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High Light-Induced Changes in the Activities of Antioxidant Enzymes and the Accumulation of Astaxanthin in the Green Alga *Haematococcus pluvialis*

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Abstract

We investigated high light-induced alterations in antioxidant enzymes by exposing green vegetative cells of the alga *Haematococcus pluvialis* to excess irradiance to induce the production of astaxanthin, a carotenoid pigment. Total activity of catalase decreased approximately 70% after high light exposure, whereas glutathione peroxidase (GPX) activity was slightly enhanced. Total activity of superoxide dismutase and ascorbate peroxidase (APX) also slightly decreased. Overall, we did not observe dramatically elevated levels of antioxidant isozymes, although APXn, GPX2, and GPX3 isozyme increased slightly. H_2O_2 content increased about sixfold after high light exposure, demonstrating severe cellular oxidative stress, whereas lipid peroxidation was notably reduced. Concomitantly, astaxanthin accumulation increased about sevenfold. This result suggests that probably massively accumulated astaxanthin may be one of the antioxidant protector against high light stress.

Keywords: *Haematococcus pluvialis*, High light, Astaxanthin, Antioxidant enzymes

The unicellular green alga *Haematococcus pluvialis* is a model organism for studying secondary carotenoid accumulation in lipid vesicles outside the plastid[1]. Primary carotenoids are those that function within the photosynthetic machinery, whereas secondary carotenoids are not required for photosynthesis and are not localized in thylakoid membranes. Upon environmental stress such as strong irradiance, *H. pluvialis* accumulates secondary carotenoids in cytosol vesicles. Green vegetative cells cultivated under optimal cell culture conditions can transform into red cyst cells under high light stress conditions and induce astaxanthin production[2].

Well known red carotenoids, astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), is used as a food additive and has potential pharmaceutical and neutraceutical applications due to its high antioxidant activity[3,4]. Unfavorable culture conditions such as strong irradiance, nitrogen limitation, pH, salinity, and some organic nutrients (e.g., acetate) are known to alter the astaxanthin concentration in *H. pluvialis*[5,6]. In addition, exposure to reactive oxygen species (ROS)-generating compounds such as Fe^{2+}, methyl viologen, and methylene blue has been shown to result in astaxanthin accumulation[7].

Electron microscopy and cytochemical investigations have shown that the *H. pluvialis* cell wall undergoes major modification in chemical composition and structural organization in response to external stimuli[2]. The extracellular matrix of *H. pluvialis* green vegetative cells is flexible and elastic, whereas the secondary wall associated with red cyst induction is thick and rigid, apparently to withstand external attacks[8]. In addition, 81 cell wall protein orthologs appear to be involved in various aspects of cell wall formation[9,10]. The red cyst cell wall is thicker than that of the green vegetative cell[8], indicating chemical and physical alteration of the cell wall in response to high light stress, but molecular information on cell wall biogenesis is still largely lacking.

Generally, high light exposure induces ROS generation and causes oxidative damage to cellular components such as DNA, proteins, lipids, and pigments[11]. ROS act as cytotoxic compounds and also mediate the induction of stress tolerance. To protect cellular membranes and organelles from the damaging effects of ROS, complex antioxidant systems are activated,

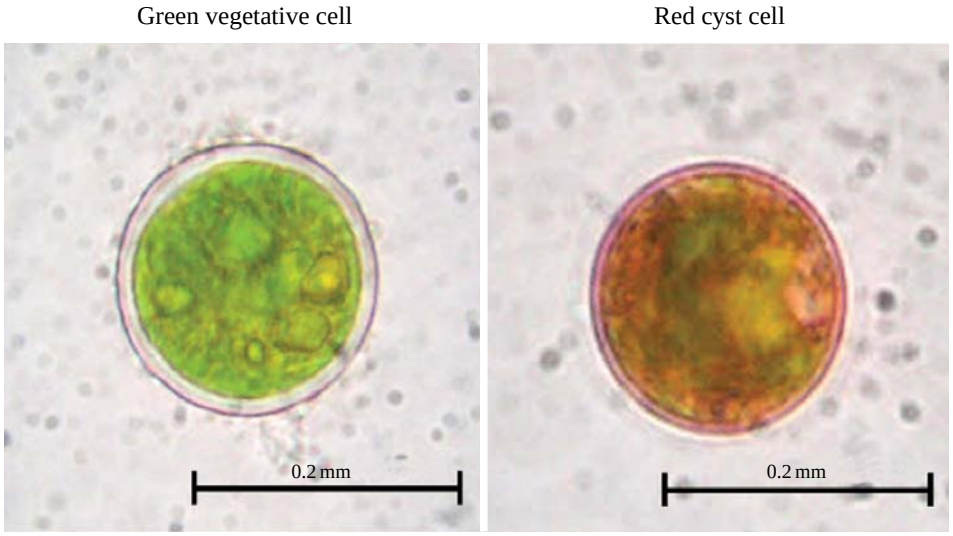

Figure 1. The light micrographs of green vegetative cell and red cyst cell of *Haematococcus pluvialis*.

including antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), ascorbate peroxidase (APX), and glutathione reductase (GR). Changes in antioxidant enzyme activities and isozyme patterns under stress conditions may serve as indicators of biotic and abiotic stresses[12,13]. SOD comprises a group of metalloenzymes that catalyzes the disproportionation of superoxide to H_2O_2 and O_2 and plays an important role in protecting against superoxide-derived oxidative stress in the cells[14-16]. The detoxification of cellular H_2O_2 through the activity of the Asada-Halliwell scavenging cycle is an important step in the defense mechanism against active oxygen species. The cycle, which occurs in the chloroplast and cytosol, involves the oxidation and re-reduction of ascorbate and glutathione through APX and GR[16,17]. APX catalyzes the reaction of ascorbic acid with H_2O_2, and GR catalyzes the regeneration of ascorbic acid[18]. Catalase can also reduce H_2O_2 to water, but it has a very low affinity for H_2O_2 compared to APX[19].

Although biotechnological production of astaxanthin has been extensively studied due to the usefulness of this secondary carotenoid, few enzymatic studies have been undertaken, especially on antioxidant enzyme responses and signal pathways to high light stress. Here, we report the changes in various antioxidant enzymes following exposure to high light stress in *H. pluvialis* to induce astaxanthin production. The interrelationships between astaxanthin accumulation and lipid peroxidation in response to high light stress were also examined.

Light Micrographs of *H. pluvialis*

Figure 1 shows the light micrographs of *H. pluvialis* at different stages and culture conditions. Green vegetative cells were cultivated under optimal cell culture conditions under a continuous light intensity of 40 E m^{-2} s^{-1} irradiance. Red cyst cells were incubated under high light stress conditions (250 E m^{-2} s^{-1} irradiance) for 72 hrs.

Table 1. Changes in the activities of various antioxidant enzymes in *Haematococcus pluvialis* following high light exposure.

	Control (%)	High light (%)
Ascorbate peroxidase	100	94 ± 0.7
Glutathione peroxidase	100	119 ± 2.5
Catalase	100	31 ± 0.9
Superoxide dismutase	100	91 ± 1.4
Glutathione reductase	100	99 ± 2.7

Effects of High Light on Antioxidant Enzyme Activities and Isozyme Patterns

We examined the activities of the antioxidant enzymes APX, GPX, CAT, SOD, and GR following high light exposure (Table 1). Upon exposure to high light, total APX activity slightly decreased. A native PAGE zymogram revealed multiple forms of APX, which we designated APX1, APX2, APX3, APX4, and APX5 (Figure 2A). Interestingly, APX5 isozyme was not observed, whereas a new band designated APXn was induced beneath APX2, possibly by the biosynthesis of APXn isozyme. Thus, APXn isozyme may play an important role in high light tolerance.

Total GPX activity increased slightly compared to the control (Table 1). GPX isozyme patterns in response to high light were also analyzed by in-gel activity staining on the native PAGE gel (Figure 2B). GPX2

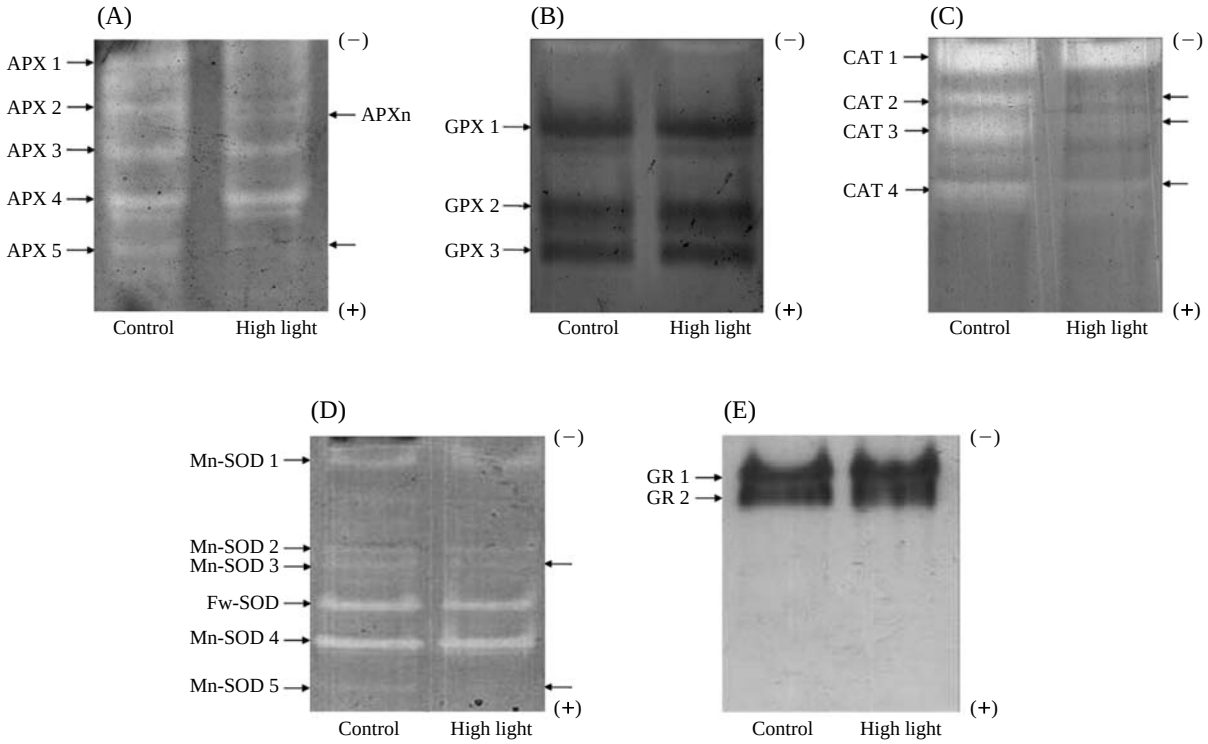

Figure 2. Alterations of the isozyme patterns of antioxidant enzymes in *Haematococcus pluvialis* following high light exposure. (A) Ascorbate peroxidase (APX), (B) Glutathione peroxidase (GPX), (C) Catalase (CAT), (D) Superoxide dismutase (SOD), (E) Glutathione reductase (GR).

and GPX3 levels were slightly elevated, as revealed by enhanced band intensity. GPX isozyme profiles showed that the slight increase in fast-migrating GPX2 and GPX3 isozymes contributed to the total GPX activity enhancement.

CAT activity decreased approximately 70% after high light treatment (Table 1). To examine whether CAT isozyme patterns might be affected by high light, we performed native PAGE on the algal extract and stained for CAT activity. The control showed four major isozyme bands: CAT1, CAT2, CAT3, and CAT4. After high light exposure, the CAT2, CAT3, and CAT4 isozyme bands were significantly reduced, whereas CAT1 remained constant (Figure 2C).

SOD activity decreased about 10% in response to high light. As shown in Figure 2D, SOD in *H. pluvialis* existed as five Mn-SOD isozymes: Mn-SOD1, Mn-SOD2, Mn-SOD3, Mn-SOD4, and Mn-SOD5, plus one Fe-SOD. Fe-SOD activity was inhibited in the presence of KCN and H_2O_2 solution (data not shown). Among the isozymes, Mn-SOD3 and Mn-SOD5 decreased after high light exposure.

GR, which regenerates reduced glutathione, is known to act in conjunction with APX to metabolize H_2O_2 to water through an ascorbate-glutathione cycle[20]. As shown in Figure 2E, under high light, GR activity and isozyme pattern were similar to those of the control. The intensities of two preexisting isozymes, GR1 and GR2, remained unaffected by high light. Overall, dramatically elevated antioxidant isozymes were not found in high light-exposed *H. pluvialis*, despite slight elevations in APXn and GPX2 and GPX3.

Effects of High Light on H_2O_2 Content and Lipid Peroxidation

H_2O_2 content and lipid peroxidation level are the markers of oxidative stress and injury and vary in response to biotic and abiotic stresses[11,13,21]. As shown in Table 2, H_2O_2 content increased about sixfold after high light exposure, indicating ROS generation and a state of severe oxidative stress. Increased levels of cellular H_2O_2 also seem to be related to reductions in catalase and ascorbate peroxidase in *H. pluvialis*.

Unsaturated membrane lipids are a primary target of oxidative attacks, and unsaturated fatty acid peroxides generate malondialdehyde (MDA), which is the most abundant individual aldehyde lipid breakdown product[22,23]. Surprisingly, the level of MDA, a major 2-thiobarbituric acid (TBA) reactive metabolite, was notably reduced after high light exposure, as

Table 2. Changes in the content of H_2O_2 and malondialdehyde in *Haematococcus pluvialis* following high light exposure.

	Control (%)	High light (%)
H_2O_2	100	596 ± 3.9
Malondialdehyde	100	30.4 ± 1.5

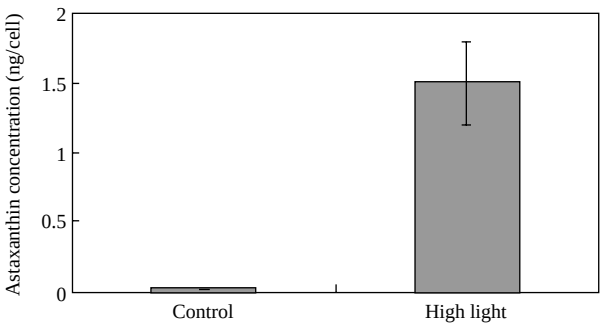

Figure 3. Astaxanthin accumulation in *Haematococcus pluvialis* following high light exposure.

shown in Table 2. Most studies have reported elevated MDA levels under external stresses[11]. The reduction of the lipid peroxidation level following high light exposure in this study may be resulted from the accumulation of strong antioxidants. In potato leaves, zeaxanthin and antheraxanthin accumulation was reported to reduce lipid peroxidation levels considerably during subsequent light stress[24].

Astaxanthin Accumulation in *H. pluvialis* under High Irradiance

Although astaxanthin was present at low levels in green vegetative cells, its accumulation in red cyst cells was striking. As shown in Figure 3, astaxanthin accumulations in red cyst cells were approximately sevenfold higher than in green vegetative cells.

Discussion

The accumulation of astaxanthin in *H. pluvialis* under stress conditions has been studied extensively; however, the information on the various antioxidant enzymes associated with astaxanthin accumulation was not available. Our investigation focused on the changes of total activities of antioxidant enzymes and isoenzyme expression patterns from astaxanthin induction condition using in-gel activity staining method. The alterations of total activity and isoenzyme profile of antioxidant enzyme in response to stress condition are a possible indicator of external stresses during ROS metabolism[12,13,18]. Studies on the several isoenzymes of antioxidant enzyme revealed that there are distinct differences in the response to oxidative stresses and physiological functions for individual isoenzymes[12,13,25,26].

In this study, total activity of multiple forms of catalase notably decreased after high light exposure, whereas glutathione peroxidase activity was slightly enhanced. Previous microarray data revealed transcript from catalase 1 gene increased substantially under stress conditions in *H. pluvialis*[5], showing discrepancy between catalase enzyme activity and the transcript. Recently, large discrepancies between mRNA and protein levels by comparison of cDNA microarray and proteomic analytical results were reported[27]. There may be a similarity and disparity between mRNA transcript and protein expression patterns because mRNA expression study provides little information about the activation state, posttranslational modification, or localization of corresponding proteins[28]. The low overlap between transcript and protein suggests the importance of considering proteins instead of transcripts when investigating the gene expression profile alteration by oxidative stress[29].

We did not find notably elevated levels of antioxidant isozymes, although APXn, GPX2, and GPX3 isozyme increased slightly, while about sixfold increase of H_2O_2 content demonstrated severe cellular oxidative stress. Thus, in *H. pluvialis*, enhanced APXn, GPX2, and GPX3 activity may function to protect the cell from oxidative stress, when CAT and SOD are inactivated by high light exposure.

However, lipid peroxidation was dramatically reduced and astaxanthin accumulation increased about sevenfold. The massive induction of astaxanthin accumulation was reported to be related to the enhanced activity of key enzymes involved in astaxanthin biosynthesis, such as a carotenoid hydroxylase[6]. Moreover, astaxanthin accumulation has been thought to be closely associated with lipid biosynthesis and cell wall formation[1]. Therefore, astaxanthin accumulation upon high light exposure seems to be involved in protecting the red cyst cell under oxidative stress conditions. This result suggests that probably massively accumulated astaxanthin may largely replace the antioxidant enzymes in protecting the red cyst cell against high light stress. Indeed, astaxanthin molecule is an excellent antioxidant molecule which may prevent lipid peroxidation. However, astaxanthin is accumulated in lipid globules in the cytoplasm far from ROS production within the chloroplast. Thus there still remains the possibility that enhanced cell lipids or other antioxidants induced by astaxanthin accumula-

tion may act in concert to exhibit antioxidant activities together with astaxanthin.

Materials & Methods

Microorganism and Culture Conditions

The unicellular green alga *H. pluvialis* UTEX 16 was obtained from the Culture Collection of Algae at the University of Texas at Austin. *H. pluvialis* was cultivated in MBBM (modified Bold's Basal Medium)[5,30]. To grow immature green cells, the cells were cultured in 2.5 Liter bubble column photobioreactors, each containing 2 Liter growth medium, at 25°C under continuous 40 E m^{-2} s^{-1} irradiance. For stress induction, exponentially growing cultures (cell density ~1 $\times 10^5$ cells mL^{-1}) were exposed to continuous 250 E m^{-2} s^{-1} irradiance for 72 hrs.

Astaxanthin Determination

Astaxanthin concentration was calculated using a calibration curve, with synthetic astaxanthin (Sigma Chemical Co., St Louis, MO) as a standard. For astaxanthin concentration < 10 mg L^{-1}, the following calibration was used: astaxanthin concentration (mg L^{-1}) $= 0.0045 \times OD_{475}$[30].

Determination of H_2O_2 and Malondialdehyde Content

H_2O_2 and malondialdehyde content were measured under control and high light illumination conditions. Algal material (1 g) was homogenized in an ice bath with 0.1% (w/v) trichloroacetic acid (TCA). After centrifugation, the supernatant was mixed with 50 mM phosphate buffer (pH 7.0) and 1 M KI. The absorbance of the supernatant was measured at 390 nm, with H_2O_2 content obtained from a standard H_2O_2 curve.

The lipid peroxidation level was determined as 2-thiobarbituric acid (TBA) reactive metabolites, expressed chiefly as malondialdehyde (MDA)[22]. The alga was extracted with 0.25% TBA in 10% TCA, and the extract was quickly cooled after heating. After centrifugation at 10,000 × g for 15 min, the absorbance of the supernatant was measured at 532 nm. The lipid peroxidation level was expressed as μmol MDA formed using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Preparation of Enzyme Extracts

Algal material was powdered in liquid nitrogen and further homogenized in 50 mM sodium phosphate buffer (pH 6.0) containing sea sand. The extract was centrifuged at 10,000 × g for 30 min, and the supernatant was used for antioxidant enzyme analysis.

Antioxidant Enzyme Assays

The cell extracts of *H. pluvialis* contained multiple forms of antioxidant enzymes, such as APX, GPX, CAT, SOD, GR. The numbers of the isoenzymes denote which isozyme migrates the greater distance to cathode. The lower the number, the smaller the migration.

CAT activity was determined spectrophotometrically by measuring the decrease in absorbance at 240 nm due to H_2O_2 decomposition[31]. APX activity was determined using ascorbate as a substrate, and the decrease in absorbance at 290 nm was measured[17]. SOD activity was determined using the xanthine/xanthine oxidase/nitroblue tetrazolium system, in which the inhibition of cytochrome *c* reduction by SOD was measured by the reduction of nitroblue tetrazolium[32]. GPX activity was determined by measuring the oxidation of NADPH at 340 nm in a coupled assay with glutathione reductase[33]. GR activity was determined by measuring the reduction of oxidized glutathione at 340 nm, as demonstrated by NADPH oxidation[32]. All determinations were expressed as the mean ± S.E. of three separate experiments.

In-gel Activity Staining of Antioxidant Enzymes

Equal amounts of algal protein extracts were subjected to native-PAGE using the discontinuous buffer system of Laemmli[34]. Antioxidant enzymes were separated on 10% separating polyacrylamide gel, with a 5% stacking gel, at 100 V for 5 h at 4°C. After the electrophoretic separation of antioxidant enzymes, the gels were stained for individual enzyme activity. Staining for CAT activity was performed as described by Woodbury[35]. The gel was incubated in 3.27 mM H_2O_2 for 25 min, rinsed in water, and soaked in a solution of 1% ferric chloride-potassium ferricyanide (III). SOD isozyme staining was performed by incubating the gel in 50 mM Tris-HCl buffer (pH 8.5) containing 10 mg methylthiazolyl tetrazolium, 6 mg phenazine methosulfate, and 15 mg $MgCl_2$[14]. For APX isozyme staining[17], the gel was incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gel was washed with 50 mM potassium phosphate buffer (pH 7.0) and soaked in a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 28 mM N,N,N′,N′-tetramethyleneethylenediamine and 2.45 mM nitroblue tetrazolium. GR activity was detected by incubating the gel in a solution of 0.25 M Tris-HCl buffer (pH 7.8) containing 0.24 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.4 mM NADPH, 0.34 mM 2,6-dichlorophenolindophenol and 3.6 mM oxidized glutathione in darkness for 1 h[18]. GPX activity was

determined by submerging the gel for 20 min twice in 50 mM Tris-HCl buffer (pH 7.9) before activity staining. The gel was soaked in the substrate solution (50 mM Tris-HCl buffer, pH 7.9, 13 mM glutathione and 0.004% H_2O_2) with gentle shaking for 10-20 min. After a brief rinse, the gel was developed in darkness at room temperature with 1.2 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and 1.6 mM phenazine methosulfate in distilled water for 10 min[36].

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