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-tetramethyl-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 dioleylophosphatidyl 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 C11) 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, 1997; Rice-Evans and Burdon, 1994; Mathews-Roth, 1991) attracted considerable interest because of its economic value as a pigment source in the aquaculture and food industries (Johnson, 1995; Kobayashi et al., 1996).

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.
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 (AUCsample) and the blank, BODIPY/AMVN system without added antioxidants (AUCblank).

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

\[
\frac{\text{relative antioxidant activity of a sample}}{[\text{AUCsample} - \text{AUCblank}]/(\text{AUC Trolox} - \text{AUC blank})} \times \frac{[\text{mol of Trolox/mole of sample}]}{0.01} \leq R \leq 95\%
\]

where (AUCsample - AUCblank) and (AUC Trolox - AUCblank) are the net protection areas under the curves of (AUC) the indicator fluorescence decay in the presence of a sample and Trolox, respectively.

(\(\Delta\text{AUC}\)) is calculated as follows:

\[
\Delta\text{AUC} = \left( f_1 f_1 + f_2 f_1 + f_3 f_1 + \ldots + f_m f_1 \right)
\]

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

In the final reaction mixture (3 mL) for the assay contained 1.3 × 10^{-9} M BODIPY 581/591 C11 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).

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 (\(y = a_0 + a_1 x\)) adequately described the data as shown by the Pearson correlation coefficient. The \(a_1\) represents the antioxidant activity relative to Trolox.

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 \(\alpha\)-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 (AUCTrolox − AUCsample)/(AUCTrolox − AUCBlank)

\[
\frac{[\text{mol of Trolox/mole of sample}]}{0.01} \leq R \leq 95\%
\]
AUCBlank) values and Trolox concentration, multiple correlation coefficient $\, R^2 = 0.99$. The antioxidant $\alpha$-tocopherol also displayed a concentration-dependent plateau followed by decay kinetics similar to those of the blank. Table 1 summarizes the results of the relative antioxidant activity of $\alpha$-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 $\alpha$-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 $\beta$-carotene; the relative antioxidant activity is 0.43 (0.06. Similar decay kinetics were obtained using the carotenoids $\alpha$-carotene, lutein, and lycopene (Figure 4). The relative peroxyl radical scavenging activities of Trolox, astaxanthin, $\alpha$-tocopherol, lycopene, $\beta$-carotene, lutein, and $\alpha$-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, $\beta$-carotene, and $\alpha$-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 $\pm$ 0.2 (Figure 6) as compared to Trolox (1.0), $\alpha$-tocopherol (0.9), $\alpha$-carotene (0.5), $\beta$-carotene (0.2), lutein (0.4) (Naguib, 1998) and lycopene (0.4).

### DISCUSSION

Fluorometric methods are widely used in immuno-assay, 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.
that interacts efficiently with peroxyl radicals. BODIPY 581/591 C11, 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, 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.

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 C11 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.

Figure 7. Chemical structures of antioxidants and BODIPY indicators.

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-aminopropane), lipid peroxidation of rat liver microsomes. 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 microsomes) 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 (Nagub, 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.

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