Appl Microbiol Biotechnol (1997) 48: 351–356

ORIGINAL PAPER

M. Kobayashi · T. Kakizono · N. Nishio · S. Nagai
Y. Kurimura · Y. Tsuji

Antioxidant role of astaxanthin in the green alga *Haematococcus pluvialis*

Received: 13 January 1997 / Received revision: 26 February 1997 / Accepted: 27 March 1997

Abstract The green unicellular alga, *Haematococcus pluvialis* has two antioxidative mechanisms against environmental oxidative stress: antioxidative enzymes in vegetative cells and the antioxidative ketocarotenoid, astaxanthin, in cyst cells. We added a reagent that generates superoxide anion radicals (O_2^-), methyl viologen, to mature and immature cysts of *H. pluvialis*. Tolerance to methyl viologen was higher in mature than in immature cysts. Mature (astaxanthin-rich) cysts showed high antioxidant activity against O_2^- in permeabilized cells, but not in astaxanthin-free cell extracts, while immature (astaxanthin-poor) cysts had very low antioxidant activities against O_2^- in both. The results suggested that astaxanthin accumulated in the cyst cells functions as an antioxidant against excessive oxidative stress. The same levels of antioxidant activities against O_2^- in both permeabilized cells and cell extracts from vegetative cells suggested the presence of antioxidative enzymes (superoxide dismutase).

Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a red ketocarotenoid that is used as a pigmentation source for marine fish aquaculture (Benemann 1992) and is also being investigated for medical applications because it is more antioxidative than β-carotene and vitamin E (Terao 1989; Miki 1991; Palozza and Krinsky 1992). The green unicellular alga, *Haematococcus pluvialis*, is a potent producer of astaxanthin (Borowitzka et al. 1991; Boussiba and Vonshak 1991; Kobayashi et al. 1991). In the algal life cycle of *H. pluvialis*, green vegetative cells with two flagellae grow autotrophically in the light (Lee and Soh 1991) and heterotrophically in the dark (Droop 1955; Kobayashi et al. 1992). Deficiency of a nutrient, such as nitrogen, induces encystment, with a morphological change from vegetative to cyst cells as the resting stage (Kakizono et al. 1992). Carotenoid biosynthesis in cyst cells is enhanced by environmental oxidative stresses (Kobayashi et al. 1993). Therefore, oxidative stress was considered to be involved in the regulation of astaxanthin biosynthesis in *H. pluvialis*. Active oxygen species regulate carotenoid biosynthesis in some microorganisms such as yeast *Phaffia rhodozyma* (Schroeder and Johnson 1993, 1995a), green algae, *H. pluvialis* (Kobayashi et al. 1993) and *Dunaliella bardawil* (Shaish et al. 1993). Furthermore, the accumulated carotenoids might function as a protective agent against oxidative stress damage (Shaish et al. 1993; Schroeder and Johnson 1995b). Although *P. rhodozyma* and *D. bardawil* are resistant to experimentally induced oxidative damage (Shaish et al. 1993; Schroeder and Johnson 1995b), the physiological role of carotenoids in vivo is not clear.

Among carotenoid-producing organisms, *H. pluvialis* has a unique morphogenetic response to environmental stress and produces carotenoid only after encystment (Kobayashi et al. 1993). Since this alga has the important features of photosynthesis, motility, and morphogenesis, it could serve as a model to investigate the functions of carotenoids in microorganisms. In this study, we measured antioxidant abilities in vivo against superoxide anion radicals (O_2^-) using the whole-cell assay system to elucidate the physiological role of astaxanthin in cyst cells of *H. pluvialis*. The results showed that *H. pluvialis* has antioxidative enzymes in vegetative cells and the antioxidative ketocarotenoid, astaxanthin, in cyst cells, that protect the alga against environmental oxidative stress.

M. Kobayashi (✉) · Y. Kurimura · Y. Tsuji
Research Laboratory of Higashimaru Shoyu Co. Ltd.,
100-3 Tominaga, Tatsuno, Hyogo, 679-41, Japan
Fax: +81-791-63-4852
e-mail: kuri@super.win.or.jp

K. Kakizono · N. Nishio · S. Nagai
Department of Fermentation Technology, Faculty of Engineering,
Hiroshima University, Higashi-Hiroshima 724, Japan

Materials and methods

Algal strain and basal culture

Haematococcus pluvialis Flotow NIES-144 was obtained from the National Institute for Environmental Studies, Tsukuba, Japan. The acetate (15 mM) basal medium was as described (Kobayashi et al. 1991). For the basal culture, a 10-ml portion of a 4-day culture was inoculated into 100 ml fresh basal medium in a 200-ml conical flask. The flask was incubated at 20 °C under a 12-h light/12-h dark illumination cycle at 20 μmol m^{-2} s^{-1} (white fluorescent lamp) as described (Kobayashi et al. 1991). The 4-day culture (vegetative cell growth phase, approx. 5.5×10^5 cells/ml) was used as the supplementation culture.

Supplementation culture

Ferrous sulfate (450 μM) or sodium acetate (45 mM) or both were added to the 4-day basal culture, to prepare *H. pluvialis* vegetative, immature cyst and mature cyst cells. Immature (astaxanthin-poor) cysts were induced by adding acetate alone (Kakizono et al. 1992; Kobayashi et al. 1993). In the presence of acetate plus Fe^{2+}, cyst formation was accompanied by enhanced astaxanthin biosynthesis (Kobayashi et al. 1993). Thus, mature (astaxanthin-rich) cysts were prepared using acetate/Fe^{2+}-supplementation cultures. When Fe^{2+} was added alone, the algal cells maintained vegetative growth until the stationary phase (Kobayashi et al. 1993). The supplementation cultures were incubated for 4 days after the additions at 20 °C under continuous illumination (120 μmol m^{-2} s^{-1}) as described (Kobayashi et al. 1993). All cultures were incubated in triplicate.

Methyl viologen exposure

Methyl viologen (MV, herbicide, Paraquat) is reduced by photosynthetic electron transport yielding the viologen radical, which rapidly autooxidizes to produce O_2^- (Rabinowitch et al. 1987). An MV solution was filtered through a membrane (Dismic-25, 0.45-μm pore size; Advantec, Tokyo, Japan), and added to 4-day supplementation cultures, which were then incubated for 3 days at 20 °C under continuous illumination (120 μmol m^{-2} s^{-1}). All cultures were incubated in triplicate.

Photosynthesis and respiration activities

The photosynthetic and respiratory rates were measured by the method of Martínez and Orús (1991) as described (Kobayashi et al. 1992). To assay the photosynthetic rate, cells were harvested by centrifugation at 2000 *g* for 10 min, and washed twice in 20 mM HEPES/NaOH buffer (pH 8.0). An O_2-free cell suspension was prepared in a water-jacketed transparent cylinder equipped with an oxygen electrode (DG-5G, Able, Tokyo, Japan) followed by N_2 bubbling for 0.5 h, then incubated at 20 °C under light at 240 μmol m^{-2} s^{-1}. After addition of 12 mM $NaHCO_3$, the O_2 evolution rate was measured as the increase in the dissolved oxygen concentration. To determine the respiratory rate, the cells were washed twice with fresh basal medium, resuspended in 22.5 mM acetate and bubbled with air for 0.5 h, then placed in a water-jacketed cylinder equipped with an oxygen electrode. The suspension was incubated at 20 °C in the dark. The O_2 uptake rate was measured as the decrease in the dissolved oxygen concentration. The cell concentration was adjusted to about 1.0 mg dry weight/ml in each experiment. All activities were assayed in triplicate cultures, and their averages are reported.

Antioxidant activity against O_2^-

The assay for superoxide dismutase was used to assess antioxidant activity against O_2^-. Toluene-treated-permeabilized cell (whole-cell) and the cell-free extract activities were determined according to Whitelam and Codd (1982). Each cell type was harvested by centrifugation at 2000 *g* for 10 min, washed and resuspended in 25 mM TRIS/HCl buffer (pH 8.0) containing 1 mM EDTA. The cell suspensions were adjusted to a concentration of 0.3–1.0 mg protein/ml. Toluene (2 ml) was added to a 4-ml aliquot of the cell suspension, and the mixture was shaken for 5 min. Thereafter, the toluene/cell mixture was allowed to stand on ice for 5 min, and the upper toluene layer was removed with a pasteur pipette. Cell-free extracts were prepared by 5×30-s bursts of ultrasonication (Sonicator W-385, Heat Systems-Ultrasonics Inc., New York, USA) on ice, followed by centrifugation (20 000 *g* for 20 min). The toluene-treated cells and the cell-free extracts were then assayed immediately for antioxidant activity against O_2^-. For the photochemical assay for antioxidant activity, a 200-μl aliquot of toluene-treated cells or cell-free extracts was added to 5 ml 25 mM TRIS/HCl buffer (pH 8.0) containing 1 mM EDTA, 10 μM flavin mononucleotide, and 5 mM hydroxylamine under aerobic/light conditions (140 μmol m^{-2} s^{-1}). At various times a 0.5-ml aliquot was removed and the nitrite formed was determined as described by Elstner and Heupel (1976). One unit of antioxidant activity against O_2^- is defined as the amount that causes a 50% decrease in the rate of nitrite formation from the oxidation of hydroxylamine by O_2^-. Bovine blood superoxide dismutase was used as the standard. Whitelam and Codd (1982) reported that the whole-cell assay system facilitated easy monitoring of superoxide dismutase sensitivity to its specific inhibitors, KCN and H_2O_2. To classify the type of superoxide dismutase of *H. pluvialis*, whole-cell activities were assayed in the presence of 1 mM KCN or H_2O_2. All activities were assayed in triplicate, and their averages are reported.

Analyses

The algal cell number and dry weight were determined as described (Kobayashi et al. 1991). For the protein assay, the algal cells were suspended in 2 M NaOH for 1 h on ice as described by Whitelam and Codd (1982), and the alkaline-solubilized protein was determined by the Bradford method (Bradford 1976), with bovine serum albumin as the standard. Intracellular carotenoid and chlorophyll were extracted with 90% acetone, and assayed as described (Kobayashi et al. 1991, 1993). The total carotenoid concentrations were determined at A_{480} using the absorption coefficient $A_{1\%} = 2500$ according to Strickland and Parsons (1968). The carotenoids were applied on silica gel plate (Kieselgel 60, 0.2 mm thickness; Merck, Darmstadt, Germany), and developed in the solvent system (acetone/*n*-hexane, 3:7 v/v) in the dark. The carotenoids were identified from their R_F values (Renstrøm et al. 1981) and by comparison with the authentic carotenoids (Kobayashi et al. 1991). The ratio of the carotenoids was determined by absorption at 545 nm with a TLC scanner (CS-930, Shimadzu, Kyoto, Japan). Thus, the concentration of astaxanthin was calculated on the basis of the proportion of the carotenoid composition (Kobayashi et al. 1993). All analyses were performed in triplicate cultures, and their averages are reported.

Chemicals

MV, bovine blood superoxide dismutase, flavin mononucleotide, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, USA, and all other reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Results

Cell damage by excessive oxidative stress

To investigate whether or not astaxanthin in the cyst cells of *H. pluvialis* protects the alga against excessive oxidative stress by O_2^-, we added MV (O_2^- generator) to

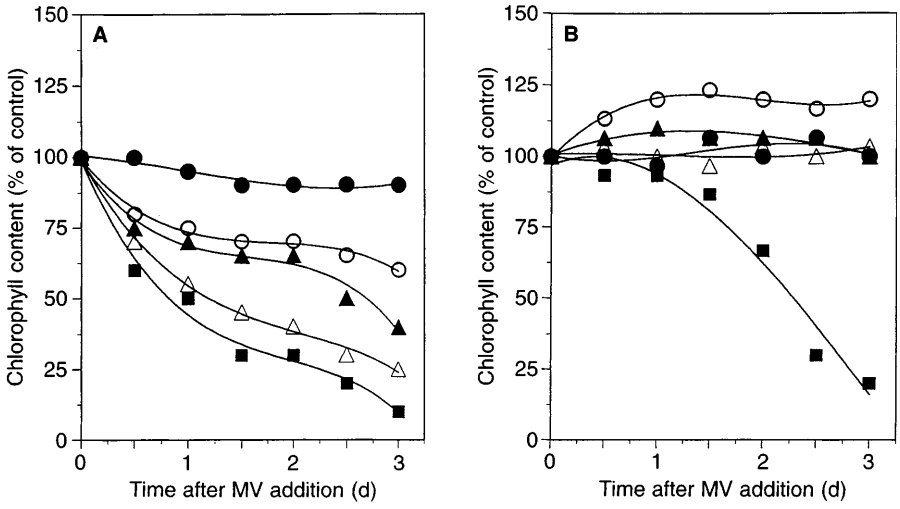

Fig. 1 Loss of chlorophyll content with time after exposure of immature (**A**) and mature (**B**) cyst cells of *Haematococcus pluvialis* to methyl viologen (*MV*). Immature and mature cysts were prepared by acetate and acetate/Fe^{2+} supplementation respectively. The zero-day control value (100%) is shown. MV concentrations: ○ 1 μM, ▲ 10 μM, △ 0.1 mM, ■ 1 mM, ● none

immature (low astaxanthin, 10 pg/cell) and mature (high astaxanthin, 50 pg/cell) cysts, then measured the residual chlorophyll content (Fig. 1). The 0-day chlorophyll content was about 10 pg/cell in both cysts. The chlorophyll content was markedly decreased in immature cysts, but was not affected in mature cysts. Chlorophyll was not degraded in mature cysts even by 0.1 mM MV as shown Fig. 1. In mature cysts, the degradation of astaxanthin did not proceed at 0.1 mM MV, and the residual astaxanthin content decreased about 50% after 3 days MV exposure at 1 mM (data not shown).

To investigate further the role of astaxanthin in preventing oxidative damage, we measured the residual metabolic activities (photosynthesis and respiration) of two types of cyst after 3 days MV exposure (Table 1). In mature cyst cells, photosynthesis was eliminated at 0.1 mM MV, whereas respiration was maintained. MV at 1 mM reduced respiration by more than 50%. In contrast, in immature cyst cells, respiratory activity was reduced by half at 1 μM MV, and both photosynthesis and respiration were eliminated by 0.1 mM MV. These results indicated that "astaxanthin-rich" red cyst cells are more tolerant towards excessive oxidative stress than are "astaxanthin-poor" cyst cells.

Antioxidant abilities in vivo against O_2^-

To investigate whether or not the astaxanthin functions as an antioxidant by reacting with active oxygen species, we studied the antioxidant ability of astaxanthin in cyst cells of *H. pluvialis* in vivo against O_2^- (Table 2). Antioxidant activity against O_2^- was estimated as units/10^7 cells as well as units/mg protein because cyst cells had a very low protein content. In green vegetative cells of *H. pluvialis*, the antioxidant activity of toluene-treated permeabilized cells (whole cells) was similar to that of cell-free extracts. In whole vegetative cells, antioxidant (superoxide-dismutase-like) activity was destroyed by boiling cells for 10 min (data not shown), and was sensitive to incubation in the presence of either KCN or H_2O_2. These results suggest the presence of antioxidant enzymes, Cu/Zn superoxide dismutase (sensitive to both KCN and H_2O_2), in vegetative cells.

Compared with vegetative cells, "astaxanthin-poor" immature cyst cells had lower antioxidant activity against O_2^- in both whole-cell and cell-free extract assays (Table 2). On the other hand, the whole-cell assay of "astaxanthin-rich" red mature cyst cells showed about 50-fold higher antioxidant activity against O_2^- than that

Table 1 Residual activities of photosynthesis and respiration in two types of cyst cell of *Haematococcus pluvialis* after exposure to methyl viologen (O_2^- generator). Immature cysts were prepared by acetate supplementation; mature cysts were prepared by acetate/Fe^{2+} supplementation. Cultures were incubated for 3 days after methyl viologen exposure, and both photosynthesis and respiratory activities were assayed. Values to the value in the absence of methyl viologen (control; 100%), are shown in parentheses

Methyl viologen (M)	O_2 turnover [μmol O_2 (mg cells)$^{-1}$ h^{-1}]			
	Immature cysts		Mature cysts	
	Photosynthesis	Respiration	Photosynthesis	Respiration
0	0.058 (100)	0.079 (100)	0.240 (100)	0.132 (100)
10^{-6}	0.061 (105)	0.039 (49)	0.247 (103)	0.208 (158)
10^{-5}	0.070 (121)	0.029 (37)	0.261 (109)	0.196 (148)
10^{-4}	0	0	0	0.146 (111)
10^{-3}	0	0	0	0.049 (37)

Table 2 Antioxidant activities against O_2^- in vegetative and cyst cells of *H. pluvialis* in assays of whole cells and cell-free extract. Vegetative cells were prepared by Fe^{2+} supplementation; immature cysts were prepared by acetate supplementation; mature cysts were prepared by acetate/Fe^{2+} supplementation. Antioxidant activities against O_2^- for whole cells were also assayed in the presence of KCN (1 mM) or H_2O_2 (1 mM). *ND* not determined

Cell type	Astaxanthin content ($mg/10^7$ cell)	Antioxidant activity against O_2^-					
		Cell-free extract assay		Whole-cell assay		Whole-cell assay ($U/10^7$ cell)	
		(U/mg protein)	($U/10^7$ cell)	(U/mg protein)	($U/10^7$ cell)	+KCN (1 mM)	$+H_2O_2$ (1 mM)
Vegetative cell	0.067	7.41	11.5	6.06	11.3	3.48	2.69
Immature cyst	0.113	2.40	2.52	6.62	6.95	ND	ND
Mature cell	0.497	3.18	0.65	163.9	33.6	33.4	33.0

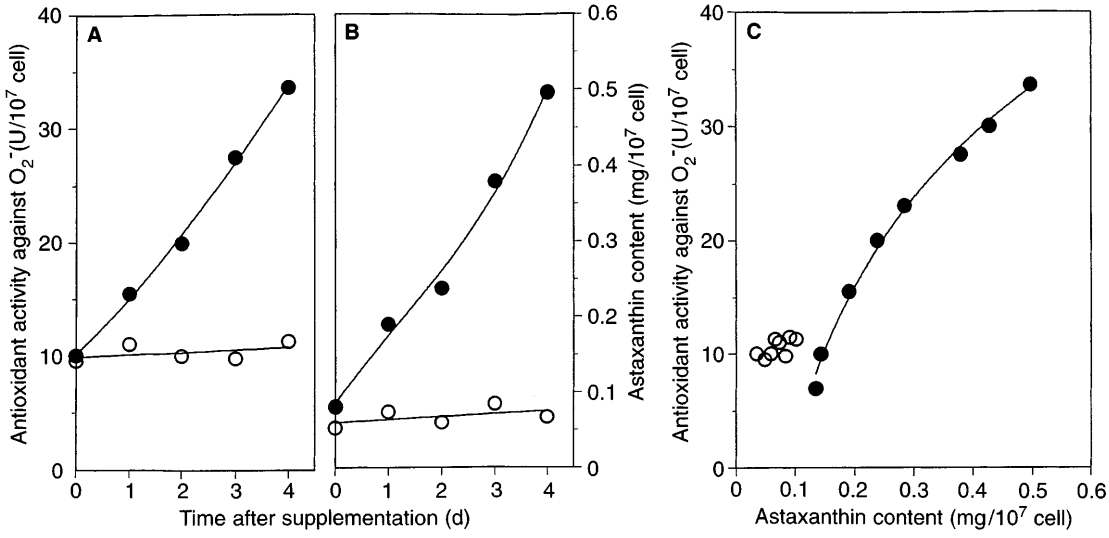

Fig. 2 Changes in antioxidant activity against O_2^- in the whole-cell assay (**A**), astaxanthin content (**B**), and correlation between antioxidant activity against O_2^- and astaxanthin content (**C**), in vegetative and cyst cells of *H. pluvialis*. Vegetative and cyst cells were prepared by Fe^{2+} and acetate/Fe^{2+} supplementation respectively. The supplementation time was scaled as day zero. ○ Vegetative cells, ● cyst cells

in the cell-free extract (astaxanthin-free). Moreover, the antioxidant activity was not destroyed by boiling whole cells of mature cysts for 10 min (data not shown), and was insensitive to both KCN and H_2O_2. The increase of antioxidant activity against O_2^- in the whole-cell assay was closely associated with maturation of cyst cells (Fig. 2A), and correlated with increased intracellular astaxanthin contents (Fig. 2B, C). These results indicate that astaxanthin in the cyst cells functions as an antioxidant against environmental oxidative stress.

Discussion

In this study, we determined the antioxidant activities of *H. pluvialis* by measuring the capacities of cell extracts or whole cells to inhibit O_2^--mediated nitrite formation. The results suggested that cyst cells might be deficient in the enzymes (superoxide dismutase) or low-molecular-mass antioxidants, such as ascorbate and glutathione, necessary to detoxify active oxygen species. It also appeared that astaxanthin located in cytosolic lipid globules and membraneous regions of cysts (Santos and Mesquita 1984) could compensate for the lack of these enzymes and antioxidants. Furthermore, it was shown that astaxanthin can prevent oxidative injury to cyst cells caused by exposure to reactive oxygen species generated by MV. Carotenoids and O_2^- do not directly react with each other without the involvement of a more reactive oxygen intermediate (Krinsky 1989). Therefore, astaxanthin might function as an antioxidant by reacting with active oxygen radicals derived from O_2^-, providing antioxidant protection in the cytoplasm and in lipid membranes.

Generally, carotenoids have two important roles in photosynthetic organisms (Young 1991). First, they act as accessory light-harvesting pigments, trapping light energy and passing it on to chlorophylls. Second, and more importantly, carotenoids protect the photosynthetic apparatus from light-mediated stress, for example by quenching singlet oxygen ($^1O_2$) generated by photooxidation. In the green alga, *D. bardawil*, it has been suggested that photosynthetically produced active oxy-

gen species, O_2^- (and its products) and $^1O_2$, are involved in triggering β-carotene biosynthesis, and the massive amount of carotenoid accumulated can protect the photosynthetic apparatus against oxidative stress (Shaish et al. 1993). Carotenoids scavenge/quench several active oxygen species such as $^1O_2$, O_2^-, H_2O_2, peroxy radicals, and hydroxyl radicals ($HO^\bullet$) both in vitro and in vivo (Burton 1989; Krinsky 1989; Miki 1991; Lim et al. 1992; Lawlor and O'Brien 1995). In the nonphotosynthetic bacterium *Deinococcus radiodurans* (Carbonneau et al. 1989), the fungus *Fusarium aquaeductuum* (Theimer and Rau 1970), yeasts *Rhodotorula mucilaginosa* (Moore et al. 1989) and *P. rhodozyma* (Schroeder and Johnson 1995b), carotenoids protect against experimentally induced oxidative damage. The red-pigmented strain of *D. radiodurans* is resistant to $HO^\bullet$, whereas the colorless strain is significantly sensitive (Carbonneau et al. 1989). In *F. aquaeductuum*, H_2O_2 induces carotenoid biosynthesis in the dark, a process that normally occurs only under illumination (Theimer and Rau 1970). In *R. mucilaginosa*, β-carotene protects the cells against O_2^- (Moore et al. 1989). In *P. rhodozyma*, Schroeder and Johnson (1995b) reported that several active oxygen species ($^1O_2$, O_2^-, H_2O_2, and peroxy radicals) regulate astaxanthin biosynthesis. Astaxanthin could play a protective role in yeast against oxidative stress, as we suggested for *H. pluvialis* in this study.

Adding azide, an inhibitor of superoxide dismutase to *D. bardawil*, greatly enhanced β-carotene biosynthesis against the accumulation of reactive oxygen species (Shaish et al. 1993). Thus, intracellular β-carotene may function together with superoxide dismutase to preserve viability in the face of the continued production of oxygen radicals in aging cells. *H. pluvialis* has two antioxidative mechanisms, involving antioxidative enzymes in vegetative cells and antioxidative astaxanthin in cyst cells, and it might change from one of these defense systems against environmental oxidative stress to the other during its life cycle. In conclusion, it seems likely that astaxanthin biosynthesis is an adaptive response against oxidative stress in cyst cells of *H. pluvialis*. The alga has developed an efficient defense system that helps it to survive under environmentally adverse conditions during evolution.

Acknowledgements We thank Takeshi Konishi for technical assistance. This work was supported in part by grant 6W04 from Hyogo Science and Technology Association, Hyogo, Japan.

References

Benemann JR (1992) Microalgae aquaculture feeds. J Appl Phycol 4: 233–245

Borowitzka MA, Huisman JM, Osborn A (1991) Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. 1. Effect of nutrients on growth and cell type. J Appl Phycol 3: 295–304

Boussiba S, Vonshak A (1991) Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Plant Cell Physiol 32: 1077–1082

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254

Burton GW (1989) Antioxidant action of carotenoids. J Nutr 119: 109–111

Carbonneau MA, Melin AM, Perromat A, Clerc M (1989) The action of free radicals on *Deinococcus radiodurans* carotenoids. Arch Biochem Biophys 275: 244–251

Droop MR (1955) Carotenogenesis in *Haematococcus pluvialis*. Nature 175: 42

Elstner EF, Heupel A (1976) Inhibition of nitrite formation from hydroxylammonium chloride: a simple assay for superoxide dismutase. Anal Biochem 70: 616–620

Kakizono T, Kobayashi M, Nagai S (1992) Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, *Haematococcus pluvialis*. J Ferment Bioeng 74: 403–405

Kobayashi M, Kakizono T, Nagai S (1991) Astaxanthin production by a green alga, *Haematococcus pluvialis* accompanied with morphological changes in acetate media. J Ferment Bioeng 71: 335–339

Kobayashi M, Kakizono T, Yamaguchi K, Nishio N, Nagai S (1992) Growth and astaxanthin formation of *Haematococcus pluvialis* in heterotrophic and mixotrophic conditions. J Ferment Bioeng 74: 17–20

Kobayashi M, Kakizono T, Nagai S (1993) Enhanced carotenoid biosynthesis by oxidative stress in acetate-induced cyst cells of a green unicellular alga, *Haematococcus pluvialis*. Appl Environ Microbiol 59: 867–873

Krinsky NI (1989) Antioxidant functions of carotenoids. Free Radic Biol Med 7: 617–635

Lawlor SM, O'Brien NM (1995) Astaxanthin: antioxidant effects in chicken embryo fibroblasts. Nutr Res 15: 1695–1704

Lee YK, Soh CW (1991) Accumulation of astaxanthin in *Haematococcus lacustris* (Chlorophyta). J Phycol 27: 575–577

Lim BP, Nagao A, Terao J, Tanaka K, Suzuki T, Takama K (1992) Antioxidant activity of xanthophylls on peroxy radical-mediated phospholipid peroxidation. Biochim Biophys Acta 1126: 178–184

Martínez F, Orús MI (1991) Interactions between glucose and inorganic carbon metabolism in *Chlorella vulgaris* strain UAM 101. Plant Physiol 95: 1150–1155

Miki W (1991) Biological functions and activities of animal carotenoids. Pure Appl Chem 63: 141–146

Moore MM, Breedveld NW, Autor AP (1989) The role of carotenoids in preventing oxidative damage in the pigmented yeast, *Rhodotorula mucilaginosa*. Arch Biochem Biophys 270: 419–431

Palozza P, Krinsky NI (1992) Astaxanthin and canthaxanthin are potent antioxidants in a membrane model. Arch Biochem Biophys 297: 291–295

Rabinowitch HD, Privalle CT, Fridovich I (1987) Effects of paraquat on the green alga *Dunaliella salina*: protection by the mimic of superoxide dismutase, desferal-Mn(IV). Free Radic Biol Med 3: 125–131

Renstrøm B, Borch G, Skulberg OM, Liaaen-Jensen S (1981) Optical purity of (3*S*, 3′*S*)-astaxanthin from *Haematococcus pluvialis*. Phytochemistry 20: 2561–2564

Santos MF, Mesquita JF (1984) Ultrastructural study of *Haematococcus lacustris* (Girod.) Rostafinski (Volvocales). I. Some aspects of carotenogenesis. Cytologia 49: 215–228

Schroeder WA, Johnson EA (1993) Antioxidant role of carotenoids in *Phaffia rhodozyma*. J Gen Microbiol 139: 907–912

Schroeder WA, Johnson EA (1995a) Singlet oxygen and peroxy radicals regulate carotenoid biosynthesis in *Phaffia rhodozyma*. J Biol Chem 270: 18374–18379

Schroeder WA, Johnson EA (1995b) Carotenoids protect *Phaffia rhodozyma* against singlet oxygen damage. J Ind Microbiol 14: 502–507

Shaish A, Avron M, Pick U, Ben-Amotz A (1993) Are active oxygen species involved in induction of β-carotene in *Dunaliella bardawil*? Planta 190: 363–368

Strickland JDH, Parsons TR (1968) IV.3. Pigment analysis. In: Strickland JDH, Parsons TR (eds) A practical handbook of seawater analysis. Fisheries Research Board of Canada, Ottawa, Ontario, Canada, pp 185–206

Terao J (1989) Antioxidant activity of β-carotene-related carotenoids in solution. Lipids 24: 659–661

Theimer RR, Rau W (1970) Light dependent carotenoid synthesis. V. Extinction of the photoinduction by reducing substances and substitution of hydrogen peroxide for light. Planta 92: 129–137

Whitelam GC, Codd GA (1982) A rapid whole-cell assay for superoxide dismutase. Anal Biochem 121: 207–212

Young AJ (1991) The photoprotective role of carotenoids in higher plants. Physiol Plant 83: 702–708