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Photochemical and Optical Properties of Water-Soluble Xanthophyll Antioxidants: Aggregation vs Complexation

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ABSTRACT: Xanthophyll carotenoids can self-assemble in aqueous solution to form J- and H-type aggregates. This feature significantly changes the photophysical and optical properties of these carotenoids, and has an impact on solar energy conversion and light induced oxidative damage. In this study we have applied EPR and optical absorption spectroscopy to investigate how complexation can affect the aggregation ability of the xanthophyll carotenoids zeaxanthin, lutein, and astaxanthin, their photostability, and antioxidant activity. It was shown that complexation with the polysaccharide arabinogalactan (AG) polymer matrix and the triterpene glycoside glycyrrhizin (GA) dimer reduced the aggregation rate but did not inhibit aggregation completely. Moreover, these complexants form inclusion complexes with both monomer and H-aggregates of carotenoids. H-aggregates of carotenoids exhibit higher photostability in aqueous solutions as compared with monomers, but much lower antioxidant activity. It was found that complexation increases the photostability of both monomers and the aggregates of xanthophyll carotenoids. Also their ability to trap hydroperoxyl radicals increases in the presence of GA as the GA forms a donutlike dimer in which the hydrophobic polyene chain of the xanthophylls and their H-aggregates lies protected within the donut hole, permitting the hydrophilic ends to be exposed to the surroundings.

1. INTRODUCTION

Carotenoids are lipophilic pigments that provide many of the colors found in nature, including the colors found in plants, flowers, and animals. The main interest in carotenoids concerns their participation in light harvesting in biological systems and prevention of light-induced oxidative damage. Another reason for the interest in the redox properties of carotenoids is related to their use as antioxidants in medicinal formulations as a result of radical-mediated processes that occur frequently in living systems.[1−7] However, practical application of carotenoids as nutritional antioxidants or components of medicinal preparations has been limited since carotenoids are highly hydrophobic, air- and light-sensitive compounds. The majority of carotenoids are lipophilic molecules with near zero inherent aqueous solubility. Moving carotenoids into a pharmaceutical application requires a chemical delivery system that overcomes the problems with parenteral administration of a highly lipophilic, low molecular weight compound. Many different methods have been developed to make the carotenoids "water dispersible", as true water solubility has not been found. Most of the attempts to increase the solubility of carotenoids depended on the preparation of cyclodextrin inclusion complexes.[8−14] However, cyclodextrin complexes demonstrate low solubility and fast aggregation in aqueous solution. Moreover, using cyclodextrin complexes does not allow control of the antioxidant activity of the carotenoids due to the fast dissociation of these complexes in biological media. Thus, identifying complexing agents without these drawbacks continues to be of considerable interest. Recently we have described the synthesis of novel carotenoid complexes with unique physicochemical properties.[15−19] In these studies we used two natural complexants derived from the plant: the triterpene glycoside glycyrrhizic acid (GA), a natural compound extracted from the licorice root,[20,21] and arabinogalactan (AG), a natural water-soluble polysaccharide extracted from Siberian larch.[22−24] (See Figure 1.) β-Glycyrrhizic acid forms a head to tail dimer containing a hydrophobic and hydrophilic component in a donutlike shape.[15] The polyene chains of the xanthophylls can reside within the hydrophobic area while allowing the hydrophilic terminal rings to stick out on each end. Arabinogalactan is a highly branched polysaccharide polymer composed of galactose and arabinose molecules in a 6:1 ratio previously reported as a complexing agent to make carotenoids water dispersible resulting in increased photostability[17] and photocatalytic activity.[18] Larch arabinogalactan is approved by the U.S. Food and Drug Administration (FDA) as a source of dietary fiber, but it also has potential therapeutic benefits as an immune stimulating agent and cancer protocol adjunct.[24] The unique properties of these complexes were demonstrated for two natural carotenoids: β-carotene and canthaxanthin.[15−19]

In this study we have investigated another class of carotenoids: xanthophylls. Xanthophylls are the carotenoids

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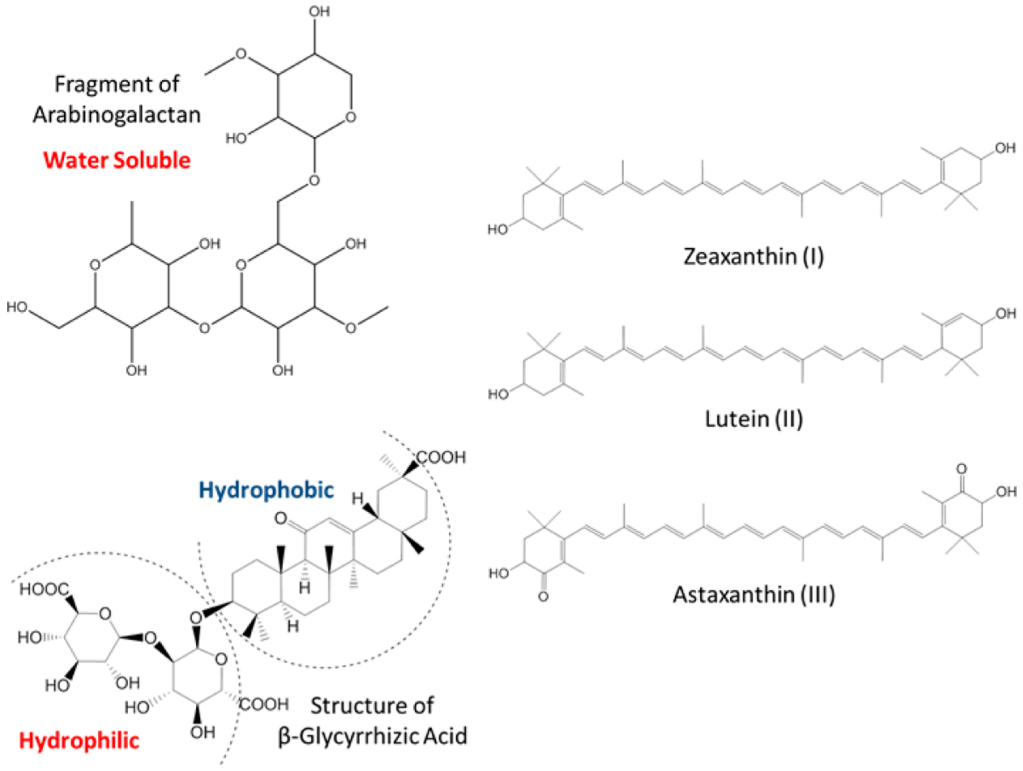

Figure 1. Molecular structures of xanthophyll carotenoids I, II, and III as well as arabinogalactan and β-glycyrrhizic acid.

which contain oxygenated substituents. Hydroxyl groups at each end of the molecule provide their unique biochemical properties. These hydroxyl groups are responsible for the carotenoids' characteristics which allow them to orient within cell membranes in ways other carotenoids cannot.[25−28] The present study is devoted to three representatives of xanthophyll carotenoids, lutein, zeaxanthin, and astaxanthin (Figure 1), which play a special role in the prevention and treatment of visual diseases. These carotenoids are not produced by the human body and must be consumed in the diet.

Lutein and zeaxanthin—dipolar, terminally dihydroxylated carotenoids—selectively accumulate at an extremely high concentration in the macula of the primate eye retina through the action of specific high-affinity binding proteins[29] from blood plasma, where more than 20 other carotenoids are available.[30,31] These two carotenoids can impede the onset of age-related macular degeneration[32,33] and have been recently added to the list of potentially beneficial nutrients provided by leafy greens. It is suggested that the role of lutein and zeaxanthin is blue light filtration and antioxidant function.[34,35] It has been shown that the high-energy, blue wavelengths of visible light are 100 times more effective at inducing free radical formation in the cells of the retina than the low-energy, red wavelengths of visible light.[36] Reacting as antioxidants with free radicals and reactive oxygen species, macular xanthophylls protect the retina against peroxidation and photodamage.[2−6] The ability of macular xanthophylls to quench singlet oxygen and triplet states of photoactive molecules is especially significant.

Astaxanthin, a carotenoid similar to zeaxanthin with a keto group in addition to the OH on the terminal cyclohexene rings, has the highest antioxidant activity among natural carotenoids. It is 10 times more effective an antioxidant than β-carotene, and >100 times more effective than vitamin E.[37−39] The two adjacent oxygen atoms on the cyclohexene ring permit formation of stable complexes with metal ions such as Ca^{2+}, Fe^{2+}, and Zn^{2+}.[40] The unusual metal complexing ability may be an important feature of astaxanthin in some organisms. For example, in some unicellular green algae, astaxanthin accumulates in huge amounts (up to 30 mg/g) under high light conditions, often in the presence of excess metal ions. This accumulation is generally thought to be a survival strategy of the algae under photooxidative and salt stress.[41] Some studies indicate also the benefit of astaxanthin for vision. In particular, it is capable in vitro of protecting porcine lens proteins from oxidative insult and degradation by calcium-induced calpain.[42] Also it is known that UVB exposure induces cell death and thinning of the corneal epithelium. However, the epithelium was morphologically well preserved after irradiation in astaxanthin-treated corneas.[43] Irradiated corneal epithelium was significantly thicker in eyes treated with astaxanthin eye drops, in a dose dependent manner. So, topical astaxanthin administration may be a candidate treatment to limit the damage by UV irradiation with wide clinical applications.

The important feature of xanthophyll carotenoids is their ability to form self-assemblies in aqueous media and even in lipid membranes.[28,44−47] Molecular self-assembly in biological systems attracts considerable attention, since it is important for the functioning of living organisms. It is well-known that carotenoids form aggregates when dissolved in hydrated polar solvents and that this aggregation is characterized by dramatic changes in their absorption spectra and photophysical properties. Two types of carotenoid aggregates can be distinguished according to their absorption spectra. The first type, associated with a large blue shift of the absorption spectrum and loss of vibrational structure of the S_2 excited state, is suggested to take the form of H-aggregates, in which the molecules are stacked with the conjugated chains oriented more or less parallel to

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each other and closely packed. The blue shift of the absorption spectrum is explained in terms of excitonic interaction between the closely packed carotenoid chromophores.[45−47] The second aggregation type, characterized by a red shift of the absorption spectrum where the resolution of vibrational bands is preserved, is attributed to J-type aggregation, in which there is a more head-to-tail organization of the conjugated chains. Self-assembly of xanthophyll carotenoids leads to new photophysical properties not available to the monomer. The photophysics that emerge upon coupling of carotenoid chromophores have an impact on various applications, in particular, to solar energy conversion.[48] One photophysical mechanism that generally requires the proximity of two chromophores is singlet fission. In this mechanism, a chromophore is photoexcited to its singlet excited state and subsequently partitions its energy over two neighboring chromophores both remaining in triplet excited states. The chromophore of zeaxanthin is favorable for the production of triplet excited states via fission, and a high yield of triplet excited states via singlet fission was found for its aggregate.[49]

However no data were found on the chemical properties of carotenoid aggregates. In the present work we have studied the reactivity of carotenoid I−III H-aggregates in some practically important processes, namely, photodegradation and antioxidant activity. In addition, the ability of water-soluble supramolecular complexes of these carotenoids with glycyrrhizic acid and arabinogalactan to form H-aggregates was investigated. A strong influence of complexation on the reactivity of carotenoid monomers and aggregates was found.

2. EXPERIMENTAL SECTION

Astaxanthin was purchased from Sigma (97%) and lutein (90%) from Kemin; zeaxanthin (90%) is from Kalsec, and its purity was checked via HPLC. At 1 mM, the maximum xanthophyll concentration used in these studies, the trace impurities were so low (0.05 mM) that they their effect was below detectability. All carotenoids were stored at −14 °C in a desiccator. Synthesis and properties of *n*-octanoic acid diester of astaxanthin is described in ref 50. The purity was checked by ^{1}H NMR spectroscopy (360 MHz, $CDCl_3$) and TLC. Glycyrrhizic acid is extracted from the Ural licorice root. For the methods of GA purification see ref 51. AG is extracted from *Larix sibirica*.[22−24] All complexes were prepared by standard liquid phase method, although a mechanicochemical method[17] has been used when high (∼5 mM) concentrations of carotenoids are required in AG. Complexes with GA were prepared by dissolving carotenoids and GA in ethanol or DMSO, and then water was added in the required proportion. Complexes with AG were prepared by mixing an ethanol solution of carotenoid with a water solution of polysaccharide in the required proportion resulting in the xanthophyll being located in the polymer matrix as part of a supramolecule.

The stoichiometry and stability of complexes were measured by conventional methods[15,52,53] and were detected by changes in the absorption spectra of carotenoids as a function of GA concentration. Absorption spectra were recorded using a Shimadzu UV−visible 1601 spectrophotometer. All measurements were performed in a 10 mm path length quartz cuvette at 293 K.

The antioxidant activity of carotenoids and their complexes was studied by the previously developed EPR spin-trapping technique.[54−56] Peroxide radicals were generated by the Fenton reaction with excess hydrogen peroxide in DMSO (99.9+%, Aldrich, ACS). The spin adduct of OOH radical with spin trap *N-tert*-butyl-α-phenylnitrone PBN (Aldrich) was recorded using a Bruker ELEXYS E540 CW X-band (9.7 GHz) EPR spectrometer at room temperature.

3. RESULTS AND DISCUSSION

3.1. Evidence of Complex Formation of Xanthophyll Carotenoids with the Triterpene Glycoside Glycyrrhizic Acid. Earlier it was found that the inclusion complexes of lipophilic carotenoid molecules formed from water-soluble oligosaccharides or polysaccharides result in significant changes in the optical (absorption wavelength and fluorescence intensity) and chemical properties of the carotenoids.[14,17−19] In the present study we used the sensitivity of the absorption wavelength of carotenoids to the surrounding environment to prove formation of the inclusion complex in a water−ethanol mixture and to measure its stoichiometry and stability constant. However it was found that even at micromolar (0.5−10 μM) concentrations the aggregation of lutein and zeaxanthin is very fast and occurs immediately after addition of water into ethanol solutions of the carotenoids. Note that these concentrations correspond to real lutein and zeaxanthin concentrations in serum and macular tissue (0.4−0.7 μM).[57] This is why we have measured the parameters of the supramolecular complexes only for astaxanthin, which shows slow aggregation over several minutes with gradual decrease of the monomer peak at 460 nm and growth of the H-aggregate at 380 nm in the absorption spectrum. It was demonstrated also that the ability to form molecular aggregates is the specific feature of OH containing carotenoids. The substitution of OH groups of astaxanthin to ester groups inhibits aggregation completely (Figure 2).

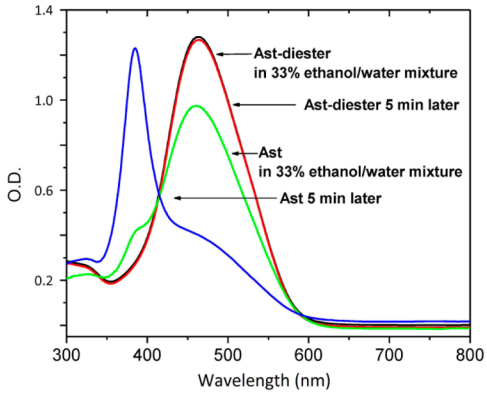

Figure 2. Optical absorption spectra of astaxanthin (7.5 μM) and its *n*-octanoic acid ester (10 μM) in 33% ethanol/water mixture immediately after mixing and after 5 min delay.

An interesting feature of the water−ethanol mixture is the dependence of the aggregation rate on the water/ethanol ratio. In 25% ethanol, aggregation of astaxanthin occurs much slower than in 33%. Figure 3 shows the Benesi−Hildebrand plot of the shift of absorption maximum of astaxanthin as a function of GA concentration in 25% ethanol where the monomer is stable for at least 10 min. The linear dependence of $1/\Delta\lambda$ vs $1/[GA]^n$ was observed only for $n = 2$. This means that astaxanthin forms a noncovalent supramolecular complex with the dimer of GA. It is evident also that the structure of the complex is different for low (<1 mM) and high (>1 mM) GA concentrations. (See Figure 3.) The same result was obtained earlier for GA complexes of the carotenoid canthaxanthin and some drug

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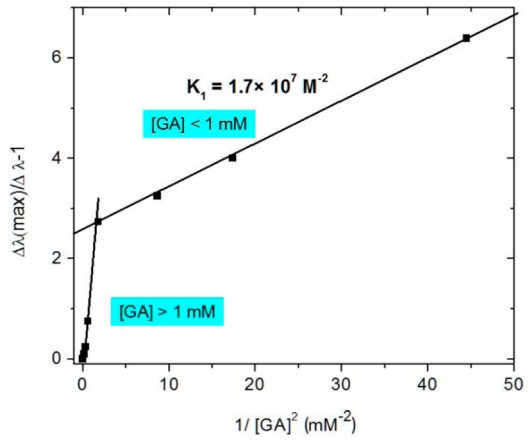

Figure 3. Benesi–Hildebrand diagram of the shift of absorption maximum of astaxanthin as a function of GA concentration in 25% ethanol.

molecules.[15,52,53,58] From the slope of the concentration dependence, the stability constant of this complex for low concentration was calculated as $k_2 = 1.7 \times 10^7 \pm 5 \times 10^5$ M^{-2}.

Figure 3 also confirms the previous conclusion that the complex stability decreases with the growth of GA concentration. It was demonstrated by various physical methods that, at concentrations higher than 1 mM, GA forms micelle-like aggregates.[58,59]

For the other two carotenoids, lutein and zeaxanthin, the absorption spectrum also changes in the presence of GA in a concentration and time dependent manner. Figure 4 shows an

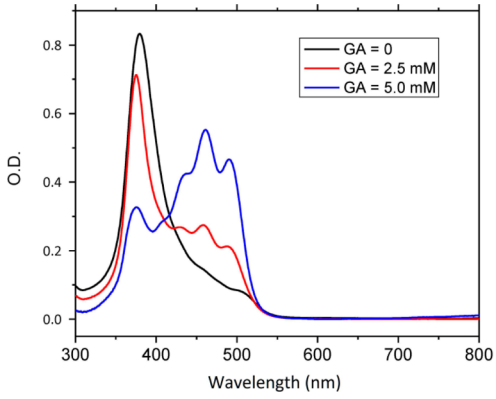

Figure 4. Optical absorption spectra of lutein (≈6 μM)in 25% ethanol/water mixture at different GA concentrations.

example of the change in the absorption spectrum of lutein in the presence of GA at 2.5 and 5 mM in a 25% ethanol solution. Increasing the GA concentration shifts the equilibrium from the H-aggregate (λ_{max} = 380 nm) to the monomer (λ_{max} = 460 nm). Note that, similarly to the astaxanthin complex, complexation of lutein with GA results in a red shift of the monomer absorption maximum relative to a pure ethanol solution (addition of water to ethanol results in a small blue shift).

Figure 5 shows the molecular structure (a) and optical spectrum (c) of the zeaxanthin H-aggregate in aqueous solution and the structure of the zeaxanthin monomer (b) as it resides in the hydrophobic environment of the GA dimer along with its optical spectrum (d). The large shift from 380 nm with the loss of vibrational structure of the S_2 excited state to 450 nm and the appearance of the vibrational bands indicates the presence of the zeaxanthin monomers.

From these experiments we can conclude that xanthophyll aggregation is a reversible process, and complexation with GA prevents aggregation.

3.2. Complexation with AG Increases Photostability of Both Xanthophyll Monomers and Their H-Aggregates. Although carotenoids are known as effective photoprotectors of living cells, in aerated aqueous solution they are unstable under light irradiation and would be unacceptable as colorants and antioxidants in foods. The reason for this is that the UV irradiation of carotenoid solutions results in a decrease of the absorption intensity with formation of reaction products which absorb light at a lower wavelength. This effect can be explained by a decrease in the length of the conjugated chain due to the addition of oxygen to the double bonds. It is known that under irradiation carotenoids can form radical cations in various media by electron transfer to the solvent molecules or to the appropriate electron acceptor. These radical cations can be reduced by back electron transfer. However this reversible process is disrupted in the presence of water molecules, which act as a proton acceptor. It results in the formation of carotenoid neutral radical, which is stabilized by the nearby proton acceptor, so a reversible electron transfer is prevented. Earlier it was demonstrated that carotenoid neutral radicals are formed from the corresponding radical cations generated electrochemically or photochemically by proton loss.[60] Although photoexcitation accelerates deprotonation of the radical cation, electrochemical measurements showed that the radical cations of a majority of carotenoids have pK values ranging between 4 and 7 and, therefore, can deprotonate spontaneously.[61,62] For symmetrical zeaxanthin radical cation,[63] proton loss occurs most favorably at the C4(4′) carbon position with loss at C5, C9, and C13 methyl less favored to form a neutral radical. For the unsymmetrical isomer lutein, the proton loss is greatly favored only at the 4 carbon position over any other position.[64] For the astaxanthin radical cation, deprotonation occurred at the C3(3′) carbon position, resulting in the lowest energy neutral radical, while proton loss at the C5, C9, or C13 methyl groups was less favored.[40] We have shown that incorporation of carotenoids into the hydrophobic polymer environment or GA micelle will reduce their interaction with water molecules.[17] Also, the decrease of electron transfer rate with electron acceptors was established.[15,17] During irradiation of aqueous solutions of the monomer carotenoids I–III by the full light of a xenon lamp, bleaching of the solutions occurs in a few seconds. On the other hand H-aggregates demonstrate a reduced degradation rate by about a factor of 10. In the present study we have investigated how the complexation with GA and AG affects monomer and H-aggregate stability.

It was found that GA does not affect the photostability of carotenoids I–III. However, the significant increase (5–10 times for full light irradiation and 2 times for λ_{irr} > 380 nm) in photostability was detected for AG complexes of these carotenoids. Moreover, the increase in photostability was detected for both monomer and H-aggregate of these xanthophylls. This means that AG can form inclusion complexes with monomers as well as with aggregates of carotenoids. As an example, Figure 6 demonstrates the difference in the photodegradation rate of astaxanthin monomers in the absence (a) and in the presence (b) of 0.4 mM arabinogalactan.

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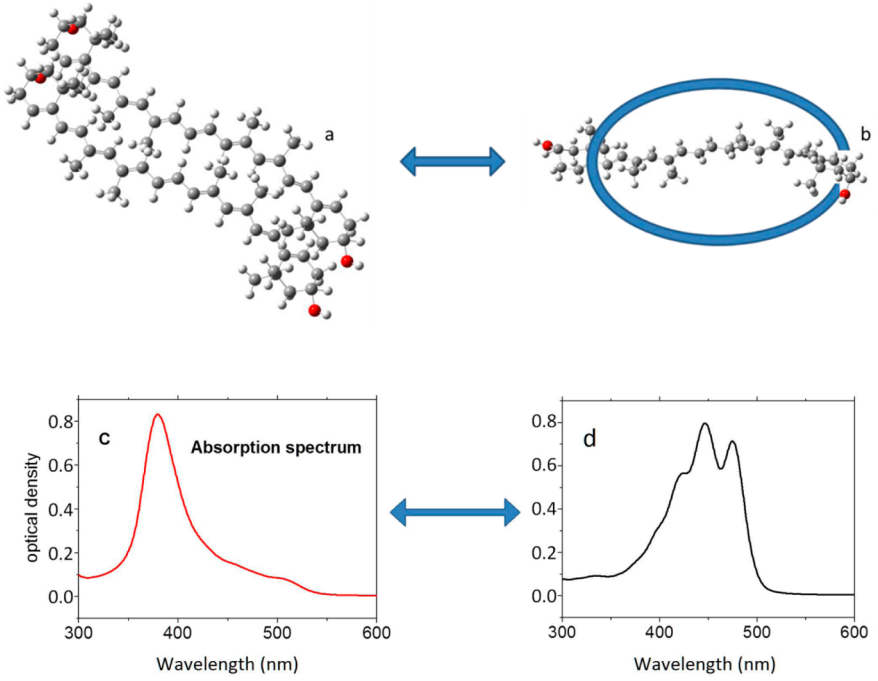

Figure 5. The structure of the H-aggregate (a) and monomer complexed with GA (b) of zeaxanthin. Also shown is the absorption spectrum of the H-aggregate (c) in aqueous solution and the zeaxanthin monomer (d) in the hydrophobic environment of the triterpene glycoside glycyrrhizic acid dimer. GA forms a head to tail donut hole dimer in which the zeaxanthin monomer can reside.

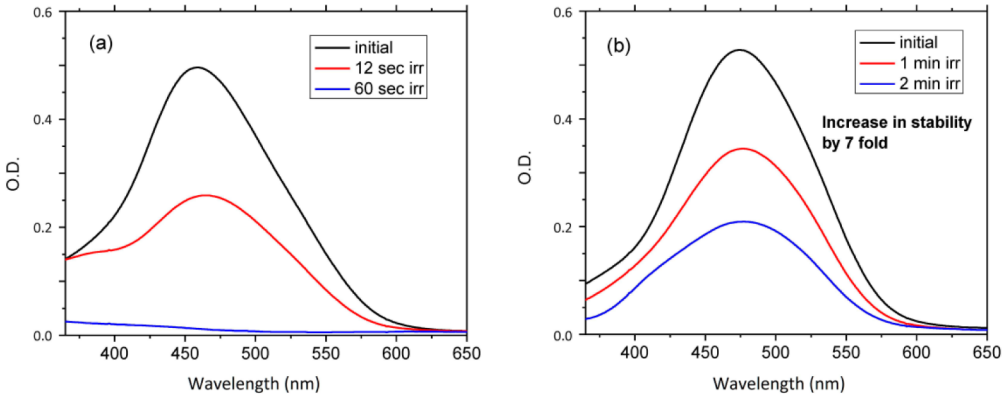

Figure 6. Photodegradation of astaxanthin monomers in 25% ethanol/water solution which forms H-aggregates. Optical absorption spectra of astaxanthin (≈4 μM) in the absence (a) and in the presence (b) of 0.4 mM arabinogalactan before and after irradiation by the full light of a xenon lamp.

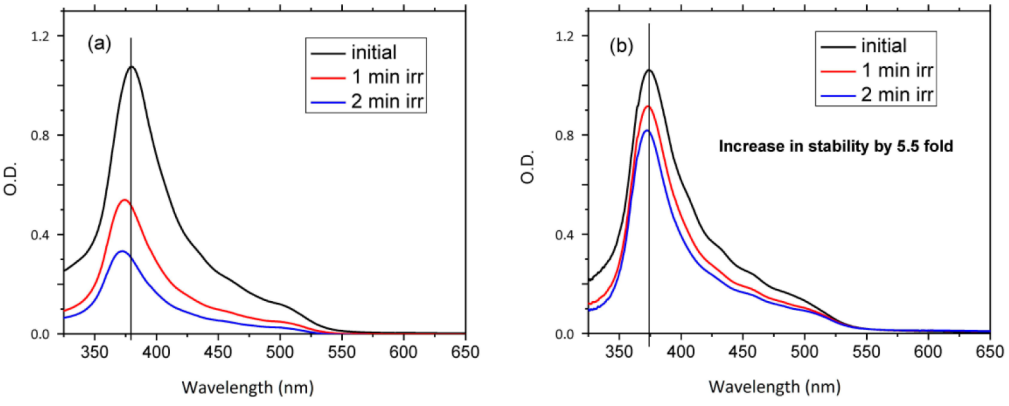

Figure 7. Photodegradation of lutein H-aggregates in 25% ethanol/water solution. Optical absorption spectra of lutein (≈8 μM) in the absence (a) and in the presence (b) of 0.4 mM arabinogalactan before and after irradiation by the full light of a xenon lamp.

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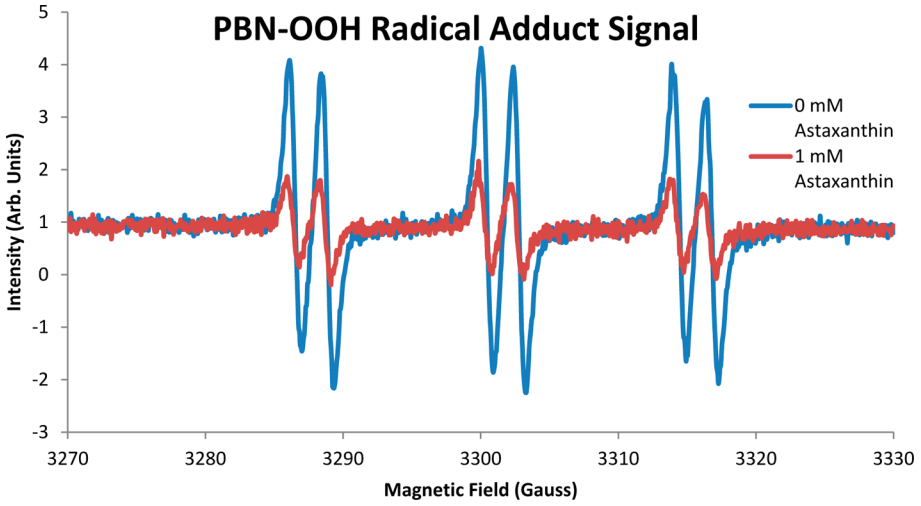

Figure 8. EPR signal of PBN−OOH radical spin adduct measured in the absence (blue) of astaxanthin and the presence (red) of 1 mM astaxanthin. The competing reactions of the carotenoid and the spin trap PBN lead to a decrease in the PBN−OOH radical spin adduct signal with increasing concentrations of carotenoids.

An increase in the photostability of H-aggregates was detected for all carotenoids I−III. As an example, Figure 7 shows the difference in photodegradation rate of lutein H-aggregates in the absence (a) and in the presence (b) of 0.4 mM arabinogalactan.

3.3. EPR Spin Trapping Measurements of the Scavenging Rate toward Peroxyl Radicals. Earlier we have developed an EPR spin-trapping technique for measurement of the relative scavenging rates toward peroxyl radicals by different carotenoids.[54,55] This method involves measuring the yield of stable spin adduct (SA) of OOH radicals with the spin trap PBN in the presence of carotenoids. It was found that as the oxidation potential of the carotenoids increased, the radical scavenging by proton abstraction from the C4 position of the cyclohexene terminal ring increased. This is consistent with the increasing acidity of the C4 proton with increasing electron accepting character of the substituents. As an example, Figure 8 shows EPR spectra of PBN−OOH radicals in the absence of astaxanthin and in the presence of 1 mM of astaxanthin.

Because of the two competing processes, i.e., reactions of the radical with carotenoid and the spin trap, the yield of the spin adduct is proportional to the carotenoid concentration. Thus, from the experimental dependence of the spin adduct yield on carotenoid concentration, the relative rate of radical scavenging (k_{Car}/k_{ST}) by carotenoid can be assessed (eq 1).[54]

$$\frac{A_0}{A} = \frac{k_{ST}[ST] + k_{Car}[Car]}{k_{ST}[ST]} \quad (1)$$

Here, k_{ST} is the rate constant of radical scavenging by the spin trap (ST), and A and A_0 are the values of the spin adduct signal intensity with and without carotenoid. For $k_{Car} \geq k_{ST}$ only an increase in radical scavenging by the carotenoid occurs (antioxidant effect). The available kinetic database (Spin Trap Data Base: http://epr.niehs.nih.gov) provides the value of the rate constant k_{ST} measured in water ($k_{ST} \leq 10^6$ M^{-1} s^{-1} for the PBN spin trap). The identification of the PBN−OOH radical spin adduct was made in the previous papers[54,55] using available literature data of the a(H) and a(N) splitting for the OOH, CH_3, and OCH_3 radicals. In addition to this assignment, some test experiments were carried out to prove our conclusion. In anaerobic conditions the peroxyl radicals were generated in DMSO via the well-known Fenton reaction (eqs 2−4).[65]

$$Fe^{2+} + H_2O_2 \rightleftharpoons Fe^{3+} + OH^{\bullet} + OH^{-} \quad (2)$$

$$OH^{\bullet} + DMSO \rightarrow CH_3^{\bullet} + CH_3(OH)SO \quad (3)$$

$$CH_3^{\bullet} + H_2O_2 \rightarrow OOH^{\bullet} + CH_4 \quad (4)$$

At low H_2O_2 concentration ($[H_2O_2] \sim [FeCl_2]$ = 1 mM) only one spin adduct PBN−CH_3 was detected with ESR parameters a(H) = 3.4 G and a(N) = 14.9 G.[54] However, at higher H_2O_2 concentration ($[H_2O_2]$ = 500 mM) the reaction of CH_3 radicals with H_2O_2 becomes important and this results in the disappearance of the PBN−CH_3 adduct, and the appearance of another adduct with higher yield which was assigned to the PBN−OOH spin adduct (a(H) = 2.3 G and a(N) = 13.9 G).[54] It is known that the $^{\bullet}$OOR spin adducts are relatively unstable especially in the presence of transition metal ions which can reduce $^{\bullet}$OOR radicals yielding the $^{\bullet}$OR spin adduct.[66,67] However, these facts are mainly related to alkyl peroxyl radicals. In our experimental conditions, with increasing H_2O_2 concentration the Fe^{2+} ions react primarily with the initial H_2O_2 and consequently the stability of the $^{\bullet}$OOH spin adduct will increase. Note that there are several examples in the literature of the observation of the PBN−OOH adducts at normal conditions.[68−70] For additional confirmation of the PBN−OOH adduct formation the superoxide dismutase (SOD) test has been made.[15] The formation of all these radical species (OOH, CH_3, and OH radicals) was also confirmed by using DMPO spin trap;[54] however, the measurements of scavenging rates by carotenoids with this spin trap have shown significant problems. First, DMPO−OOH adduct is unstable and transforms to DMPO−OH adduct via reaction with Fe^{2+}.[71] Second, due to higher spin trapping rates of DMPO compared with some carotenoids, we have detected pro-oxidant effect instead of antioxidant in this reaction.[55] This is why PBN was chosen for kinetic measurements in the previous and present studies.

In the present work, using the same approach, we have compared the reaction rates of the OOH radical with monomers in DMSO solution and H-aggregates of carotenoids

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I and II in an aqueous DMSO solution. The formation of aggregates under these experimental conditions was confirmed by optical absorption measurement. It was found that astaxanthin does not form aggregates in aqueous DMSO. In addition, the influence of glycyrrhizic acid on the scavenging ability of lutein and zeaxanthin has been investigated in an aqueous DMSO solution. Figure 9 shows the time dependence of the PBN−OOH spin adduct EPR intensity in the absence of carotenoids, and in the presence of 1 mM lutein and zeaxanthin monomers.

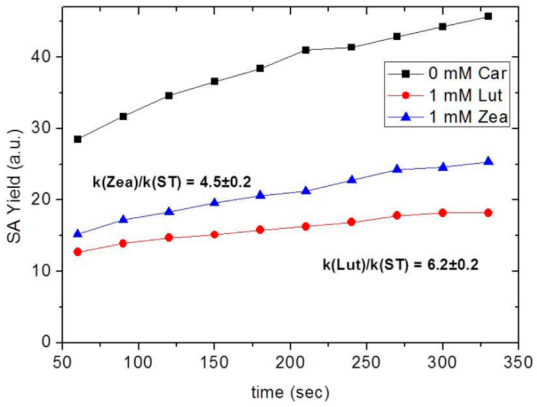

Figure 9. PBN−OOH SA yield in EPR spin trapping experiment at different times after the start of the reaction in DMSO solution: PBN = 5 mM, Fe^{2+} = 1 mM, H_2O_2 = 500 mM, Car = 1 mM.

The initial yield of spin adduct corresponds to the free radicals produced in the Fenton reaction (k = 76 M^{-1} s^{-1}), but the further slow growth is due to reaction of excess hydrogen peroxide with the reaction product (eqs 5 and 6), Fe^{3+} ($k \sim$ 0.02 M^{-1} s^{-1}).[72,73]

$$Fe^{3+} + H_2O_2 \rightleftharpoons FeOOH^{2+} + H^+ \quad (5)$$

$$FeOOH^{2+} \xrightarrow[\text{slow}]{} Fe^{2+} + OOH^{\bullet} \quad (6)$$

The significant decrease of SA yield in the presence of carotenoids is due to the reaction of these carotenoids with $^{\bullet}OOH$. The relative scavenging rates calculated for the initial SA concentrations are equal to 4.5 ± 0.2 for zeaxanthin and 6.2 ± 0.2 for lutein. These rates also reflect the different preferred proton abstraction locations of the isomers. These values were slightly changed with time due to secondary reactions of carotenoids with Fe^{3+}[55] and carotenoid metabolites with free radicals. In this study we did not analyze these reactions.

As it was shown earlier, at equal concentrations, $[H_2O_2]$ = $[FeCl_2]$ = 1 mM, only the spin adduct PBN−CH_3 was detected by EPR.[54] In these experimental conditions, the SA signal was stable over 5 min, and no growth was observed as in the case of excess H_2O_2. However, the SA yield decreases in the presence of carotenoids I and II in a concentration dependent manner. The calculated relative scavenging rates toward methyl radicals are 0.7 ± 0.2 for zeaxanthin and 1.2 ± 0.2 for lutein. Taking into account the known rate constant for the reaction PBN with CH_3 radical ($k_{ST} \sim 10^7$ M^{-1} s^{-1})[74] one can estimate the absolute rate constant for trapping C-centered radicals by lutein and zeaxanthin ($k \sim 10^7$ M^{-1} s^{-1}).

Figure 10a shows that the formation of H-aggregates in 80% DMSO/water solution significantly reduces the scavenging ability of these xanthophyll carotenoids. Moreover, the increase of SA yield (pro-oxidant effect!) was observed in the presence of zeaxanthin aggregates as indicated by the negative value of −0.4 ± 0.2 for the relative ratio of k_{Zea}/k_{ST} as deduced from eq 1. This occurs when $(A_0/A - 1)$ is less than 1. Earlier[55] it was demonstrated that a pro-oxidant effect of carotenoids (an increase in the amount of free radicals formed) arises from the reduction of Fe^{3+} to Fe^{2+} by the carotenoids according to the reaction shown in eq 7, which then proceeds back to eqs 2−4:[55]

$$Car + Fe^{3+} \rightarrow Car^{\bullet+} + Fe^{2+} \quad (7)$$

This effect increases with decreasing oxidation potential of the carotenoids, with increasing H_2O_2 concentration, or with a decrease in the scavenging rate of the carotenoid. Another condition is that if $k_{Car} \geq k_{ST}$, then no pro-oxidant effect occurs. However, if there occurs any decay channel for free radicals, this enhances the pro-oxidant effect of carotenoids, such as reactions with lipids. For β-carotene,[55] k_{Car}/k_{ST} = 0.65. which is a condition where the pro-oxidant effect is possible. β-Carotene exhibits a low oxidation potential, measured to be the same as zeaxanthin.[61] This then provides a reason for observing a pro-oxidant effect for zeaxanthin aggregates. On the other hand, there is no pro-oxidant effect observed from lutein aggregates, indicating a greater scavenging rate (k_{Lut}/k_{ST} = 0.5 ± 0.2) than zeaxanthin aggregates. This may be due to the greater proton donating rate of the unsymmetrical lutein aggregates compared to the symmetrical zeaxanthin aggregates.

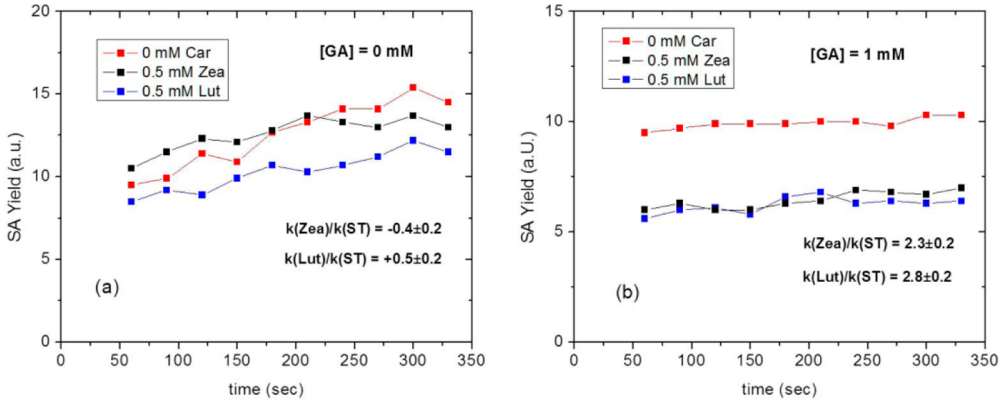

Figure 10. PBN−OOH SA yield in the EPR spin trapping experiment where H-aggregates are formed at different moments after the start of the reaction in 80% DMSO/water solution: PBN = 2 mM, Fe^{2+} = 0.5 mM, H_2O_2 = 500 mM, (a) in the absence of GA and (b) with 1 mM GA.

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We can conclude from the EPR experiments that H-aggregates demonstrate much lower spin trapping ability (Figure 10a). The reaction of zeaxanthin with Fe^{3+} results in additional radical production at the beginning of the reaction (pro-oxidant effect), but then this reaction reduces the efficiency of Fe(III) + H_2O_2→ Fe(II) + OOH reaction, which becomes the major way for free radical formation. Glycyrrhizic acid does not affect the yield of spin adduct in the absence of carotenoids (Figure 10b) but increases significantly the carotenoid spin trapping ability: k_{Lut}/k_{ST} = 2.8 ± 0.2 and k_{Zea}/k_{ST} = 2.3 ± 0.2. We suppose that the effect of the GA dimer is due to the reduced aggregation rate of the carotenoids, as well as the oxidation of carotenoids by Fe(III) ions and by increasing the available location of the C4 proton of the carotenoid for H abstraction by $OOH^{\bullet}$. The GA dimer also shields the carotenoid polyene chain from reaction with Fe(III) ions. The latter process was investigated by optical absorption methods in this study.

3.4. Optical Absorption Study of the Reaction Kinetics of Monomers and H-Aggregates with Fe^{3+} Ions and OOH Radicals. In order to explore the mechanism of antioxidant vs pro-oxidant activity of the xanthophyll carotenoids in more detail, the decay kinetics of carotenoids lutein and zeaxanthin in the presence of Fe^{3+} ions and H_2O_2 were analyzed using optical absorption spectroscopy.

In this study we consider two main reaction ways (eqs 8 and 9) which result in decrease of carotenoid absorption intensity, namely, reaction of carotenoid with Fe^{3+} ion and with OOH radical.

$$\text{Car} + Fe^{3+} \xrightarrow{k_1^*} \text{Car}^{\bullet+} + Fe^{2+} \quad (8)$$

$$\text{Car} + OOH^{\bullet} \xrightarrow{k_2^*} \text{Products} \quad (9)$$

As it was shown in our previous study, the first reaction can play a key role in pro-oxidant activity of some carotenoids.[55] Hydroperoxyl radicals in this reaction system are formed via interaction of hydrogen peroxide with Fe^{3+} and Fe^{2+} ions, as described earlier. The measurements were made with $Fe(ClO_4)_3$ in the absence and in the presence of hydrogen peroxide in DMSO aqueous solutions: with 5% water to measure monomers (470 nm) and with 25% of water to form H-aggregates (380 nm). The decay kinetics were measured over 10 min, and the decay rates were calculated from the initial slopes (see Figure 11 as an example). The values k_1 and k_2 are related to the reaction of H-aggregates with Fe^{3+} ion and OOH radical, and the values k_{1a} and k_{2a} are related to the reactions of carotenoid monomer with these species. Note that the calculated decay rates of absorption intensity are not equal to the corresponding reaction rates k_1* and k_2* of carotenoid with Fe^{3+} ions and OOH radicals, since the reaction products can absorb light at the same wavelength as the initial carotenoid. These decay rates might be considered as the lower limit of the reaction rates.

One can see a 3-fold decrease of the zeaxanthin decay rate with Fe^{3+} ions (k_1 and k_{1a}), and complete inhibition of the reaction with OOH radical for H-aggregates (k_2) as compared with carotenoid monomers (k_{2a}). Decay rates were estimated using stationary radical concentration [OOH] ≈ 1 nM calculated from known reaction rates of the main reaction processes:[72,73] $k(Fe^{3+} + H_2O_2) = 0.02\ M^{-1}\ s^{-1}$; $k(Fe^{3+} + OOH) = 2 \times 10^5\ M^{-1}\ s^{-1}$. We can conclude from these measurements that the pro-oxidant effect of zeaxanthin H-aggregates can arise from the reaction with Fe^{3+} ions under suppression of its ability to trap free radicals.

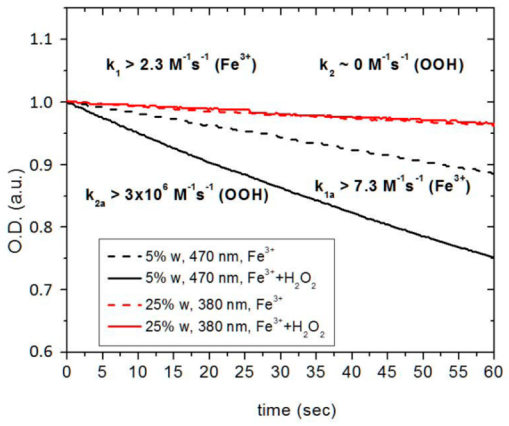

Figure 11. Decay kinetics of zeaxanthin detected at absorption maximum of the monomer at 470 nm and the H-aggregate at 380 nm in 5% and 25% water/DMSO solutions respectively: Fe^{3+} = 0.25 mM, H_2O_2 = 125 mM.

We have obtained the same decay rate values for the lutein monomer ($k_{2a} \geq 2.5 \times 10^6\ M^{-1}\ s^{-1}$), but only a factor of 5 times decrease in the decay rate with $OOH^{\bullet}$ for H-aggregates. This may be a result of the unsymmetrical lutein forming a different and more open H-aggregate structure than the symmetrical zeaxanthin (Figure 5) resulting in a greater proton donation rate. It is noticeable that very close reaction rates were obtained from EPR spin trapping and optical absorption experiments.

CONCLUSION

Formation of H-aggregates is an exclusive property of carotenoids which contain OH groups in a cyclohexene ring, as substitution of an OH with an OR group prevents the aggregation. Formation of H-aggregates significantly changes the optical and chemical properties of these carotenoids, in particular their light harvesting ability and antioxidant activity. Complexes of these carotenoids with GA demonstrate higher antioxidant activity in aqueous solution. Reactions of the xanthophyll carotenoid inclusion complexes with OOH radicals occur by proton loss from the most acidic proton of the carotenoid which is exposed to the environment when complexed with the donut hole GA complex as depicted in the graphical abstract. In the presence of water, lutein shows much lower antioxidant activity due to formation of H-aggregates, demonstrated by EPR spin trapping experiments using the model Fenton reaction. Zeaxanthin shows a pro-oxidant effect in the same conditions. Taking into account the important role of these carotenoids in eye and skin health, glycyrrhizin and arabinogalactan can be considered as perspective delivery systems which provide enhanced stability and solubility of xanthophyll carotenoids.

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Notes

The authors declare no competing financial interest.

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