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, and on the surfaces of invertebrates. 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 (1O2), an important active oxygen species. Di Mascio et al. have examined the quenching activities of carotenoids against 1O2 by using a thermodissociable endoperoxide of a naphthylidene derivative, named NDPO2 and a germanium photodiode detection system. They recognized the difference in the results for 1O2 quenching constants between Conn et al., Lee and Min, and themselves 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 has also revealed that astaxanthin shows a strong quenching activity against 1O2, 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 1O2 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, according to a newly developed method involving R•-mediated lipid peroxidation. In this study, we sought to determine the second-order rate constants for 1O2 quenching activities of representative carotenoids in marine organisms using a thermodissociable endoperoxide of 1,4-dimethylnaphthalene as a 1O2 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. 1H-NMR (500 MHz) spectra were recorded with a Varian Unity 500 instrument in CDC13. Visible absorption spectra (VIS) were recorded on a Shimadzu UV-2100S recording spectrophotometer in hexane. Chemiluminescence emissions from excited 1O2 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 1O2 Generator

Thermodissociable endoperoxide of 1,4-dimethylnaphthalene (EDN) was prepared as a 1O2 generator from 1,4-dimethylnaphthalene (DN) as described in the method of Wasserman and Larsen. DN was dissolved in a mixture of dichloromethane and ethanol (4:1), which was maintained at 0°C. After addition of 0.1% of methylene blue as a photosensitizer, the solution was irradiated with 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 1O2 generator, which could release molecular oxygen in the singlet state at 37°C.
Carotenoids

Carotenoids used here were prepared as follows: (3S, 3\(^{-}S\))-Astaxanthin (1) and (3R, 3\(^{-}R\))-zeaxanthin (2) were extracted and purified by HPLC from the culture broth of the marine bacterium *Agrobacterium aurantiacum*\(^{12} \text{)} and the ovaries of mackerel *Pneumatophorus japonicus*,\(^{11} \text{)} respectively. Lutein B (3) and tunaxanthin C (4) were obtained from the integuments of rainbow trout *Oncorhynchus mykiss* (Salmo gairdnerii irideus)\(^{13} \text{)} and yellowtail *Seriola quinquergadiata*,\(^{14} \text{)} 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} \text{)} and the bryozoa *Zoobotryon pellucidum* (N. Shimidzu and W. Miki: MBI, unpubl. data), respectively, by extracting with acetone and isolating by HPLC. \(^{-}\text{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\text{H}-\text{NMR, monitored by TLC, and stored below -18 \text{°C until used. The structures of these carotenoids are shown in Fig. 1. (a-Tocopherol, a reference 1\text{›}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\(_3\)OD (2:1) containing 10\(^{-}2\) to 10\(^3\) \(\mu\text{M}\) of each carotenoid was placed in a thermostated glass tube (100 x 75 mm) at 37 \text{°C}. Chemiluminescence counting was started just after adding the endoperoxide of 1,4-dimethyl-naphthalene (EDN) at the final concentration of 50 mm, and counted for 60 s. Chemiluminescence counts of both a control (designated as So), without any sample, and with a sample (designated as S) were recorded. The total quenching constant (\(k_q + k_r\)) was analyzed on a Stern-Volmer plot, which is based upon the following equation,\(^8\)

\[
S_o/S = 1 + (k_q + k_r)kd^{-1}[Q]
\]

(1)

where \(k_q\) is the physical quenching rate constant, \(k_r\) is the chemical reaction rate constant, \(kd\) is the \(1\text{O}_2\) lifetime constant in the solvent, and [Q] is the concentration of the carotenoids. When the quenching activity is great enough, then \(k_q \gg k_r\) and the contribution of the chemical reaction to the \(1\text{O}_2\) quenching procedure expressed as \(k_r\) can be neglected. Thus, eq. (1) can be simplified into the following equation,

\[
S_o/S = 1 + k_qkd^{-1}[Q] = 1 + \kappa[Q].
\]

(2)

Taking the inverse of eq. (2) yields

\[
S/S_o = 1/(1 + k_qkd^{-1}[Q]) = 1/(1 + \kappa[Q])
\]

(3)

where the parameter \(\kappa\) can be estimated by the non-linear least squares parameter estimation method of calculating the \(S/S_o\) value from the chemiluminescence counts and of [Q]. Since \(\kappa = k_qkd^{-1}\), the \(k_q\) value can be obtained by multiplying the \(\kappa\) value by the \(kd\) value. The \(k_q\) value is found as the second-order rate constant for the \(1\text{O}_2\) quenching activity of each carotenoid from eq. (3). The quenching activity of each carotenoid was assessed by comparing the \(k_q\) value with that of \(\alpha\)-tocopherol.

Results and Discussion

The \(k_q\) values of the carotenoids with that of \(\alpha\)-tocopherol in both CDCl\(_3\) and CD\(_3\)OD (2:1) are shown in Table 1. The strong \(1\text{O}_2\) quenching activity of \(\beta\)-carotene (7) has been known for over twenty years,\(^{16} \text{)} and has been found to be more efficient under low partial pressures of oxygen (15 torr).\(^{17} \text{)} 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} \text{)}

\[
1\text{O}_2 + \text{Car} \rightarrow 1\text{O}_2 + ^3\text{Car}
\]

Triplettarotenoids reverted to the ground state with the liberation of a small amount of heat.\(^{16,10} \text{)} It is generally accepted that an increasing number of conjugated double bonds is associated with a greater quenching activity against \(^1\text{O}_2\).\(^{4,19} \text{)} 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 \(k_q\) 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 \(\beta\)-end groups, a phenomenon which was also recognized by Conn et al.\(^{18} \text{)} using benzene as the solvent to
measure the difference in $k_q$ between that of 1, $17 \times 10^9$, and that of 2, $14 \times 10^9$. However, the $k_q$ value of 1 was much greater than that of 2, when measured in a mixture of CDCl$_3$ and CD$_3$OD (2:1), a solvent with less hydrophobicity. A similar relationship was also observed between the $k_q$ values of 8 and 7, which showed a similar tendency as reported by Di Mascio et al.\(^9\) in the case between that of 1, $(24 \times 10^9)$, 2, $(10 \times 10^9)$, 8, $(21 \times 10^9)$, and 7, $(14 \times 10^9)$, with a mixture of C$_2$H$_5$OH, CHCl$_3$ and H$_2$O (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 $k_q$ 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 $k_q$ values between 1 and 7 in CDCl$_3$, they are very close to each other, whereas the $k_q$ value of 1 is approximately 40 times greater than that of 7 in a mixture of CDCl$_3$ and CD$_3$OD (2:1). One of the authors\(^6\) 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 $\alpha$-tocopherol. This result suggests that 1O$_2$ is mainly quenched not by $\alpha$-tocopherol but by carotenoids in marine organisms.

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References

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