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Quenching of singlet oxygen by carotenoids produced in *Escherichia coli* – attenuation of singlet oxygen-mediated bacterial killing by carotenoids

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Abstract We examined the viability of *Escherichia coli* transformants harboring various carotenoids synthesizing genes in a medium containing an enzymatic singlet oxygen generating system, which contained myeloperoxidase, hydrogen peroxide and Br^- at pH 4.5. Singlet oxygen quenching activities of various carotenoids in phosphatidyl choline micelles in aqueous media were also studied using the same enzymatic singlet oxygen generating system. Viability of the transformants producing carotenoids was higher than that of the wild type *E. coli* in the singlet oxygen generation mixture. Of the transformants tested, the viability of zeaxanthin-diglucoside producing transformant was the highest. Carotenoids in increasing order of k_q values were β-carotene, a cyclic carotene < zeaxanthin with hydroxy groups ≤ lycopene, an acyclic carotene < canthaxanthin and astaxanthin with keto groups ≪ zeaxanthin-diglucoside. The k_q value of zeaxanthin-diglucoside was 3.5 times higher than that of β-carotene. These results suggest that orientation of the carotenoids in lipid layers of micelles and also in phospholipid membrane of bacteria is important for quenching of singlet oxygen. Furthermore, the viability of transformants producing lycopene and phytoene was almost as high as that of the transformant producing zeaxanthin-glucoside.

Key words: Carotenoid; Singlet oxygen; Transformant; Phosphatidyl choline; Micelle; Myeloperoxidase; *Escherichia coli*

1. Introduction

Carotenoids are considered to protect plants, animals, and microorganisms from the destructive effects of activated oxygen species, such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxy radical ($^\bullet OH$), [18,19,22] and are known to quench $^1O_2$ [9,21]. It has been widely accepted that $^1O_2$ quenching activity of carotenoids in organic solvents is mainly dependent on the number of conjugated double bands [12,13]. $^1O_2$ was recognized as a product in both biochemical and photochemical systems [8]. $^1O_2$ readily oxidizes biological molecules including DNA [14,40] and is responsible for the cell destruction caused by light and certain photosensitizers [12,41]. In 1974, Krinsky [20] first reported that neutrophils killed a colorless mutant strain of *Sarcina lutea* more readily than a carotenoid-containing strain and suggested that $^1O_2$ generated in neutrophil phagosomes participates in bacterial killing. Similarly, Dahl et al. [5] have reported that carotenoid-containing Gram-positive bacteria, such as *S. lutea* and *Staphylococcus aureus*, are more resistant to the lethal effects of gas phase $^1O_2$, compared with their colorless mutant strains. However, the bactericidal action of $^1O_2$ generated in aqueous media has not yet been evaluated due to lack of specific $^1O_2$ generating systems in aqueous media.

Recently, Nakano's group found that wild type *Escherichia coli* were killed easily by $^1O_2$ generated in a myeloperoxidase (MPO)-based enzymatic $^1O_2$ generating system, which is very similar to the intracellular condition of polymorphonuclear leukocytes [37] or by $^1O_2$ generated by the thermal decomposition of a naphthalene endperoxide in buffer solution and such a $^1O_2$-mediated bacterial killing was significantly attenuated by lycopene in the transformant *E. coli* [28]. Whether or not the antibactericidal activity of bacterial carotenoids results from quenching $^1O_2$ or another mechanism is currently unknown.

The present work was undertaken to examine the behavior of carotenoid in bacterial membrane against $^1O_2$ toxicity, using carotenoid producing transformant *E. coli* and wild type *E. coli*, carotenoid–phospholipid micelles, and the MPO–H_2O_2–Br^- system as pure $^1O_2$ generating system [37].

2. Materials and methods

2.1. Expression of carotenoid producing genes in E. coli

Plasmids used in this study are shown in Table 1. These plasmids carry the carotenoid biosynthesis genes, which are derived from the epiphytic bacterium *Erwinia uredovora* [24] or the marine bacterium *Agrobacterium aurantiacum* [25]. Plasmid pCRT-XYZ, which carries the *E. uredovora crtX, crtY* and *crtZ* genes, was constructed as follows: the 1.30-kb *Ava*I (995)–*Sal*I (2295) fragment of pCAR16 [24], whose *Ava*I site was changed to the *Kpn*I site, and the 2.48-kb *Sal*I–*Eco*RI fragment derived from pCRT-YZ [36] were ligated to the

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Abbreviations: ETPs, electron transport particles; H_2O_2, hydrogen peroxide; $^\bullet OH$, hydroxy radical; HOCl, hypochlorous acid; $^1O_2$, singlet oxygen; O_2^-, superoxide anion; MCLA, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-a] pyrazin-3-one; MPO, myeloperoxidase; SOD, superoxide dismutase

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Table 1
The major carotenoids accumulated in *E. coli* (JM101) transformants carrying plasmids

Transformant	Plasmids	Carotenoids accumulated in *E. coli* transformants
Astaxanthin-T	pACCAR16ΔcrtX[b], pAK96K[b]	Astaxanthin (88%), echinenone (3%), canthaxanthin (9%)
Zeaxanthin-glc-T	pACCRT-EIB[b], pCRT-XYZ[a]	Zeaxanthin β-diglucoside (78%), β-cryptoxanthin (16%), β-carotene (6%)
Zeaxanthin-T	pACCAR16ΔcrtX[b], pAK96NK[b]	Zeaxanthin
Canthaxanthin-T	pACCAR16ΔcrtX[b], pAK916[b]	Canthaxanthin
Carotene-T	pACCAR16ΔcrtX[b]	β-Carotene
Lycopene-T	pACCCRT-EIB[b]	Lycopene
Phytoene-T	pACCCRT-EIB[b]	Phytoene
Wild type	pACYC184, pBluescript SK$^+$	–

[a]This work.
[b]Misawa et al. [27].

*Kpn*I–*Eco*RI site of the *E. coli* vector pUC19 (Takarashuzo, Kusatsu, Japan).

E. coli JM101 was used as a host for the plasmids. Total carotenoid contents in the transformants were approximately 0.02 fmol/cell. Carotenoid compositions of the transformants are shown in Table 1.

2.2. The viability of the E. coli transformants in the $^1O_2$ generation mixture

The *E. coli* transformants producing various carotenoids were cultured in LB medium [31] including ampicillin (150 μg/ml) and/or chloramphenicol (30 μg/ml) at 27°C with shaking (80 min) in test tubes. In the present paper, the carotenoid producing transformant is abbreviated as carotenoid-T, e.g. astaxanthin-T. Growth was monitored by measuring optical density at 600 nm. Cells at early stationary phase were harvested by centrifugation, washed and suspended in the minimal medium [11] without vitamin B_{12}. The cell suspensions were dispensed in a polypropylene vial (∅17×51 mm) and mixed with the standard $^1O_2$ generation mixture described below. Final bacterial cell density in the reaction mixture was adjusted to 10^8 cells/ml. After the specified period at 25°C in the $^1O_2$ generating system, samples were taken, washed and the viable cells were enumerated by spreading in triplicate on an LB agar plate after appropriate dilutions. Colonies were counted after 24 h aerobic incubation at 37°C. The viability was expressed as percentage of viable cells of transformants exposed to $^1O_2$ generation medium relative to that of wild type.

2.3. Analysis of carotenoid compositions in E. coli transformants

Carotenoids were extracted from the transformant cells with $CHCl_3$–CH_3OH (1:2, v/v) according to Takaichi and Shimada [35]. The extracts were dried in vacuo, dissolved in acetone or MeOH, filtered through polycarbonate 0.22 μm filter and applied to a Tosoh high performance liquid chromatography system equipped with a photodiode array detector. A prepacked Radial-Pak Nova-Pak C_{18} cartridge (100×8 mm) installed in an RCM radial compression module was used for the analysis with methanol as a solvent (2.0 ml/min). Carotenoids were identified by their absorption spectra and comparing R_t values with those of authentic standards. Quantitative analysis was done by calculating peak areas of the chromatogram.

2.4. Estimation of oxidase activity in electron transport particles (ETPs)

ETPs from *E. coli* were prepared as described by Rakita et al. [30]. ETPs were suspended in 30 mM Tris buffer, pH 8.0, at a protein concentration of 0.05 mg/ml, and oxygen consumption was determined with a Clark-type oxygen electrode following addition of 31 mM sodium succinate, 62 mM D,L-lactate, 62 mM glycerol-3-phosphate, 0.94 mM NADH in a total volume of 1 ml. Results are expressed as nmol of O_2 consumed per mg protein on the basis of 215 μM O_2 in air-saturated buffer at 37°C. Protein concentration was determined by Bio-Rad Protein Assay kits (Bio-Rad Laboratories, CA, USA).

Determination of O_2 consumption or ATP synthesis from ADP by ETPs are described previously [37]. In all assays, electron donors including sodium succinate, NADH, D,L-lactate and glycerol-3-phosphate were used. Oxidase activity was expressed as nmol of O_2 consumed per minute per mg protein.

2.5. Determination of ATP from ADP by ETPs

The reaction mixture was essentially the same as that described in Section 2.4, except that 0.365 mM ADP and a kit of luciferin–luciferase containing Mg^{2+} (Lot.: 3971017, Kikkoman, Chiba, Japan) were included. The reaction was initiated by the addition of the electron donor and continued at 37°C with gentle agitation in a luminescence reader (Aloka BLR-301, Aloka, Japan). At 5 min after the initiation of the reaction, the luciferin–luciferase agent (0.1 ml) was injected into the reaction mixture and a brief intense emission was recorded with the luminescence reader. The intensity of the emission originating from the luciferin–luciferase–ATP reaction was calibrated with known concentrations of ATP and used for the quantification of ATP formed in the experimental system. The results were expressed as nmol of ATP formed per minute.

2.6. Analysis of $^1O_2$ quenching activity of carotenoids in micelles

Phytoene and zeaxanthin glucosides were purified from the corresponding transformants. Other carotenoids and α-tocopherol were purchased from Funakoshi (Tokyo, Japan) and Hoffman-La Roche (Switzerland). A *Cypridina luciferin* analog, 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-a] pyrazin-3-one (MCLA) and bovine

Table 2
Slope for plot of S_0/S versus carotenoids concentration and relative apparent k_q values

	N[a]	OH[b]	Slope×10^{-5} (M^{-1})	k_q^{rel}	
				Micelle	Org. solv.[c]
Phytoene	3	0	1.15	0.2	–
Lycopene	11	0	11.8	1.85	2.2
β-Carotene	11	0	6.2	1	1
Canthaxanthin	11+2	0	14.2	2.2	1.5
Astaxanthin	11+2	0	13.6	2.2	1.7
β-Cryptoxanthin	11	1	9.14	1.5	0.4
Zeaxanthin	11	2	6.45	1	0.7
Zeaxanthin-diglucoside	11	2	21.3	3.5	–
α-Tocopherol			0.66	0.1	0.02

k_q^{rel}: relative apparent k_q values.
[a]Number of conjugated C=C double bonds+number of C=O double bonds.
[b]Number of hydroxyl groups.
[c]Di Mascio et al. [6].

erythrocyte superoxide dismutase (SOD) were purchased from Tokyo Kasei (Tokyo, Japan) and Sigma, respectively. Bovine heart phosphatidyl choline (PC) (freeze-dried powder) was purchased from Funakoshi. MPO was extracted from human peripheral granulocytes and purified by column chromatography according to Morita et al. [26]. MPO activity was assayed and calculated according to Klebanoff et al. [15]. The standard $^1O_2$ generation mixture contained 0.0125 U/ml MPO, 5 mM KBr, 20 μM desferrioxamine, 0.5 μM SOD, 0.5 mM H_2O_2 and 0.1 M acetate buffer at pH 4.5 in a total volume of 2 ml [27,39]. $^1O_2$ concentration in the standard mixture was determined by MCLA-dependent luminescence. 15 μM MCLA was added to the generation mixture and $^1O_2$ concentration was calculated from a calibration line obtained using the HOCl+H_2O_2+MCLA as a standard $^1O_2$-mediated luminescence assay [16]. PC micelles were obtained by mixing PC in ethanol with carotenoids or α-tocopherol (in acetone) in acetate buffer. The reaction mixture for assay of the $^1O_2$ quenching activity contained all components of the standard reaction mixture, 580 μM PC and 15 μM MCLA in a total volume of 2 ml. The reaction was initiated by the addition of MPO and maintained at 25°C for 3 min. Quenching experiment of MCLA chemiluminescence with carotenoids was conducted in the presence of large excess H_2O_2 and photons emitted were counted during the initial 3 min with gentle agitation in a luminescence reader (Aloka BLR-301, Aloka, Japan). The photon count correlates linearly with concentration of MPO or HOCl. Namely, MCLA consumption is small so that MCLA concentration is approximated as constant, and singlet oxygen concentration is at the steady state under the condition. Therefore, assuming (a) carotenoids do not quench singlet excited state of emitter of MCLA chemiluminescence; (b) kinetics of reactions can be treated by kinetic equations that are applied for reactions in homogeneous solution, we obtain Eq. 1. S_0 and S denote photon count in the absence and the presence of polyene Q, during the initial 3 min. The k_q is the apparent second order rate constant for quenching of singlet oxygen quenching with polyene. The k_d, pseudo-first order rate constant for quenching of singlet oxygen with all components contained in the solution, is defined by Eq. 2, where k_{d1}–K_{d6} are second order rate constants for bimolecular quenching of singlet oxygen with components contained in the solution.

$$S_0/S = 1 + (k_q/k_d)[Q] \quad (1)$$

$$k_d = k_{d1}[MCLA] + k_{d2}[H_2O] + k_{d3}[SOD] + k_{d4}[buffer] + k_{d5}[desferrioxamine] + k_{d6}[MPO] \quad (2)$$

When polyene concentration is less than 1.25 μM, the plot of S_0/S versus [Q] was found to be linear. Values of k_q/k_d are obtained from the slope of this plot for each carotenoid (Table 2). Relative k_q values for carotenoids were calculated from k_q/k_d values (Table 2).

To obtain k_q, we have to estimate the k_d value. The k_{d1}[MCLA] in pH 7.12 phosphate buffer at 37°C is calculated to be 10^4 s^{-1} from $k_{d1} = 6.96\times10^8$ M^{-1} s^{-1} [10]. The k_{d1}[MCLA] in pH 4.5 and 25°C should be less than 10^4 s^{-1}, because MCLA reactivity depends on pH and temperature [1]. The $k_{d2}[H_2O] = 2.47\times10^5$ s^{-1} [10]. The $k_{d3}[SOD] = 1.4\times10^3$ s^{-1} from $k_{d3} = 2.7\times10^9$ M^{-1} s^{-1} [34]. The k_{d4}[buffer] was not determined during the present study and a value of 0.15×10^5 s^{-1} [10] was assumed from published literature. The k_{d5}[desferrioxamine] and k_{d6}[MPO] are also unknown. If we assume k_d is the same as that in 0.1 M sodium buffer solution, i.e. 2.62×10^5 s^{-1} [10], we obtain apparent k_q values for carotenoids by multiplying this value by the slope of the plot of S_0/S versus [Q] (Table 2).

3. Results and discussion

As shown in Table 3, viability of all the transformants in the standard $^1O_2$ generation mixture was higher than that of wild type *E. coli*. In transformants, viabilities of phytoene-T, lycopene-T, and zeaxanthin-glc-T were higher than those of other transformants (seven times higher than that of wild type cells). However, the viability of β-carotene-T was almost equal to that of wild type cells. Zeaxanthin with hydroxyl group increased viability more than canthaxanthin and astaxanthin with enolic keto group in *E. coli*.

The relative apparent k_q values (k_q^{rel}) for the $^1O_2$ quenching activity of the carotenoid micelle are shown in Table 2. The k_q^{rel} values of carotenoid micelles were approximately 10 times higher than that of α-tocopherol. The k_q^{rel} value of lycopene, an acyclic carotene, was higher than that of β-carotene, a cyclic carotene. Further, the k_q^{rel} values of canthaxanthin and astaxanthin with keto groups were higher than that of zeaxanthin with hydroxy groups. The k_q^{rel} value of zeaxanthin glucoside with several polar groups was the highest in the micelle.

The three types of carotenoids, phytoene, lycopene and zeaxanthin glucoside, showed powerful protective activity in *E. coli* against $^1O_2$ toxicity. It has been widely accepted that $^1O_2$ quenching activity of carotenoids in organic solvent is mainly dependent on the number of conjugated double bonds and is influenced to a lesser extent by carotenoid end groups (cyclic or acyclic) [6] or the nature of substituents in carotenoids containing cyclic end groups [3,4,9]. In the organic solvent, in which carotenoids are distributed uniformly, $^1O_2$ could easily attack the carotenoid molecule. On the other hand, carotenoids in micelle or phospholipid membrane are restricted in their mobility, thereby reducing the chance to react with $^1O_2$.

From the experimental results obtained with monolayer vesicles (a phospholipid liposome), Milon et al. [23] and Qurisson and Nakatani [29] have suggested that the carotenoids with two polar hydroxy groups, such as zeaxanthin, lie across the monolayer membrane with the chromophore in the lipid layer and both polar heads in water. Alternatively, β-carotene without polar head may be completely embedded in the lipid layer of the membrane [33]. In the present work with carotenoid–phospholipid micelles (Table 2), the relative apparent k_q values (k_q^{rel}) of lycopene micelle were higher than that of β-carotene. The k_q^{rel} value of hydroxy carotenoids in the micelle is higher than those in the organic solvent. The k_q^{rel} value of zeaxanthin glucoside was the highest of all in the micelle. A good correlation between viability of carotenoid transformant (Table 3) and $^1O_2$ quenching activity of carotenoid PC micelles can be seen in the case of zeaxanthin-glc. If a carotenoid is located at the surface of a bacterial membrane, it can easily quench a short-lived $^1O_2$ and then it could efficiently protect against $^1O_2$-mediated bacterial killing.

In contrast to this assumption, phytoene, which had very weak $^1O_2$ quenching activity in phospholipid micelles, exerted

Table 3
The viability of transformant and control *E. coli* in $^1O_2$ generation medium (10 μM)

Strains	Viability (%) ± S.E.M.[a]	The ratio of viability[b]
Control	5.4 ± 0.57	1
Phytoene-T	38.9 ± 2.88	7.2
Lycopene-T	39.5 ± 2.71	7.3
β-Carotene-T	8.0 ± 1.70	1.5
Canthaxanthin-T	16.0 ± 2.09	2.9
Astaxanthin-T	11.9 ± 1.98	2.2
Zeaxanthin-T	25.7 ± 3.27	4.7
Zeaxanthin-glc-T	38.7 ± 3.36	7.1

[a]Standard error of mean ($n = 12$).
[b]The percentage of transformant *E. coli* viability/control *E. coli* viability.

Table 4
Wild type and transformant *E. coli* oxidase activities[a] and ATP formation[b]

Type of *E. coli*	Substrates	Oxidase activities	ATP formation
Wild type	Succinate	736.8 ± 46.3	39.8
	NADH	485.2 ± 32.1	84.2
	D,L-Lactate	102.3 ± 7.97	39.1
	Glycerol-3-phosphate	58.8 ± 0.98	13.5
Phytoene-T	Succinate	64.0 ± 4.67	36.4
	NADH	58.1 ± 6.23	76.5
	D,L-Lactate	36.1 ± 3.00	44.7
	Glycerol-3-phosphate	31.9 ± 3.33	17.1
Lycopene-T	Succinate	249.3 ± 10.3	25.1
	NADH	195.9 ± 16.4	59.5
	D,L-Lactate	48.5 ± 3.00	22.2
	Glycerol-3-phosphate	30.3 ± 6.54	9.6

[a]The oxidase activity of ETPs prepared from wild type and transformant *E. coli* was measured with substrates indicated. The oxidase activity was expressed as nmol of O_2 consumed per minute per mg protein.
[b]The formation of ATP from ADP of ETPs was expressed as nmol of ATP formed per minute per mg protein.

a powerful antibactericidal effect on this transformant (Table 3). It seemed to exert a powerful antibactericidal effect in *E. coli* by some unknown mechanisms other than quenching $^1O_2$. It was reported that isoprenoids, a precursor of carotenoid, incorporated in liposomes of phospholipid increase the rigidity of the liposome vesicles [17]. In this respect, phytoene which incorporated in a bacterial membrane may also have a stabilizing effect on the membrane. Such an antibactericidal activity of phytoene may arise from stabilizing the bacterial membrane by the interaction of membrane phospholipid with isoprenoid chain of phytoene to protect against $^1O_2$ toxicity. In the previous report, we suggested that lycopene causes structural changes in the membrane of transformant *E. coli* [37]. As reported previously [37], bacterial lycopene significantly influenced respiratory chain enzymes involved in the electron transport system. This is clear evidence that bacterial carotenoid brings about a structural change in the bacterial cytoplasmic membrane in which an electron transport system is present.

O_2 consumption and ATP formation in ETPs from wild type *E. coli*, phytoene-T and lycopene-T are shown in Table 4. O_2 consumption for succinate oxidation and for NADH oxidation in the wild type *E. coli* were several times higher than those of transformants. However, ATP formation for these substrates in wild type *E. coli* was almost identical to that in transformants. Since ATP formed from ADP in our system would be hydrolyzed by the ATPase system in ETPs, it is likely that the amount of ATP measured is less than the true amount of ATP formed. According to the data shown in Table 4, phytoene also causes structural change in the membrane of transformant.

Tuveson and Sandmann [38] have reported that lycopene transformant *E. coli* were more sensitive to a photosensitizer which generates $^1O_2$ by energy transfer than a control *E. coli*. The authors have suggested that acyclic carotenoids do not exhibit a protective effect against photosensitizers in triplet states or $^1O_2$. However, under their experimental conditions, free radicals such as O_2^-, $^\bullet OH$ and sensitizer radicals, which are generated by type 1 reaction [7], may oxidize lycopene to lycopene radicals, such as β-carotene [2], thereby promoting membrane damage in *E. coli*. Sandmann et al. also reported [32] that zeaxanthin protected against the photosensitized UV-B treatment in *E. coli* for direct interaction with sensitizing molecules.

Further testing will be required to elucidate the bactericidal mechanism of $^1O_2$ and the protective function of isoprenoids, such as carotenoids, in the prokaryotic cell against $^1O_2$.

References

[1] Akutsu, K., Nakajima, H. and Fujimori, K. (1995) J. Chem. Soc. Perkin Trans. 2, 1699–1706.
[2] Burton, G.W. and Ingold, K.U. (1984) Science 224, 569–573.
[3] Dahl, T.A., Midden, W.R. and Hartman, P.E. (1987) Photochem. Photobiol. 46, 345–352.
[4] Dahl, T.A., Midden, W.R. and Hartman, P.E. (1988) Mutat. Res. 201, 127–136.
[5] Dahl, T.A., Midden, W.R. and Hartman, P.E. (1989) J. Bacteriol. 171, 2188–2194.
[6] Di Mascio, P., Kaiser, S. and Sies, H. (1989) Arch. Biochem. Biophys. 274, 532–538.
[7] Foote, C.S. (1946) in: Free Radicals in Biology, Vol. 2, pp. 85–133, Academic Press, New York.
[8] Foote, C.S. (1968) Science 162, 963–970.
[9] Foote, C.S., Chang, Y.C. and Denny, R.W. (1970) J. Am. Chem. 92, 5216–5218.
[10] Fujimori, K., Komiyama, T., Tabata, H., Nojima, T., Ishiguro, K., Sawaki, Y., Tatsuzawa, H. and Nakano, M. (1998) Photochem. Photobiol. 68, 143–149.
[11] Hassan, H.M. and Fridovich, I. (1978) J. Biol. Chem. 22, 8143–8148.
[12] Ito, T. (1978) Photochem. Photobiol. 28, 493–508.
[13] Kanofsky, J.R. (1989) Chem. Biol. Interact. 70, 1–28.
[14] Kawanishi, S., Inoue, S., Sano, S. and Aiba, H. (1986) J. Biol. Chem. 261, 6090–6095.
[15] Klebanoff, S.J., Waltersdorph, A.M. and Rosen, H. (1984) Methods Enzymol. 105, 399–403.
[16] Koga, S. and Nakano, M. (1991) Arch. Biochem. Biophys. 289, 223–229.
[17] Krajewski-Bertrand, M.A., Hayer, M., Wolff, G., Milon, A., Albrecht, A.M., Heissler, D., Nakatani, Y. and Qurisson, G. (1990) Tetrahedron 46, 3143–3154.
[18] Krinsky, N.I. (1968) in: Photophysiology – Current Topics (Giese, A.C., Ed.), p. 123, Academic Press, New York.
[19] Krinsky, N.I. (1971) in: Carotenoids (Isler, O., Gutman, H. and Solms, U., Eds.), p. 669, Birkhauser, Basel.
[20] Krinsky, N.I. (1974) Science 186, 363–365.
[21] Krinsky, N.I. (1979) Pure Appl. Chem. 51, 649–660.
[22] Mathews-Roth, M.M. and Micheline, M. (1984) Photochem. Photobiol. 40, 63–67.
[23] Milon, A., Wolff, G., Ourisson, G. and Nakayama, Y. (1986) Helv. Chim. Acta 69, 12–24.
[24] Misawa, N., Nakagawa, M., Kobayashi, K., Yamano, S., Izawa, Y., Nakamura, K. and Harashima, K. (1990) J. Bacteriol. 172, 6704–6712.
[25] Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A., Kajiwara,

S., Saito, T., Ohtani, T. and Miki, W. (1995) J. Bacteriol. 177, 6575–6584.
[26] Morita, Y., Iwamoto, H., Aibara, S., Kobayashi, T. and Hasegawa, E. (1986) J. Biochem. 99, 761–770.
[27] Nakano, M. (1990) Methods Enzymol. 186, 585–591.
[28] Nakano, M., Kambayashi, Y., Tatsuzawa, H., Komiyama, T. and Fujimori, K. (1998) FEBS Lett. 432, 9–12.
[29] Ourisson, G. and Nakatani, Y. (1990) in: Carotenoids – Chemistry and Biology (Krinsky, N.I., Mathews-Roth, M.M. and Taylor, R.F., Eds.), pp. 237–245.
[30] Rakita, R.M., Michel, B.R. and Rosen, H. (1990) Biochemistry 29, 1075–1080.
[31] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning: a Laboratory Manual, 2nd edn., Appendix A, CSH press.
[32] Sandmann, G., Kuhn, S. and Boger, P. (1998) Appl. Environ. Microbiol. 64, 1972–1974.
[33] Strzalka, K. and Gruszecki, W.I. (1994) Biochim. Biophys. Acta 1194, 138–142.
[34] Suzuki, N., Mizumoto, I., Toya, Y., Nomoto, T., Mashiko, S. and Inaba, H. (1990) Agric. Biol. Chem. 54, 2783–2787.
[35] Takaichi, S. and Shimada, K. (1988) Methods Enzymol. 213, 374–385.
[36] Takaichi, S., Sandmann, G., Schunrr, G., Satomi, Y., Suzuki, A. and Misawa, N. (1996) Eur. J. Biochem. 241, 291–296.
[37] Tatsuzawa, H., Maruyama, T., Misawa, N., Fujimori, K., Hori, K., Sano, Y., Kanbayashi, Y. and Nakano, M. (1998) FEBS Lett. 439, 329–333.
[38] Tuveson, R.W. and Sandmann, G. (1993) Methods Enzymol. 214, 323–330.
[39] Uehara, K., Hori, K., Nakano, M. and Koga, S. (1991) Anal. Biochem. 199, 191–196.
[40] Wefers, H., Schulte-Frohlinde, D. and Sies, H. (1987) FEBS Lett. 211, 49–52.
[41] Weishaupt, K.R., Gomer, C.J. and Dougherty, T.J. (1976) Cancer Res. 36, 2326–2329.