

**Proton-Donative Antioxidant Activity of Fucoxanthin with
1,1-Diphenyl-2-Picrylhydrazyl (DPPH)**

Takaharu Nomura*¹, Masako Kikuchi², Akiko Kubodera¹, and
Yasushi Kawakami²

- 1) Department of Radiopharmacy, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Ichigayafunagawara-machi, Shinjuku, Tokyo 162, Japan
2) Department of Biotechnology, Institute of Research and Innovation, 1201 Takada, Kashiwa, Chiba. 277, Japan

Received March 26, 1997

Summary: Reactions between a carotenoid, fucoxanthin and 1,1-diphenyl-2-picrylhydrazyl were investigated both under anoxic and aerobic conditions. Fucoxanthin equimolarly reacted with 1,1-diphenyl-2-picrylhydrazyl under anoxic conditions. Under aerobic conditions, only a part of fucoxanthin consumed 1,1-diphenyl-2-picrylhydrazyl and the degree of reaction fluctuated with repeated trials. β -Carotene or other carotenoids, β -cryptoxanthin, zeaxanthin, lycopene and lutein, were also examined in the reaction with 1,1-diphenyl-2-picrylhydrazyl under anoxic conditions. All these compounds scarcely reacted with 1,1-diphenyl-2-picrylhydrazyl.

Key Words: fucoxanthin / carotenoids / antioxidant / 1,1-diphenyl-2-picrylhydrazyl / anoxic conditions

Polyunsaturated fatty acids are reported to share more than 30% of total fatty acids in diatoms or brown algae. The crude extract of a diatom, *Phaeodactylum tricorutum*, was demonstrated to have antioxidant activity against the oxidation of polyunsaturated fatty acids [1]. We previously identified the antioxidant substance in the crude extract as fucoxanthin [2]. Fucoxanthin is known to be one of the carotenoids contained in Chromophyta, diatoms and brown algae, of which the distinctive feature is the presence of allene bond and oxygenic

Abbreviation

DPPH, 1,1-diphenyl-2-picrylhydrazyl

* To whom correspondence should be addressed.:

Department of Biotechnology, Institute of Research and Innovation, 1201 Takada, Kashiwa, Chiba 277, Japan

Phone: 81-471-46-0011, Fax. 81-471-44-8944

functional groups, -hydroxyl, carbonyl, epoxy and carboxyl moieties (Fig. 1.) [3]. Typical antioxidants are usually proton donors; ascorbic acid, α -tocopherol, glutathione or L-cysteine are all known to be reductive antioxidants that scavenge superoxide, hydroxyl radical or hydroperoxy radical [4-9]. Some carotenoids are shown to serve as singlet oxygen quenchers [10,11]. Although reductive antioxidative activity of carotenoids has not yet explicitly evidenced, repressive activity of fucoxanthin against the oxidation of fatty acids suggests that the pigment has the proton donative ability to the radicals in fatty acid peroxidation as well as the ability of singlet oxygen quenching. Many researchers examine the capability of proton donative antioxidants as free radical scavengers. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) in ethanol or chloroform solution shows strong absorption at around 520 nm, which gives deep violet color to the solution. This compound can accept an electron or a hydrogen radical to become a stable irreversible product. The decolorization is stoichiometric to the numbers of electrons taken up [4,12-14].

Our previous report showed that fucoxanthin had an antioxidant activity against 2,2'-azobisisobutyronitrile (AIBN)-induced oxidation of linoleic acid [2]. The details of the antioxidative activities, however, have not yet been studied. We investigated in this paper the reactions between fucoxanthin and DPPH.

Materials and Methods

Materials. Fucoxanthin was isolated and purified from a culture of *Phaeodactylum tricornutum* as previously reported [2]. β -Carotene, DPPH and α -tocopherol were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). β -Cryptoxanthin, zeaxanthin and lycopene were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Lutein was obtained from SIGMA Chemical Company (St. Louis, USA). All the carotenoids and DPPH were stored at -80 °C in the nitrogen-packed vials and the solutions were made immediately before use. The concentration of fucoxanthin in chloroform solution was determined from the maximum absorbance at 461 nm and 483 nm by dividing with the molar absorption coefficients. Evstigeev and Paramonova reported the coefficients: $E_{1\text{cm}}^{1\%} = 1140$ in ethanol solution (maximum absorbance at 452nm); $E_{1\text{cm}}^{1\%} = 1080$ in chloroform solution [15], which were in good agreement with ours, 1133 in ethanol and 1082 in chloroform. We thus employed the values of our measurement in the following experiments.

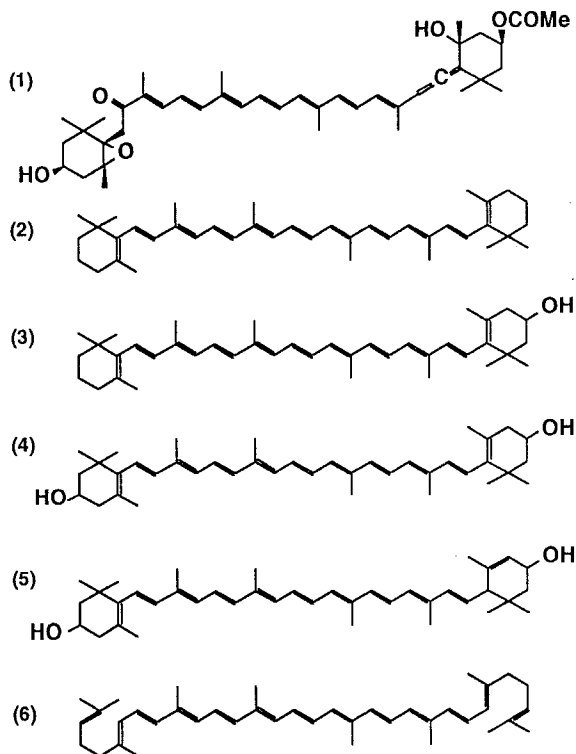


Fig. 1. Structure of Carotenoids.

(1) fucoxanthin, (2) β -carotene, (3) β -cryptoxanthin, (4) zeaxanthin, (5) luteone, and (6) lycopene.

Sample Preparation and Examination of DPPH-Quenching Activities of Carotenoids. The reaction of fucoxanthin with DPPH was conducted by the method of Blois, with a slight modification [4]. Briefly, a glass vial (50 ml) that contained 45 ml chloroform was tightly closed with a screw cap that had a septum. Nitrogen gas was bubbled with shaking to exclude oxygen in the vial. The bubbling was repeated five times or more. DPPH and carotenoids were packed into a micro glass vial (2 ml) under nitrogen atmosphere, which was also tightly packed with a screw cap with a septum. The appropriate volume of the chloroform bubbled with nitrogen, was injected in the microvial to dissolve DPPH and carotenoids. The reaction was done with another screw-capped vial within which DPPH and carotenoid solutions were mixed together. All vials were shielded from light with aluminum foil and all the experiments were performed in a dark room. The vials were kept at room temperature for 20 min. The reaction mixture was drawn and transferred to quartz cells by a syringe for measurement. The consumption of DPPH were determined by the decrease of the maximum absorption at 526 nm using a corresponding carotenoid as a blank with a Shimadzu UV-1600 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The correlation between absorption at 526 nm and the amount of DPPH was determined by reaction of various amounts of α -tocopherol with DPPH.

Results

Figure 2. shows the correlation between decrease of concentrations of DPPH and concentrations of carotenoids added to the reaction mixture under anoxic conditions. The decrease of the absorption at 526 nm which was characteristic to DPPH was measured to quantify the concentration of DPPH. The absorption of both DPPH and fucoxanthin contributed to that of the reaction mixture at 526 nm. The absorption of fucoxanthin after the reaction at 526 nm unchanged, however, though fucoxanthin must be somewhat modified by the reaction with DPPH (data not shown). The slope of the regression curve was 1.045 for fucoxanthin, which indicates that DPPH reacted with equivalent moles of fucoxanthin. It means in other words that fucoxanthin donates an electron or a hydrogen radical to DPPH under anoxic conditions.

Similar reactions were carried out for β -carotene, β -cryptoxanthin, zeaxanthin, luteine and lycopene as well as fucoxanthin. Among these, β -carotene and lycopene are hydrocarbons, β -cryptoxanthin has a hydroxy group and zeaxanthin and luteine have two hydroxy groups at both ends (Fig. 1.). Decrease of DPPH concentrations was negligible for the carotenoids tested except fucoxanthin. We can thus conclude that these five carotenoids scarcely reacted with DPPH under anoxic conditions.

Reactions of fucoxanthin with DPPH under aerobic conditions were also carried out (Fig. 3.). Various amount of fucoxanthin was added to 100 μ M DPPH to see the stoichiometry of the reactions. In contrast to the anoxic reactions, fucoxanthin incompletely reacted with DPPH; the decrease of DPPH concentrations was smaller than those under anoxic conditions by around an order. The results, moreover, fluctuated considerably with repeated runs. Two lines for the aerobic reaction shown in Fig. 3. indicates the higher and the lower limits of the fluctuation. This partial and unstable reactivity of fucoxanthin with DPPH under aerobic conditions suggests that fucoxanthin was partially oxidized by molecular oxygen with the aid of DPPH. The unstable reaction was presumably due to the change of concentrations of dissolved oxygen in the solvent.

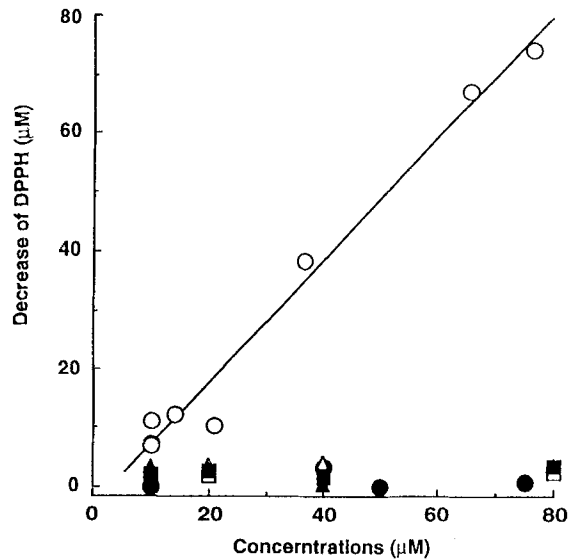


Fig. 2. Quenching of DPPH with Various Carotenoids under Anoxic Conditions. Various amount of carotenoids were added to the mixture containing 150 μM DPPH. Slope of the regression curve; 1.045 ($r^2=0.980$) for fucoxanthin (\circ), 0.005 ($r^2=0.068$) for β -carotene (\bullet), 0.010 ($r^2=0.204$) for β -cryptoxanthin (Δ), 0.001 ($r^2=0.000$) for zeaxanthin (\blacktriangle), 0.006 ($r^2=0.184$) for luteine (\square), and 0.017 ($r^2=0.380$) for lycopene (\blacksquare).

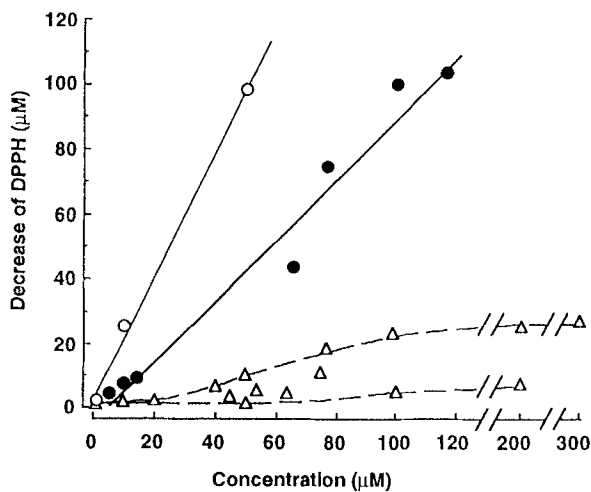


Fig. 3. Quenching of DPPH with Fucoxanthin and α -Tocopherol.

Slope of the regression curve; 0.950 ($r^2=0.906$) for fucoxanthin under anoxic conditions (\bullet) and 1.926 ($r^2=0.996$) for α -tocopherol under aerobic conditions (\circ). (Δ) for fucoxanthin under aerobic conditions. Two lines for the aerobic conditions of fucoxanthin indicate the higher and the lower limits of the fluctuation.

Figure 3. also shows the aerobic reactions of α -tocopherol with 100 μ M DPPH. As Blois reported [4], 50 μ M α -tocopherol reacted with 100 μ M DPPH, which indicates that one molecule of α -tocopherol donated two electrons to a molecule of DPPH. α -Tocopherol stoichiometrically reacted with DPPH both under aerobic and anoxic conditions (data not shown for anoxic conditions). Decrease of DPPH in the reaction of α -tocopherol over 50 μ M completely leveled off, which means that all of the DPPH was consumed by the reaction. Fucoxanthin anaerobically reacted with DPPH and the decrease of DPPH were about half of those in α -tocopherol.

All the experiments so far were performed only for 20 min. Although the reactions with DPPH apparently undergo almost instantaneously, whether 20 min incubation is sufficient for complete reaction is not explicitly verified, especially for aerobic reactions with fucoxanthin which gave quite unstable results. The reactions with the other carotenoids which showed very poor reactivity within 20 min might also require longer incubation. Long term incubation up to 24 hours was then conducted to see the advancement of the reactions for fucoxanthin and β -carotene both under aerobic and anoxic conditions (Fig. 4.). The 40 ml samples were stood for up to 24 hours under room temperature and 1 ml of the samples was taken for colorimetry with appropriate intervals. Figure 4(A). shows the change of O.D. of the samples stood for under anoxic conditions. The O.D.s of the chloroform solutions of DPPH, fucoxanthin and β -carotene did not change during the period. The values in Figs 4(A). and 4(B). are the direct reading of O.D.s of the solutions and fucoxanthin or β -carotene has some absorption at 526 nm as tails of their respective peaks. Both the mixtures of DPPH / fucoxanthin and DPPH / β -carotene were also stable though fucoxanthin reacted with DPPH under anoxic conditions. The O.D. of the mixture of DPPH and fucoxanthin was also measured back to about 2 min after the onset of the reaction to see the short term process. The value at $t=0$ is not a measured one but a simple sum of the absorption of reactants measured separately. The decrease of O.D. at the vicinity of $t=0$ was the same as a those at prolonged incubation, which indicated that the reaction was almost instantaneous in these intervals.

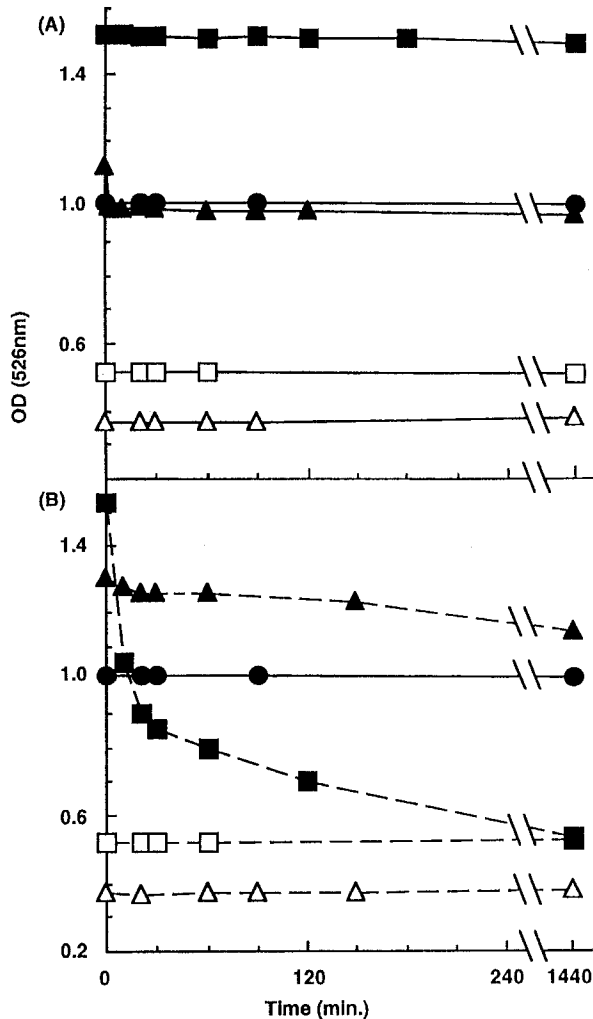


Fig. 4. Time Course of Reactions of Carotenoids with DPPH.

Quenching of DPPH with fucoxanthin or β -carotene under anoxic conditions (A), or aerobic conditions (B). The initial concentration of DPPH was $100\mu\text{M}$ and these of fucoxanthin and β -carotene were $50\mu\text{M}$. The symbols were DPPH (●), fucoxanthin (Δ), β -carotene (\square), DPPH and fucoxanthin (\blacktriangle), and DPPH and β -carotene (\blacksquare).

The results of the aerobic reactions were shown in Fig. 4(B). The O.D. of the mixture of DPPH / fucoxanthin was not stable. It gradually decreased by 26 % within 24 hours. The change of the mixture of DPPH / β -carotene was even more significant; the O.D. dropped sharply from 0.82 to 0.57 within an hour and reduced to nearly 40 % of the original value after 24 hours. The chloroform solutions of respective chemicals were all stable within 24 hours even under aerobic conditions.

Discussion

The above results show that fucoxanthin equimolarly reacts with DPPH as radical quencher under anoxic conditions whereas the other carotenoids have practically no quenching activities. This might simply suggest that fucoxanthin is more reactive to radicals than the other carotenoids tested here. All these carotenoids, however, were reported to have antioxidative activity against lipid peroxidation [17-20] or quenching ability of singlet oxygen [10,21-23]. β -Carotene was relatively more sensitive than fucoxanthin under aerobic conditions in our experiments of long term incubation. Kikuchi *et al.* also reported that the antioxidant activity of β -carotene was twice as effective as that of fucoxanthin against the peroxidation of polyunsaturated lipid [2]. This difference in the reactivity of these carotenoids with radicals probably depends first on the presence of oxygen in the reaction. Under anoxic conditions, fucoxanthin, having six oxygen atoms in the molecule, may generally be more sensitive to radicals. Many researchers show that one of the difference in the antioxidant activity in carotenoids is closely correlated with the presence of intramolecular oxygen atoms [11,16,24-26]. When molecular oxygen is present in the reaction with DPPH, however, the reactivity appears more complex because antioxidants react not only with DPPH but also with oxygen. DPPH, in this case apparently promoted the reaction.

Low oxygen partial pressure is found in most tissues under physiological conditions. Barton and Ingold pointed that high quenching ability are required for antioxidants under these conditions to remove radicals of extremely small quantities and that good radical-trapping antioxidants should be active even at low oxygen partial pressure and reported that β -carotene had an antioxidative activity reversely correlated with oxygen tension [27]. Our results under anoxic conditions infer that fucoxanthin may possibly be a more potent antioxidant than β -carotene or other carotenoids if we intrapolate the above tendency to the much lower oxygen partial pressure.

Antioxidative agents quench radicals usually through the liberation of hydrogen radicals. It results in the transient radicalization of the compounds themselves. The activated compounds then need stabilization of the radical moieties through several modes: dimerization or polymerization; intramolecular quenching of the radical moieties; radical transfer to the other compounds. L-Cysteine and glutathione are the examples for the first case. Their sulphhydryl groups donate a hydrogen radical to DPPH by their own radicalization. The radicalization is then stabilized by the dimerization of the two radicalized antioxidative compounds. Examples for the second case are α -tocopherol and ascorbic acid. They stabilize radicals by donating two hydrogen radicals or two electrons to them at the expense of their own activation. The activation makes two radical moieties within their molecules and the two moieties are stabilized through the formation of quinone (α -tocopherol) or dehydro-compounds (ascorbic acid).

Fucoxanthin, in this case, donated an electron to DPPH. Similar mode of one-electron donation was also observed in 2-O-octadecylascorbic acid (CV-3611 for commercial name) [28], in which one of the two hydroxyl moiety of ascorbic acid was inactivated by replacing with octadecyl group. Preliminary analysis showed neither dimer nor polymer formation like above (data not shown). Complex fragmentation of the carotenoid seems as likeliest results as in CV-3611 (S. Terao, personal communication), though no definite products have not identified.

Acknowledgments

This work was financially supported in point by grants from Central Research Institute of Electric Power Industry.

REFERENCES

1. Uchida, M., Japan Kokai Tokkyo Koho, 1343496 (Oct. 29, 1986).
2. Kikuchi, M., Hirano, A., Kunito, S., and Kawakami, Y. (1995) *J. Mar. Biotechnol.* 3, 132-135.
3. Straub, O. (1987) in *Key to Carotenoids (2ed)* (Pfander, H., Gerspacher, M., Rychener, M., and Schwabe, R., Eds.), pp. 1-296, Birkhauser Verlag, Basel, Boston,.

4. Blois, M. S. (1958) *Nature* 181, 1199-1200.
5. Sies, H., Stahl, W., and Sunquist, A. R. (1992) *Ann. NY Acad. Sci.* 669, 7-20.
6. Tolbert, B. M. (1985) *Int. J. Vit. Nutr. Res.* 27, 121-138.
7. Bendich, A., Machlin, L. J., Scandurra, O., Burton, G. W., and Wayner, D. D. M. (1986) *Adv. Free Rad. Biol. Med.* 2, 419-444.
8. Craw, M. T., and Depew, M. C. (1985) *Rev. Chem. Intermed.* 6, 1-31.
9. Niki, E. (1987) *Chem. Phys. Lipid* 44, 227-253.
10. Sunquist, A. R., Brivida, K., and Sies, H. (1994) in *Methods in Enzymology* (Packer, L. Ed.), 234, pp.384-388, Academic Press, New York.
11. Shimadzu, N., Goto, M., and Miki, W. (1996) *Fisheries Sci.* 62, 134-137.
12. Iwatsuki, M., Niki, E., and Kato, S. (1993) *BioFactors* 4, 123-128.
13. Hiramatsu, M., Packer, R., and Packer, L. (1990) in *Methods in Enzymology* (Packer, L. Ed.), 190, pp.273-280, Academic Press, New York 1990.
14. Hiramatsu, M., Edamatsu, R., Kohno, M., and Mori, A. (1988) in *Recent Advances in the Pharmacology of Kampo Medicines* (Hosoya, E., and Yamamoto, Y., Eds.), pp.120-127.
15. Evstigeev, V. B., and Paramonova, L. I. (1974) *Biokhimiya* 39, 394-400.
16. Hirayama, O., Nakamura, K., Hamada, S., and Kobayashi, Y. (1994) *Lipids* 29, 149-150.
17. Kennedy, T. A., and Liebler, D. C. (1992) *J. Biol. Chem.* 267, 4658-4663.
18. Miki, W., Otaki, N., Shimidzu, N., and Yokohama, A. (1994) *J. Mar. Biotechnol.* 2, 35-37.
19. Oshima, S., Ojima, F., Sakamoto, H., Ishiguro, Y., and Terao, J. (1995) *J. Nutr. Sci. Vitaminol.* 39, 607-615.
20. Lim, B. D., Nagao, A., Terao, J., Tanaka, K., Suzuki, T., and Takuma, K. (1992) *Biochim. Biophys. Acta* 1126, 178-184.
21. Foote, C. S., and Denny, R. W. (1968) *J. Am. Chem. Soc.* 90, 6233-6235.
22. Truscott, T. G., Land, E. J., and Sykes, A. (1973) *Photochem. Phtobiol.* 17, 43-51.
23. Conn, P. F., Schlaoh, W., and Truscott, T. G. (1991) *J. Phtpchem. Phtobiol. B. Biol.* 11, 41-47.
24. Mascio, P. D., Kaiser, S., and Sies, H. (1989) *Arch. Biochem. Biophys.* 274, 532-538.
25. Terao, J. (1989) *Lipids* 24, 659-661.
26. Miller, N. J., Sampson, J., Candeias, L. P., Bramley, P. M., and Rice-Evans, C. A. (1996) *FEBS Lett.* 384, 240-242.
27. Burton, G. W., and Ingold, K. U. (1984) *Science* 224, 569-573.
28. Shimamoto, N., Ohtsuki, H., Imamoto, T., Kitayoshi, T., Hirata, M., Kato, K., and Terao, S. (1987) in *Free Radicals in Clinical Medicine* (Kondo, M. Ed.), *Nihon-Igakukan*, pp.91-95, Tokyo, Japan.