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Carotenoids as Singlet Oxygen Quenchers in Marine Organisms

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To understand the roles of carotenoids as singlet oxygen quenchers in marine organisms, quenching activities of eight major carotenoids, astaxanthin, canthaxanthin, β-carotene, zeaxanthin, lutein, tunaxanthin, fucoxanthin and halocynthiaxanthin were examined according to the method using a thermodissociable endoperoxide of 1,4-dimethylnaphthalene as a singlet oxygen generator. The second-order rate constant for the singlet oxygen quenching activity by each carotenoid was determined, suggesting that an increasing number of conjugated double bonds in carotenoid was proportional to greater quenching activity. The quenching activity of each carotenoid was found to be approximately 40 to 600 times greater than that of α-tocopherol. The potency of these carotenoids suggests that they may play a role in protecting marine organisms from active oxygen species.

Key words: carotenoid, active oxygen species, singlet oxygen quencher, thermodissociable endoperoxide, chemiluminescence

Carotenoids are widely distributed in marine organisms, especially in the integuments and ovaries in fish and shellfish,[1,2] and on the surfaces of invertebrates.[1,3] They are estimated to play an important role as antioxidants for protecting these organisms from injuries caused by free radicals and active oxygen species, such as singlet molecular oxygen ($^1O_2$), an important active oxygen species.[4–6] Di Mascio *et al.*[7,8] have examined the quenching activities of carotenoids against $^1O_2$ by using a thermodissociable endoperoxide of a naphthylidene derivative, named $NDPO_2$ and a germanium photodiode detection system. They[8] realized that the difference in the results for $^1O_2$ quenching constants between Conn *et al.*,[9] Lee and Min,[10] and themselves[7] was not due to the solvent systems used in their experiments, but to the recording techniques used, and recognized the large quenching constant values for lycopene, astaxanthin and several oxycarotenoids. One of the authors[4] has also revealed that astaxanthin shows a strong quenching activity against $^1O_2$, which is approximately one-hundred times stronger than that of α-tocopherol, a common antioxidant in plants and animals. Although a lot of carotenoids thus show effective $^1O_2$ quenching activities, little information has been obtained on the activities of the carotenoids in marine organisms. We have already reported the scavenging effects of major carotenoids in marine animals against organic free radicals, common radical species,[4,5] according to a newly developed method involving R•-mediated lipid peroxidation.[5] In this study, we sought to determine the second-order rate constants for $^1O_2$ quenching activities of representative carotenoids in marine organisms using a thermodissociable endoperoxide of 1,4-dimethylnaphthalene as a $^1O_2$ generator in order to better understand the role of the carotenoids.

Materials and Methods

General Procedure

Structural confirmation of each agent was carried out mainly by spectroscopic methods. ^{1}H-NMR (500 MHz) spectra were recorded with a Varian Unity 500 instrument in $CDCl_3$. Visible absorption spectra (VIS) were recorded on a Shimadzu UV-2100S recording spectrophotometer in hexane. Chemiluminescence emissions from excited $^1O_2$ were counted with an Aloka BLR-201 Chemiluminescence detector using a Hamamatsu Photonics R464 photomultiplier tube (280–690 nm, max 400 nm).

Preparation of Thermodissociable $^1O_2$ Generator

Thermodissociable *endoperoxide of 1,4-dimethylnaphthalene* (EDN) was prepared as a $^1O_2$ generator from *1,4-dimethylnaphthalene* (DN) according to the method of Wasserman and Larsen[11] described briefly as follows: DN was dissolved in a mixture of dichloromethane and ethanol (4:1), which was maintained at 0°C. After addition of 0.018% of methylene blue as a photosensitizer, the solution was irradiated in the presence of oxygen using 30,000 lx of white light at 0°C for 30 min while stirring; this was then concentrated under nitrogen gas and purified by column chromatography on silica gel (Silica gel 60, 70–230 mesh, Nacalai tesque) using a suitable ratio mixture of hexane and benzene as a solvent below 4°C by monitoring with silica gel TLC to give EDN. Compound EDN was stored at 0°C until used as a thermodissociable $^1O_2$ generator, which could release molecular oxygen in the singlet state at 37°C.[7,8]

Carotenoids

Carotenoids used here were prepared as follows: (3*S*, 3′*S*)-Astaxanthin (**1**) and (3*R*, 3′*R*)-zeaxanthin (**2**) were extracted and purified by HPLC from the culture broth of the marine bacterium *Agrobacterium aurantiacum*[12] and the ovaries of mackerel *Pneumatophorus japonicus*,[1] respectively. Lutein B (**3**) and tunaxanthin C (**4**) were obtained from the integuments of rainbow trout *Oncorhynchus mykiss* (*Salmo gairdnerii irideus*)[13] and yellowtail *Seriola quinqueradiata*,[14] respectively, by extracting with acetone, saponifying with methanolic potassium hydroxide and isolating by HPLC. Fucoxanthin (**5**) and halocynthiaxanthin (**6**) were obtained from the brown alga *Undaria pinnatifid*[15] and the bryozoa *Zoobotryon pellucidum* (N. Shimidzu and W. Miki: MBI, unpubl. data), respectively, by extracting with acetone and isolating by HPLC. β-Carotene (**7**) and canthaxanthin (**8**) were purchased from Wako Pure Chemical Ind. and Extrasynthese Co., respectively, and purified by HPLC. All carotenoid purities were ascertained by VIS and ^{1}H-NMR, monitored by TLC, and stored below −18°C until used. The structures of these carotenoids are shown in Fig. 1. α-Tocopherol, a reference $^1O_2$ quencher, was purchased from Sigma Chemical Co. and purified by HPLC. The purity of each reagent was found to be greater than 99%.

Measurement of Singlet Oxygen Quenching Activity

One hundred microliters of $CDCl_3$ or a mixture of $CDCl_3$ and CD_3OD (2:1) containing 10^{-2} to 10^3 μM of each carotenoid was placed in a thermostated glass tube (10φ × 75 mm) at 37°C. Chemiluminescence counting was started just after adding the endoperoxide of 1,4-dimethylnaphthalene (EDN) at the final concentration of 50 mM, and counted for 60 s. Chemiluminescence counts of both a control (designated as S_0), without any sample, and with a sample (designated as S) were recorded. The total quenching constant ($kq+kr$) was analyzed on a Stern-Volmer plot, which is based upon the following equation,[8]

$$S_0/S=1+(kq+kr)kd^{-1}[Q] \quad (1)$$

where kq is the physical quenching rate constant, kr is the chemical reaction rate constant, kd is the $^1O_2$ lifetime constant in the solvent, and [Q] is the concentration of the carotenoids. When the quenching activity is great enough, then $kq \gg kr$ and the contribution of the chemical reaction to the $^1O_2$ quenching procedure expressed as kr can be neglected. Thus, eq. (1) can be simplified into the following equation,

$$S_0/S=1+kq\,kd^{-1}[Q]=1+\kappa[Q]. \quad (2)$$

Taking the inverse of eq. (2) yields

$$S/S_0=1/(1+kq\,kd^{-1}[Q])=1/(1+\kappa[Q]) \quad (3)$$

where the parameter κ can be estimated by the non-linear least squares parameter estimation method of calculating the S/S_0 value from the chemiluminescence counts and of [Q]. Since $\kappa=kq\,kd^{-1}$, the kq value can be obtained by multiplying the κ value by the kd value. The kq value is found as the second-order rate constant for the $^1O_2$ quenching activity of each carotenoid from eq. (3). The quenching activity of each carotenoid was assessed by comparing the kq value with that of α-tocopherol.

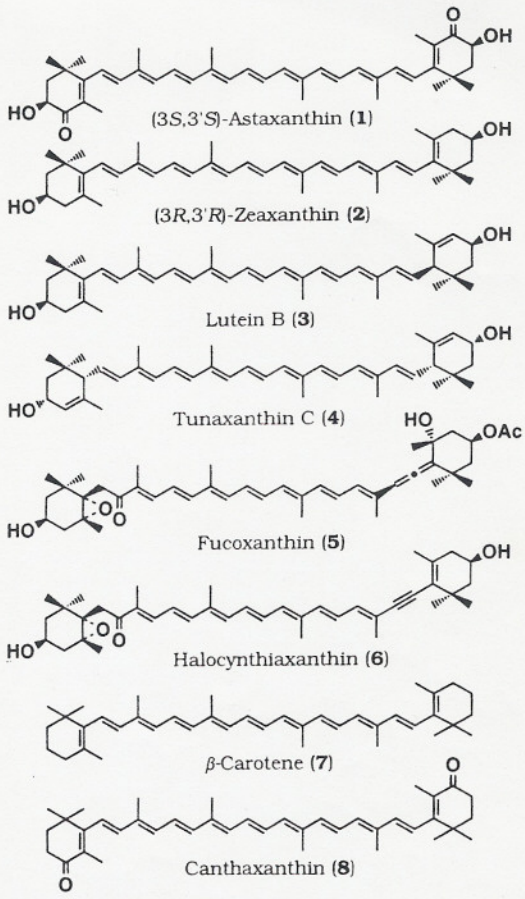

Fig. 1. Structures of carotenoids examined for their activities as singlet oxygen quenchers.

Results and Discussion

The kq values of the carotenoids with that of α-tocopherol in both $CDCl_3$ and a mixture of $CDCl_3$ and CD_3OD (2:1) are shown in Table 1. The strong $^1O_2$ quenching activity of β-carotene (**7**) has been known for over twenty years,[16] and has been found to be more efficient under low partial pressures of oxygen (15 torr).[17] Carotenoids showed remarkable effectiveness for inactivating the active oxygen species and in the process forming triplet state carotenoids very rapidly, as shown in the following equation,[16]

$$^1O_2+Car \rightarrow {}^3O_2+{}^3Car$$

Triplet carotenoids reverted to the ground state with the liberation of a small amount of heat.[16,18] It is generally accepted that an increasing number of conjugated double bonds is associated with a greater quenching activity against $^1O_2$.[4,19] This can also be recognized in this case using a $CDCl_3$ solvent system (Table 1) from the relationship between the N value of **2** which possesses eleven conjugated double bonds (N=11), with that of **3** which has ten (N=10), and that of **4** which has nine (N=9).

The kq value of **1** was slightly greater than that of **2** in $CDCl_3$. This seemed to be due to a small contribution to the quenching activity by the carbonyl groups at allylic positions in β-end groups, a phenomenon which was also recognized by Conn *et al.*[9] using benzene as the solvent to

Table 1. The second-order rate constants (kq) calculated for the $^1O_2$ quenching activities of the carotenoids in $CDCl_3$ and in a mixture of $CDCl_3/CD_3OD$ (2:1)

Compounds	N[a]	OH[b]	$kq \times 10^{-9}$ ($M^{-1}s^{-1}$)	
			$CDCl_3$	$CDCl_3/CD_3OD$ (2:1)[d]
Astaxanthin (**1**)	11+2	2	2.2	1.8
Zeaxanthin (**2**)	11	2	1.9	0.12
Lutein (**3**)	10	2	0.80	—[e]
Tunaxanthin (**4**)	9	2	0.15	—[e]
Fucoxanthin (**5**)	9+1	2	—[e]	0.005
Halocynthiaxanthin (**6**)	9+1	2	—[e]	0.002
β-Carotene (**7**)	11	0	2.2	0.049
Canthaxanthin (**8**)	11+2	0	—[e]	1.2
α-Tocopherol			0.004	—[e]

[a] Number of conjugated C=C double bonds + number of C=O double bonds.
[b] Number of hydroxyl groups.
[c] $kd = 0.33 \times 10^4$ from Bellus.[27]
[d] $kd = 0.36 \times 10^4$ calculated from the kd value of each solvent from Bellus[27] and Rodgers.[28]
[e] Not examined.

measure the difference in kq between that of **1**, 17×10^9, and that of **2**, 14×10^9. However, the kq value of **1** was much greater than that of **2**, when measured in a mixture of $CDCl_3$ and CD_3OD (2:1), a solvent with less hydrophobicity. A similar relationship was also observed between the kq values of **8** and **7**, which showed a similar tendency as reported by Di Mascio *et al.*[7] in the case between that of **1**, (24×10^9), **2**, (10×10^9), **8**, (21×10^9), and **7**, (14×10^9), with a mixture of C_2H_5OH, $CHCl_3$ and H_2O (50:50:1) as the solvent. These results suggest that the carbonyl groups of carotenoids in hydrophilic solvent play a role in enhancing the quenching activity by increasing the chance of direct contact with $^1O_2$.

The contribution of the hydroxyl groups in the carotenoids to quenching activity was observed in the mixed solvent system. Carotenoid **1**, possessing two carbonyl groups, showed a larger kq value than that of **8**, which possesses the same number of double bonds. A similar relationship was also recognized between **2** and **7**; comparing the kq values between **1** and **7** in $CDCl_3$, they are very close to each other, whereas the kq value of **1** is approximately 40 times greater than that of **7** in a mixture of $CDCl_3$ and CD_3OD (2:1). One of the authors[4] has previously reported the carbonyl and hydroxyl groups of carotenoids to be important for these quenching activities, which were presumed to be based on the affinities between carotenoids and the $^1O_2$ generator, methylene blue, or the solvent used in the experiment, depending on its hydrophobicity. The relationship between **1** and **7** mentioned above is thought to be based on the affinity in the latter case. In the case of marine invertebrates, most dietary carotenoids are bioconverted into oxidized forms by increasing the number of C=C or C=O double bonds conjugated, or by increasing the number of hydroxyl groups in order to lower the hydrophobicities. It is presumed that the oxidative bioconversion of the carotenoids is effective for the animals in protecting themselves from active oxygen species by enhancing the carotenoid $^1O_2$ quenching activities.

Allene or acetylene groups in the polyene chain of **5** and **6** did not appear to have any influence on quenching activity. Recently, Nishino's group[20–22] reported the effectiveness of carotenoids, **1**, **2**, **5**, **6**, and others, as anti-cancer agents. They revealed that these carotenoids showed greater anti-cancer activities than that of **7**. The activity of **1** or **2** can be accounted for on the basis of quenching and/or scavenging activity against active oxygen species, whereas the activities of **5** and **6** are ambiguous for understanding the mechanisms of the anti-cancer activity. Tomita's group[23–26] also revealed the anti-cancer activities of **1** and other carotenoids, and proposed a mechanism for enhancing the immunological activities. It seems that the quenching and/or scavenging activity of carotenoids against active oxygen species is thus independent from their anti-cancer activity.

All the carotenoids tested here indicated approximately 35 to 540 times greater quenching activities than α-tocopherol. This result suggests that $^1O_2$ is mainly quenched not by α-tocopherol but by carotenoids in marine organisms.

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References

1) W. Miki, K. Yamaguchi, and S. Konosu: Comparison of carotenoids in the ovaries of marine fish and shellfish. *Comp. Biochem. Physiol.*, **71B**, 7–11 (1982).
2) T. Matsuno: Animal carotenoids, in "Carotenoids. Chemistry and biochemistry" (ed. by N. I. Krinsky, M. M. Mathews-Roth, and R. F. Taylor), Plenum press, New York, 1989, pp. 59–74.
3) T. Matsuno: New structures of carotenoids in marine animals. *Pure Appl. Chem.*, **57**, 659–666 (1985).
4) W. Miki: Biological functions and activities of animal carotenoids. *Pure Appl. Chem.*, **63**, 141–146 (1991).
5) W. Miki, N. Otaki, N. Shimidzu, and A. Yokoyama: Carotenoids as free radical scavengers in marine animals. *J. Mar. Biotechnol.*, **2**, 35–37 (1994).
6) W. Miki, N. Otaki, A. Yokoyama, H. Izumida, and N. Shimidzu: Okadaxanthin, a novel C_{50}-carotenoid from a bacterium, *Pseudomonas* sp. KK10206C associated with marine sponge, *Halichondria okadai*. *Experientia*, **50**, 684–686 (1994).
7) P. Di Mascio, S. Kaiser, and H. Sies: Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch. Biochem. Biophys.*, **274**, 532–538 (1989).
8) P. Di Mascio, A. R. Sundquist, T. P. A. Devasagayam, and H. Sies: Assay of lycopene and other carotenoids as singlet oxygen quenchers. *Methods Enzymol.*, **213**, 429–438 (1992).
9) P. F. Conn, W. Schalch, and T. G. Truscott: The singlet oxygen and carotenoid interaction. *J. Photochem. Photobiol.*, **B11**, 41–47 (1991).
10) S.-H. Lee and D. B. Min: Effects, quenching mechanisms, and kinetics of carotenoids in chlorophyll-sensitized photooxidation of soybean oil. *J. Agric. Food Chem.*, **38**, 1630–1634 (1990).
11) H. H. Wasserman and D. L. Larsen: Formation of 1,4-endoperoxides from the dye-sensitized photo-oxygenation of alkylnaphthalenes. *J. Chem. Soc., Chem. Comm.*, 253–254 (1972).
12) A. Yokoyama, H. Izumida, and W. Miki: Production of astaxanthin and 4-ketozeaxanthin by a marine bacterium, *Agrobacterium aurantiacum* sp. nov. *Biosci. Biotech. Biochem.*, **58**, 1842–1844 (1994).
13) K. Yamaguchi and W. Miki: Comparison of pigments in the integument of cobalt, albino, and normal rainbow trout, *Salmo gairdnerii irideus*. *Comp. Biochem. Physiol.*, **68B**, 517–520 (1981).
14) W. Miki, K. Yamaguchi, S. Konosu, T. Takane, M. Satake, T. Fujita, H. Kuwabara, S. Shimeno, and M. Takeda: Origin of tunaxanthin in the integument of yellowtail (*Seriola quinqueradiata*).

Comp. Biochem. Physiol., **80B**, 195–201 (1985).
15) P. Karrer, A. Helfenstein, H. Wehrli, B. Pieper, and R. Morf: The carotenes, the xanthophylls, fucoxanthin and capsanthin. *Helv. Chim. Acta*, **14**, 614–632 (1931).
16) C. S. Foote and R. W. Denny: Chemistry of singlet oxygen. VIII. Quenching by β-carotene. *J. Am. Chem. Soc.*, **90**, 6233–6235 (1968).
17) G. W. Burton and K. U. Ingold: β-Carotene: An unusual type of lipid antioxidant. *Science*, **224**, 569–573 (1984).
18) N. I. Krinsky: Carotenoids in medicine, in "Carotenoids. Chemistry and biochemistry" (ed. by N. I. Krinsky, M. M. Mathews-Roth, and R. F. Taylor), Plenum press, New York, 1989, pp. 279–291.
19) T. G. Truscott: The photophysics and photochemistry of the carotenoids. *J. Photochem. Photobiol.*, **B6**, 359–371 (1990).
20) J. Okuzumi, H. Nishino, M. Murakoshi, A. Iwashima, Y. Tanaka, T. Yamane, Y. Fujita, and T. Takahashi: Inhibitory effects of fucoxanthin, a natural carotenoid, on N-myc expression and cell cycle progression in human malignant tumor cells. *Cancer Lett.*, **55**, 75–81 (1990).
21) J. Okuzumi, T. Takahashi, T. Yamane, Y. Kitao, M. Inagake, K. Ohya, H. Nishino, and Y. Tanaka: Inhibitory effects of fucoxanthin, a natural carotenoid, on N-ethyl-N′-nitro-N-nitrosoguanidine-induced mouse duodenal carcinogenesis. *Cancer Lett.*, **68**, 159–168 (1993).
22) H. Nishino: Anti-carcinogenic activity of carotenoids in foodstuffs. *Nippon Nogei Kogaku Kaishi*, **67**, 39–41 (1993) (in Japanese).
23) Y. Tomita: Immunological role of vitamin A and its related substances in prevention of cancer. *Nutr. Cancer*, **5**, 187–194 (1983).
24) H. Jyonouchi, R. J. Hill, Y. Tomita, and R. A. Good: Studies of immunomodulating actions of carotenoids. I. Effects of β-carotene and astaxanthin on murine lymphocyte functions and cell surface marker expression in *in vivo* culture system. *Nutr. Cancer*, **16**, 93–105 (1991).
25) H. Jyonouchi, L. Zhang, and Y. Tomita: Studies of immunomodulating actions of carotenoids. II. Astaxanthin enhances *in vivo* antibody production to T-dependent antigens without facilitating polyclonal B-cell activation. *Nutr. Cancer*, **19**, 269–280 (1993).
26) H. Jyonouchi, L. Zhang, M. Gross, and Y. Tomita: Immunomodulating actions of carotenoids: Enhancement of *in vivo* and *in vitro* antibody production to T-dependent antigens. *Nutr. Cancer*, **21**, 47–58 (1994).
27) D. Bellus: Physical quenchers of singlet molecular oxygen. *Adv. Photochem.*, **11**, 105–202 (1979).
28) M. A. J. Rodgers: Solvent-induced deactivation of singlet oxygen: Additivity relationships in nonaromatic solvents. *J. Am. Chem. Soc.*, **105**, 6201–6205 (1983).