Antioxidant Activities of Astaxanthin and Related Carotenoids

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The antioxidant activities of astaxanthin and related carotenoids have been measured by employing a newly developed fluorometric assay. This assay is based on 4,4-difluoro-3,5-bis(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene (BODIPY 665/676) as an indicator; 2,2′-azobis-2,4-dimethylvaleronitrile (AMVN) as a peroxyl radical generator; and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) as a calibrator in an organic and liposomal media. By employing this assay, three categories of carotenoids were examined: namely, the hydrocarbon carotenoids lycopene, α-carotene, and *β*-carotene; the hydroxy carotenoid lutein; and the α-hydroxy-ketocarotenoid astaxanthin. The relative peroxyl radical scavenging activities of Trolox, astaxanthin, α-tocopherol, lycopene, *β*-carotene, lutein, and α-carotene in octane/butyronitrile (9:1, v/v) were determined to be 1.0, 1.0, 1.3, 0.5, 0.4, 0.3, and 0.2, respectively. In dioleoylphosphatidyl choline (DOPC) liposomal suspension in Tri-HCl buffer (pH 7.4 at 40 °C), the relative reactivities of astaxanthin, *β*-carotene, α-tocopherol, and lutein were found to be 1.00, 0.9, 0.6, and 0.6, respectively. When BODIPY 665/676 was replaced by 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (BODIPY 581/591 C_{11}) as an indicator, astaxanthin showed the highest antioxidant activity toward peroxyl radicals. The relative reactivities of Trolox, astaxanthin, α-tocopherol, α-carotene, lutein, *β*-carotene, and lycopene were determined to be 1.0, 1.3, 0.9, 0.5, 0.4, 0.2, and 0.4, respectively.

Keywords: *Antioxidants; fluorescence; carotenoids; astaxanthin; α-tocopherol, liposomes*

INTRODUCTION

There is currently considerable interest in the role of carotenoids in delaying or preventing degenerative diseases such as atherosclerosis, cancer, aging (Halliwell, 1997; Rice-Evans and Burdon, 1994; Mathews-Roth, 1991) and eye diseases (Pratt, 1999; Kirschfeld, 1982). A number of studies have shown that carotenoids act as antioxidants by quenching singlet oxygen and free radicals (Tsuchiya et al., 1992; Palozza and Krinsky, 1992a).

Although earlier research on antioxidant activity of carotenoids was focused on *β*-carotene, other carotenoids have recently been shown to be more effective antioxidants than *β*-carotene (Tanaka et al., 1995). The red carotenoid astaxanthin (3,3′-dihydroxy-*β*,*β*-carotene-4,4′-dione) attracted considerable interest because of its potent antioxidant activity and also because of its economic value as a pigment source in the aquaculture and food industries (Johnson, 1995; Kobayashi et al., 1997). The antioxidant activity of astaxanthin has been reported to be 10 times stronger than that of other carotenoids, namely, zeaxanthin, lutein, canthaxanthin, and *β*-carotene (Miki, 1991).

The major carotenoids in human serum are α- and *β*-carotenes, cryptoxanthin, lycopene, zeaxanthin, and lutein. Only lutein and zeaxanthin are found in the human retina in appreciable amounts. It has been suggested that lutein and zeaxanthin are concentrated in the retina because of their ability to cross the blood retinal barrier and their ability to quench singlet oxygen and scavenge free radicals, thus protecting the retina (Tso and Lam, 1996). *β*-Carotene is reported to be relatively inaccessible to the retina because of its inability to cross the blood retinal barrier. Other carotenoids, such as astaxanthin and canthaxanthin, were also found to selectively cross the blood retinal barrier. However, canthaxanthin was reported to cause adverse effects on the retina. Astaxanthin has been associated with reduced risk of diseases such as age-related macular degeneration and ischemic diseases, effects attributed to its potent antioxidant activity (Tso and Lam, 1996).

Several direct and indirect methods for assaying the antioxidant activity of carotenoids in vitro have been developed (Punchard and Kelly, 1996). Almost all of these methods are based on inhibition of free radical-induced oxidation reactions or quenching of stable free radicals by antioxidants. Important considerations in developing an assay to measure peroxyl radical scavenging activity are sensitivity and reliability. Fluorometry is a particularly important analytical technique due to its extreme sensitivity, which is orders of magnitude greater than that of spectrophotometric methods. The widely used fluorometric assay reported in the literature that measures activity of lipophilic antioxidants in a lipid environment is based on the use of lipid-soluble *cis*-parinaric acid as a probe. *cis*-Parinaric acid loses its fluorescence upon interaction with peroxyl radicals and retains its fluorescence in the presence of antioxidants (Kuypers et al., 1987; Packer and Wirtz, 1995). *cis*-Parinaric acid is, however, air-sensitive and photolabile and absorbs in the UV region at 320 nm (Haugland, 1996), where test compounds also absorb. To overcome these problems, the development of a fluorometric assay based on visible-absorbing fluorescent probes that belong to the BODIPY class of dyes to measure antioxidant activities of carotenoids in a lipid environment is presented in this paper.

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MATERIALS AND METHODS

Chemicals. The fluorescent indicators BODIPY 581/591 C_{11} and BODIPY 665/676 were purchased from Molecular Probes (Eugene, OR). 2,2′-Azobis-2,4-dimethylvaleronitrile (AMVN) and 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) were obtained from Wako Chemicals (Richmond, VA). Antioxidants were of high purity and used as received. Astaxanthin (98%, commercial astaxanthin contains an impurity that interacts rapidly with the indicator, resulting in some loss of its initial fluorescence signal), lycopene (90−95%), α-carotene (96%), and β-carotene (95%) were purchased from Sigma (St. Louis, MO). Lutein was obtained from Fluka (St. Louis, MO). α-Tocopherol was obtained from Supelco (Bellefonte, PA). The solvents were of analytical grade and used without further purification.

Fluorometric Assay. Fluorescence measurements were performed in a 1-cm quartz cuvette using a Shimadzu RF1501 spectrofluorometer equipped with a stirrer and a temperature-controlled cell holder. The fluorescence signal of the indicator was monitored over a period of specified time to evaluate its thermal and photochemical stability in the absence of test samples. The fluorescence signal of the indicator decays upon addition of peroxyl radicals, generated at a controlled rate by thermal decomposition of AMVN in air-saturated solutions. The fluorescence decay was monitored to completion. The antioxidant activity of a sample was determined from its ability to retain the fluorescence signal of the indicator in the presence of peroxyl radicals.

All fluorescence measurements have been expressed relative to the reading of the fluorescence signal at 1 min incubation before addition of AMVN for octane/butyronitrile and after 5 min incubation in the presence of AMVN (5.3 mM) for the DOPC system. The fluorescence measurements were recorded every minute at the 600-nm emission wavelength with excitation at 570 nm for BODIPY 581/591 C_{11}, at the 675-nm emission wavelength with excitation at 620 nm for BODIPY 665/676 in octane/butyronitrile (9:1, v/v), and at the 700-nm emission wavelength with excitation at 600 nm for DOPC. The net protection area provided by an antioxidant sample was calculated using the difference between the areas under the curves (AUC) (Naguib, 1998) of the indicator fluorescence decay in the presence of an antioxidant sample (AUC_{Sample}) and the blank, BODIPY/AMVN system without added antioxidants (AUC_{Blank}).

The peroxyl radical scavenging activity of a sample relative to Trolox is given by

$$\text{relative antioxidant activity of a sample} = [(AUC_{Sample} - AUC_{Blank})/(AUC_{Trolox} - AUC_{Blank})] \times [(\text{mol of Trolox/mol of sample})] \quad (1)$$

where $(AUC_{Sample} - AUC_{Blank})$ and $(AUC_{Trolox} - AUC_{Blank})$ are the net protection areas under the curves of (ΔAUC) the indicator fluorescence decay in the presence of a sample and Trolox, respectively.

(ΔAUC) is calculated as follows:

$$\Delta AUC = (f_1/f_1 + f_2/f_1 + f_3/f_1 + \ldots f_t/f_1) \quad (2)$$

where f_1 is the initial reading of the fluorescence signal at 1 min and f_t is the fluorescence signal at time t. Data are expressed as mean ± standard deviation (SD).

The final reaction mixture (3 mL) for the assay contained 1.3×10^{-9} M BODIPY 581/591 C_{11} or 1.1×10^{-7} M BODIPY 665/676 in octane/butyronitrile (9:1, v/v). Solutions containing Trolox in final concentrations of 267 and 105 μM were used as standards for BODIPY 581/591 and BODIPY 665/676, respectively. Appropriate amounts of stock solutions of test samples in chloroform were added to the reaction mixtures. Chloroform (100 μL) was shown to have no effect on the kinetic analysis of the fluorescence decay of the indicators.

In DOPC liposomal medium, the reaction mixture (3 mL) contained 5.3 mM AMVN, 0.8 mM DOPC liposomes, 1.1×10^{-6} M BODIPY 665/676, and an appropriate amount of antioxidant in 20 mM Tris-HCl buffer (pH 7.4).

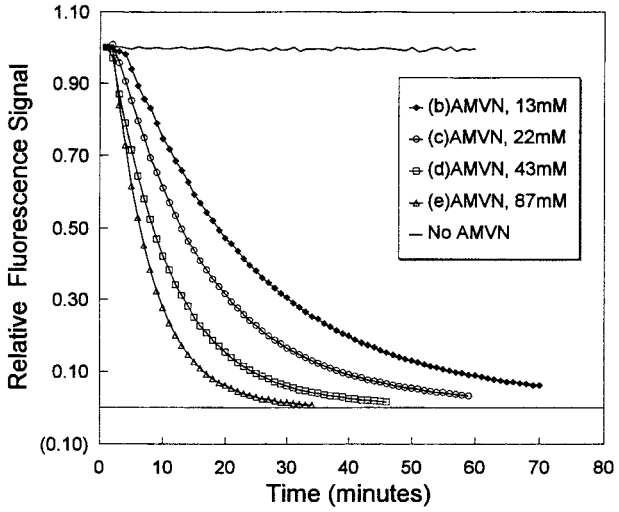

Figure 1. Time course of changes in BODIPY 665/676 fluorescence in octane:butyronitrile (9:1) on incubation at 39 °C: alone (—) or in the presence of varying amounts of added AMVN.

Statistical Analysis. Linear regression analysis of antioxidant activity (y) versus antioxidant concentrations (x) were computed using the regression procedure in Microsoft Excel 97 for Windows. A linear fit ($y = a_0 + a_1x$) adequately described the data as shown by the Pearson correlation coefficient. The a_1 represents the antioxidant activity relative to Trolox.

RESULTS

Figure 1 shows that the fluorescent indicator BODIPY 665/676 in octane:butyronitrile (9:1, v/v) is both thermally and photochemically stable in the absence of peroxyl radicals. In the presence of peroxyl radicals derived from AMVN, the indicator BODIPY 665/676 gradually loses its fluorescence, and the decay rate increases as the concentration of added AMVN increases (Figure 1, curves b−e).

The antioxidant activity of a substance is measured by its ability to retain the fluorescence of the indicator in the presence of AMVN. The net protection of the fluorescence of BODIPY 665/676 provided by an antioxidant sample is calculated using the difference between AUC of the indicator fluorescence decay in the presence and absence of an antioxidant sample (Naguib, 1998). This technique combines both inhibition time and inhibition degree into one parameter, namely, the AUC. Thus, the activity of an antioxidant that lacks a clear plateau region can easily be calculated.

The antioxidant activity of a sample is measured relative to that of Trolox on a molar basis, i.e., the net protection area provided by 1 mol of an antioxidant relative to that provided by 1 mol of Trolox. Table 1 shows that using different concentrations of an antioxidant yields the same antioxidant activity relative to that of Trolox on a molar basis.

Trolox, an analogue of α-tocopherol, was used as a calibrator. Upon addition of Trolox and in the presence of the peroxyl radical generator AMVN, the fluorescence decay of BODIPY 665/676 showed a plateau region followed by a decrease in the fluorescence signal in a manner similar to that of the blank BODIPY 665/676 and AMVN system (Figure 2). The initial plateau phase increased as the concentration of Trolox increased, and a linear correlation was found between $(AUC_{Trolox} -$

Table 1. Peroxyl Radical Scavenging Activities of α-Tocopherol and Astaxanthin[a]

concn (μM)	ΔAUC[b]	relative antioxidant activity[c]
	α-Tocopherol	
55[d]	2.95	1.35
	2.95	1.35
	2.85	1.31
	2.58	1.17
	2.80	1.27
	2.59	1.17
110	5.58	1.28
220	12.0	1.37
	Astaxanthin[e]	
147	6.55	1.02
147	6.50	1.02
153	5.92	0.89
145	5.67	0.98
145	7.17	1.24

[a] BODIPY 665/676 (1.1×10^{-7} M), AMVN (43 mM), and Trolox (105 μM) in octane:butyronitrile (9:1). [b] $\Delta AUC = AUC_{Sample} - AUC_{Blank}$. [c] Relative peroxyl radical scavenging activity was calculated according to eq 1. [d] First three data of 55 μM were obtained in the same day, and the other data were obtained at different days using same stock solution of α-tocopherol. [e] Data were obtained at different days using fresh stock solutions.

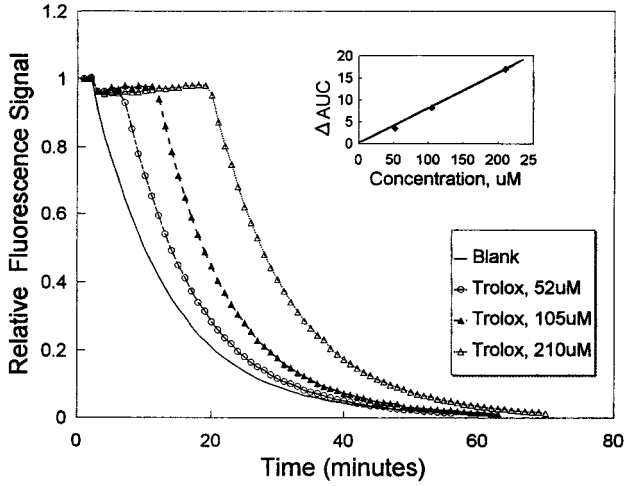

Figure 2. Time course of changes in BODIPY 665/676 fluorescence in octane:butyronitrile (9:1) on incubation at 39 °C with 43 mM AMVN: alone (—) or in the presence of varying amounts of Trolox. Insert is a plot of ΔAUC versus Trolox concentration, multiple correlation coefficient = 0.999. $\Delta AUC = AUC_{Sample} - AUC_{Blank}$.

AUC_{Blank}) values and Trolox concentration, multiple correlation coefficient = 0.99. The antioxidant α-tocopherol also displayed a concentration-dependent plateau region followed by decay kinetics similar to those of the blank. Table 1 summarizes the results of the relative antioxidant activity of α-tocopherol as a function of different concentrations and within same day and from day to day variation. The mean ± SD of the antioxidant activity of α-tocopherol is 1.29 ± 0.08, and the coefficient of variation (CV) is <10%. The mean ± SD for the relative antioxidant activity of astaxanthin is 1.03 ± 0.12 (Table 1). Figure 3 shows the net protection of the fluorescence of BODIPY 665/676 in the presence of AMVN provided by varying amounts of β-carotene; the relative antioxidant activity is 0.43 ± 0.06. Similar decay kinetics were obtained using the carotenoids α-carotene, lutein, and lycopene (Figure 4). The relative peroxyl radical scavenging activities of Trolox, astaxanthin, α-tocopherol, lycopene, β-carotene, lutein, and

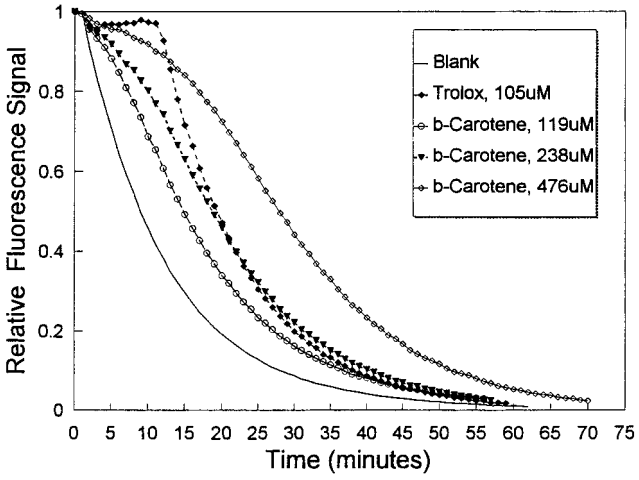

Figure 3. Time course of changes in BODIPY 665/676 fluorescence in octane:butyronitrile (9:1) on incubation at 39 °C with 43 mM AMVN: alone (—), in the presence of 105 μM Trolox (♦), or varying amounts of β-carotene.

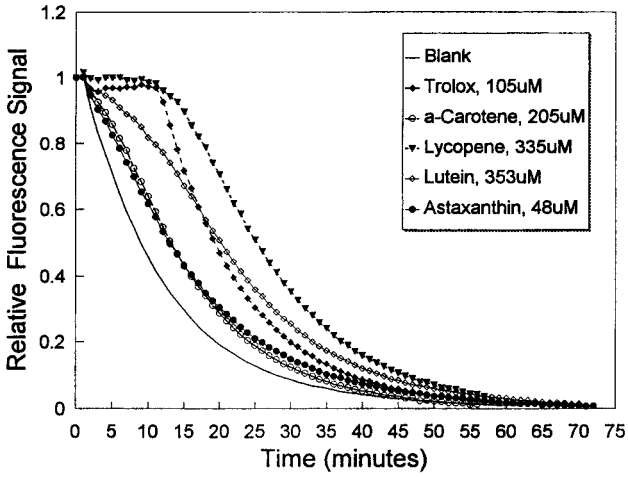

Figure 4. Time course of changes in BODIPY 665/676 fluorescence in octane:butyronitrile (9:1) on incubation at 39 °C with 43 mM AMVN: alone (—), in the presence of 105 μM Trolox (♦), 205 μM α-carotene, 335 μM lycopene, 353 μM lutein, or 48 μM astaxanthin.

α-carotene in octane/butyronitrile (9:1, v/v) were determined to be 1.0, 1.0, 1.3, 0.5, 0.4, 0.3, and 0.2, respectively.

In the DOPC liposomal system containing BODIPY 665/676 and AMVN, astaxanthin, lutein, β-carotene, and α-tocopherol provided protection of the indicator from peroxyl radicals attack (Figure 5).

By employing BODIPY 581/591 as an indicator and in the presence of AMVN, astaxanthin showed the highest relative antioxidant activity, 1.3 ± 0.2 (Figure 6) as compared to Trolox (1.0), α-tocopherol (0.9), α-carotene (0.5) , β-carotene (0.2), lutein (0.4) (Naguib, 1998) and lycopene (0.4).

DISCUSSION

Fluorometric methods are widely used in immunoassay, analytical, and clinical chemistry (van Dyke, 1990; Wolfbeis, 1993). A fluorescence-based assay for measuring activity of lipophilic antioxidants in a lipid environment requires three basic components: (a) a radical initiator (azo or peroxide type) to generate oxygen radicals; (b) an indicator that loses its fluorescence upon oxidation by free radicals; and (c) a calibrator

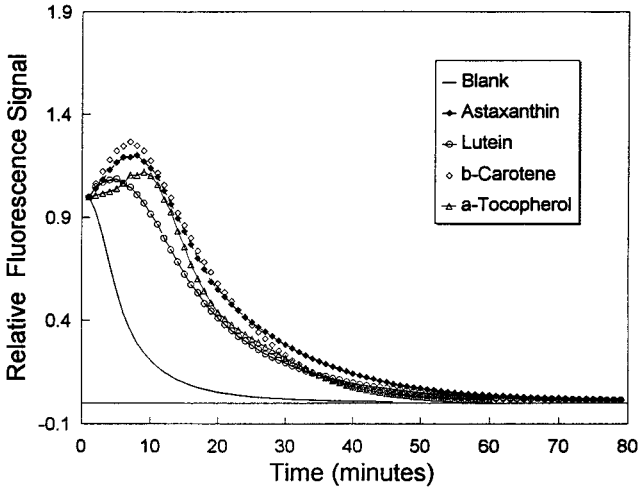

Figure 5. Time course of changes in BODIPY 665/676 fluorescence in DOPC (0.8 mM) in 20 mM Tris-HCl (pH 7.4) on incubation at 39 °C with 5.3 mM AMVN in the presence of astaxanthin (11.7 μM), lutein (15.3 μM), β-carotene (12.3 μM), and α-tocopherol (16.7 μM).

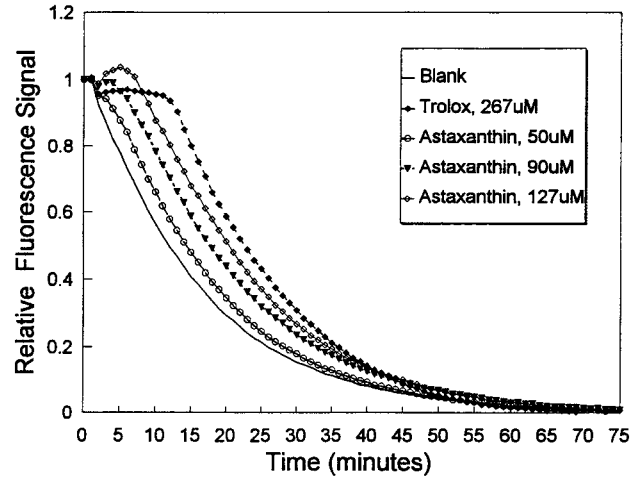

Figure 6. Time course of changes in BODIPY 581/591 C_{11} fluorescence in octane:butyronitrile (9:1) on incubation at 41 °C with 0.26 M AMVN: alone (—), in the presence of 0.267 mM Trolox (♦), or varying amounts of astaxanthin.

that interacts efficiently with peroxyl radicals. BODIPY 581/591 C_{11}, which belongs to the BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene) class of dyes, has previously been employed (Naguib, 1998) as an indicator to measure antioxidant activities of carotenoids in a lipid environment. BODIPY dyes are characterized by a combination of high extinction coefficients, good fluorescence quantum yields, nonpolar structures, high photostabilities, and absorption in the visible region of the electromagnetic spectrum.

Another member of this class of dyes, BODIPY 665/676, has the following advantages as an indicator: (a) it has a highly conjugated polyene system, making it easily susceptible to oxidation by peroxyl radicals; (b) it absorbs at a longer wavelength (main absorption at 665 nm) than BODIPY 581/591 (580 nm), which makes it suitable for test samples that absorb appreciably in the visible region; and (c) it has a relatively large Stokes shift (15 nm) as compared to that of BODIPY 581/591 C_{11} (10 nm) (Haugland, 1996), resulting in less interference of scatter to its emission. The lipid-soluble AMVN, which thermally decomposes in air-saturated solutions to generate peroxyl radicals, was chosen as an initiator. Trolox, an analogue of vitamin E, was used as a calibrator.

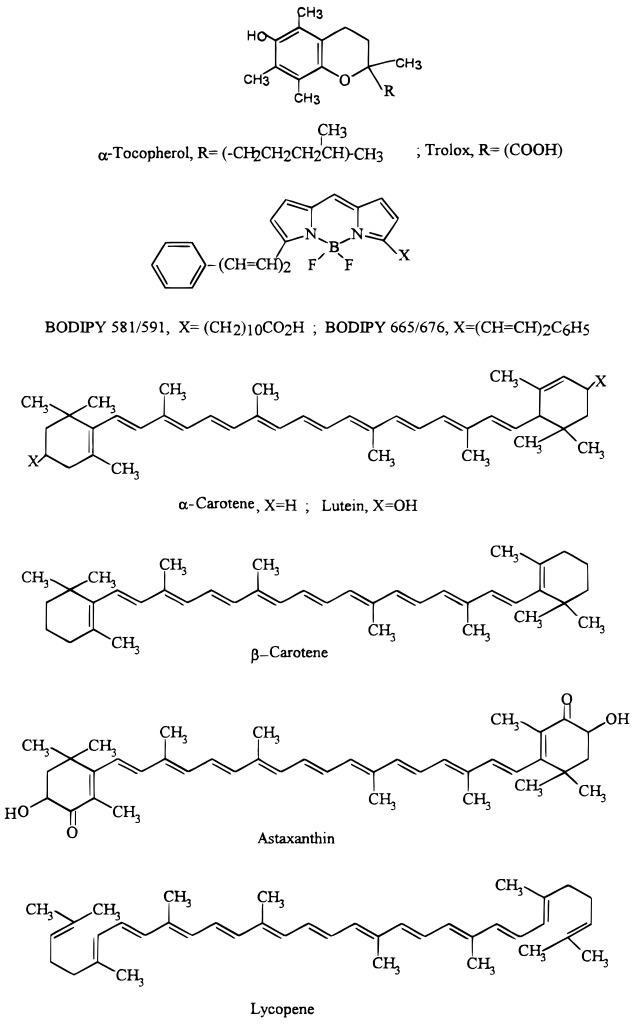

Figure 7. Chemical structures of antioxidants and BODIPY indicators.

BODIPY 665/676 loses its fluorescence in the presence of peroxyl radicals and retains its signal in the presence of an antioxidant. The antioxidant activities of carotenoids and α-tocopherol employing BODIPY 665/676 and AMVN assay relative to Trolox were calculated from the fluorescence decay data and eq 1. Both α-tocopherol and Trolox act as antioxidants via a hydrogen atom transfer from the phenolic group to a peroxyl radical. They are structurally similar, with the long-chain alkyl group of α-tocopherol replaced by a carboxyl group in Trolox. Among the hydrocarbon carotenoids (α-carotene, β-carotene, and lycopene), the latter showed a slightly high antioxidant activity. There is considerable evidence that β-carotene acts as an antioxidant by forming an adduct with peroxyl radicals and/or by transfer of an electron to peroxyl radicals to yield the corresponding radical cation (Liebler and McClure, 1996). Introduction of a hydroxyl group into the carotene structure, such as lutein, resulted in no significant change in its antioxidant activity as compared to hydrocarbon carotenoids. The α-hydroxyketocarotenoid astaxanthin, on the other hand, showed a high antioxidant activity. Presumably, the keto group activates the hydroxyl group and hence facilitates hydrogen transfer to the peroxyl radicals. To explain the high antioxidant activity of the α-hydroxyketocarotenoid astaxanthin as com-

pared to other carotenoids, it is proposed that in solution astaxanthin exits in an equilibrium, the extent of which depends on the solvent, with the enol form of the ketone, thus the resulting ortho-dihydroxy-conjugated polyene system possess a hydrogen atom capable of acting as a chain breaking in free radical reaction in a way similar to the hydroxyl group of α-tocopherol. This is also in agreement with previous results obtained by Terao (1989), who found that astaxanthin is more effective than β-carotene and zeaxanthin in inhibiting free radical oxidation of methyl linoleate in solution.

In the DOPC liposomal system containing BODIPY 665/676 and AMVN, astaxanthin showed a relatively high antioxidant activity as compared to α-carotene, β-carotene, and lutein. Palozza and Krinsky (1992b) reported that astaxanthin is as effective as α-tocopherol in inhibiting radical initiated, by either chelated iron (Fe^{3+}/ADP) and a reducing component (NADPH) or the water-soluble azo initiator 2,2′-azobis(2-amidinopropane), lipid peroxidation of rat liver microcosms. β-Carotene was reported to be a much less potent antioxidant in this system. However, Nakagawa et al. (1997) reported that both β-carotene and astaxanthin provide antioxidant protection against NADPH-dependent microsomal (rat liver microcosms) lipid peroxidation induced by chelated iron (Fe^{3+}/ADP) and NADPH. These discrepancies may be due to different experimental conditions.

By employing BODIPY 581/591 as an indicator and in the presence of peroxyl radicals derived from AMVN, astaxanthin showed relatively high antioxidant activity as compared to α-carotene, β-carotene, and lutein. These results again confirm that the α-hydroxyketocarotenoid astaxanthin is a more effective antioxidant as compared to hydrocarbon and hydroxy carotenoids.

In summary, the antioxidant activities of the α-hydroxyketocarotenoid astaxanthin, the hydroxyl carotenoid lutein, hydrocarbon carotenoids lycopene and α- and β-carotenes, and α-tocopherol were determined by employing novel fluorometric assays based on BODIPY class of dyes as indicators, a lipophilic peroxyl radical generator AMVN, and lipophilic media. The α-hydroxyketocarotenoid astaxanthin was found to exhibit the highest antioxidant activity of the selected carotenoids tested.

It is worth mentioning that BODIPY 665/676 is more sensitive than BODIPY 581/591 toward peroxyl radicals. The later requires twice the amounts of AMVN (Naguib, 1998) as that of BODIPY 665/676 for similar completion decay time of a comparable fluorescence signal. However, BODIPY 581/591 based assay is suitable for samples that display no absorption between 550 and 600 nm but show long wavelength absorption and/or background fluorescence above 620 nm.

ABBREVIATIONS USED

AMVN, 2,2′-azobis-2,4-dimethyl valeronitrile; AUC, area under curve; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacene; BODIPY 665/676, 4,4-difluoro-3,5-bis-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene; BODIPY 581/591 C_{11}, 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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