear relation in the range 0.2–1 indicaxanthin/vitamin E molar ratio, whereas a drop was observed when indicaxanthin exceeded α-tocopherol concentration (Figure 7).

Discussion

Indicaxanthin, a phytochemical of the betalain class, has recently been investigated as a radical scavenger, in either aqueous solutions or in lipid systems [12,16–18]. This paper expands knowledge on the antioxidant capacities of this compound, evaluating the kinetic parameters for the reaction with lipoperoxyl radicals generated in solution, and assessing the antioxidant activity of the molecule in an aqueous liposomal system.

Indicaxanthin exhibits a classical chain-breaking antioxidant behavior when included in methanol solutions of methyl linoleate submitted to oxidation by AMVN. Since the reactivity of indicaxanthin with

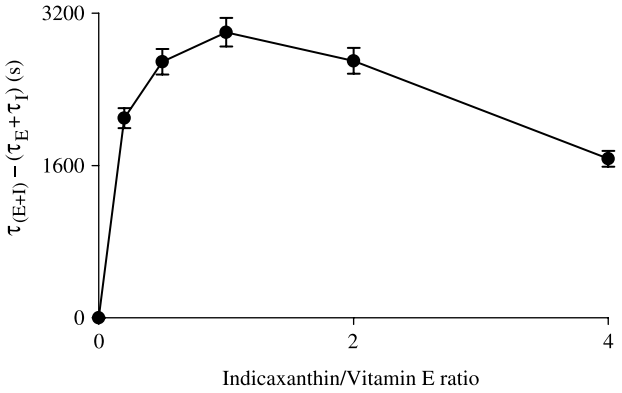

Figure 7. Dependence of synergistic antioxidant activity between α-tocopherol and indicaxanthin on the indicaxanthin/α-tocopherol ratio. Each point represents the mean ± SD of three determinations carried out with different soybean PC liposomal preparations.

AMVN-derived peroxyl radicals has appeared negligible in this system, the prevention of lipid oxidation can be ascribed to scavenging of lipoperoxyl radicals. Mechanistic molecular aspects of the antioxidant activity of indicaxanthin, as well as other betalains [8–10], have not entirely been clarified. Peak potentials indicating reducing power at 0.611 and 0.895 V have been measured by cyclic voltammetry [12], then is expected that indicaxanthin would efficiently inactivate alkylperoxyl radicals (1.0 V) [28]. Our kinetic measurements in solution showed that the effectiveness of the molecule as a lipoperoxyl radical-scavenger is comparable to that of a lipid antioxidant such as vitamin E, with a stoichiometric factor approaching 2, and an inhibition constant of the same order as that reported for α-tocopherol under similar conditions [24]. The cyclic amine of betalamic acid, the building block of the betalain pigments may be envisaged as a site in the molecule active in reducing lipoperoxyl radicals, by acting similarly to the amine group of ethoxyquin, a potent chain-breaking antioxidant in lipid systems [14,15] and *in vivo* [29]. After H-atom donation, the polyene system of indicaxanthin may allow the formation of a resonance-stabilised aminyl radical whose reactivity will be affected by the environment. In methanol solution of peroxidizing methyl linoleate, the observed stoichiometry suggests reaction with a second lipoperoxyl radical.

As shown by a number of studies, indicaxanthin exhibits an amphyphilic character being capable of binding to LDL [16], as well as partitioning between plasma and red blood cells [17,18]. The amphyphilic nature of the molecule has appeared further evident in the present study. When submitted to dialysis either in the absence or in the presence of liposomes, indicaxanthin showed affinity for bilayers of either saturated or unsaturated phosphatidyl cholines.

Indicaxanthin was quite effective in preventing lipid oxidation, when incorporated in soybean PC liposomes submitted to aqueous radicals from AAPH. However, its activity was not consistent with the antioxidant activity observed in solution. The inhibitory effect occurred through two different actions affecting both the duration of the lag phase and the propagation rate. Although concentration-dependent, the inhibition periods and the decrease of the kinetic chain length were not linearly related to the concentration, rather a decrease of relative efficiency was observed by increasing the indicaxanthin amounts. In addition, indicaxanthin paradoxically remained in its native state for a long period during lipid oxidation, the lower the concentration the longer the time-interval before a decline of concentration was evident. These observations suggested a mechanism more complex than that of a classical chain-terminating lipoperoxyl radical-scavenger.

Recycling of indicaxanthin through reduction of a short-lived radical intermediate formed after reaction with a lipid peroxyl radical, may be hypothesized to rationalize the antioxidant activity and consumption kinetics of the pigment in liposomes. The liposomal oxidation model of soybean PC limits the potential regenerating substrate to unsaturated lipids. The reduction potential of the couple PUFA·/PUFA-H (0.6 V) [28,30] would make the regeneration of indicaxanthin a feasible reaction. Accordingly, the abstraction of a bis-allylic hydrogen atom from linoleic acid would generate a carbon-centered radical capable of initiating a new oxidation chain (Figure 8). Thus, the initial antioxidant event would be converted in a "prooxidant" insult, which would be consistent with the drop of the relative effectiveness at higher indicaxanthin concentrations. However, since an antioxidant effect is evident, the rate at which the betalain radical intermediate starts oxidation events must be lower than that of the lipid peroxyl radical. Regeneration reactions are in competition with reactions of termination, in which complete oxidation of the molecule will occur, through scavenging of a second peroxyl radical (Figure 8). Termination reactions will prevail as

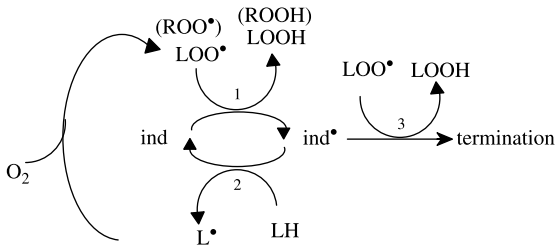

Figure 8. Proposed scheme of inhibition of azo-initiator-induced lipid oxidation in aqueous soybean PC liposomes by indicaxanthin. Reaction of indicaxanthin (ind) with lipoperoxyl or aqueous peroxyl radicals (LOO˙ or ROO˙) generates an intermediate radical (ind˙) and hydroperoxide (LOOH or ROOH) (reaction 1). We propose that ind˙ is reduced by reaction with polyunsatured fatty acid (LH) to re-generate ind and a carbon centered radical (L˙) that in turn reacts with oxygen (O_2) to form LOO˙ (reaction 2). This process competes with termination reactions which would result in consumption of the phytochemical (reaction 3).

liposome oxidation proceeds, i.e. when the decrease of native fatty acids will make hardly effective the regeneration process. This implies that the ratio indicaxanthin/lipid is critical in determining the recycling effectiveness, as well as the actual equilibrium between antioxidant (Figure 8, reactions 1 and 3) and pro-oxidant (Figure 8, reaction 2) activity of indicaxanthin. The evidence that the consumption of indicaxanthin incorporated in an aqueous liposomal system of DPPC in the presence of AAPH started immediately, may support the hypothesis that the unsaturated lipids may condition the fate of the molecule in peroxidizing lipid bilayers. Regeneration of polyphenol antioxidants by unsaturated lipids has been described [31]. Our data suggest that such reactions may be part of the antioxidant behavior of other reductants.

The antioxidant activity in liposomes depends both on the radical scavenging ability of a compound and also its location and orientation in the system. It has been observed that hydrophilic antioxidants, including thiols, cysteine, and glutathione, which reside wholly in the aqueous phase are usually not so effective in inhibiting the first stages of oxidation and do not produce clear inhibition periods [32,33]. While the experiments with DPPC liposomes provide evidence of the reaction of indicaxanthin with AAPH-derived radicals, the affinity for the bilayer and the net lag phases in peroxidising soybean PC liposomes containing indicaxanthin suggest that the compound can reside at the polar surface region of the phospholipid bilayer. Such a location can allow indicaxanthin to effectively retard lipid oxidation initiated by free radicals from the aqueous phase, or eventually interact with lipid-derived radicals floating at the lipid-water interface [28], and favour the reactions with native lipid to be regenerated. Apparently, the molecular dispersion of indicaxanthin in the peroxidising LAME solution prevents indicaxanthin recycling and results in a rapid consumption of the molecule.

The interaction of antioxidant phytochemicals with other antioxidants may be important for the beneficial effects of these compounds in reducing oxidative stress *in vivo*. In the present study synergistic effects between indicaxanthin and vitamin E have been observed in the soybean-PC liposomal oxidation model. Cooperative interactions between two antioxidants, one of which is vitamin E, are usually due to re-generation of α-tocopheroxyl radicals [34–37]. Phenolic compounds [38] can serve as scavengers of aqueous peroxyl radicals near the membrane surface and regenerate α-tocopherol which acts as a second line of defense against aqueous peroxyl radicals and as a scavenger of chain propagating lipid peroxyl radicals within membranes. The regeneration of α-tocopherol, however, cannot be postulated to explain our findings, though the consumption kinetics showed reciprocal protective interactions when indicaxanthin and α-tocopherol acted in combination. According to the proposed scheme (Figure 8), the lipoperoxyl radical-scavenging activity of α-tocopherol will preserve unsaturated fatty acids thus supporting the indicaxanthin regeneration. This would extend the indicaxanthin effect and delay its consumption through termination reactions. Moreover, vitamin E activity can contribute to counteract the prooxidant events promoted by indicaxanthin recycling. In this light the drop of synergism at the increase of the indicaxanthin/α-tocopherol ratio may easily be rationalized. On the other hand, the slower consumption of vitamin E during liposomal oxidation in the presence of indicaxantin, can result from the concurring effective trapping of peroxyl radicals by the betalain.

In conclusion, indicaxanthin is a quite effective chain-terminating lipoperoxyl radical-scavenger in solution, with antioxidant effects in liposomes that appear to depend on its interactions with the phospholipid bilayer, and to be enhanced by the concurrent action of α-tocopherol. Indicaxanthin is a bioavailable phytochemical in humans [39], and accumulates in red blood cells [17] and LDLs [39]. The data presented suggest that it might effectively participate in the antioxidative protection of lipid moieties *in vivo*, and support the possible significance of this compound for human health.

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