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Antioxidative capacity and enzyme activity in *Haematococcus pluvialis* cells exposed to superoxide free radicals*

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Abstract The antioxidative capacity of astaxanthin and enzyme activity of reactive oxygen eliminating enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) were studied in three cell types of *Haematococcus pluvialis* exposed to high concentrations of a superoxide anion radical (O_2^-). The results show that defensive enzymes and astaxanthin-related mechanisms were both active in *H. pluvialis* during exposure to reactive oxygen species (ROS) such as O_2^-. Astaxanthin reacted with ROS much faster than did the protective enzymes, and had the strongest antioxidative capacity to protect against lipid peroxidation. The defensive mechanisms varied significantly between the three cell types and were related to the level of astaxanthin that had accumulated in those cells. Astaxanthin-enriched red cells had the strongest antioxidative capacity, followed by brown cells, and astaxanthin-deficient green cells. Although there was no significant increase in expression of protective enzymes, the malondialdehyde (MDA) content in red cells was sustained at a low level because of the antioxidative effect of astaxanthin, which quenched O_2^- before the protective enzymes could act. In green cells, astaxanthin is very low or absent; therefore, scavenging of ROS is inevitably reliant on antioxidative enzymes. Accordingly, in green cells, these enzymes play the leading role in scavenging ROS, and the expression of these enzymes is rapidly increased to reduce excessive ROS. However, because ROS were constantly increased in this study, the enhance enzyme activity in the green cells was not able to repair the ROS damage, leading to elevated MDA content. Of the four defensive enzymes measured in astaxanthin-deficient green cells, SOD eliminates O_2^-, POD eliminates H_2O_2, which is a by-product of SOD activity, and APX and CAT are then initiated to scavenge excessive ROS.

Keyword: astaxanthin; *Haematococcus pluvialis*; lipid peroxidation; reactive oxygen species (ROS)

1 INTRODUCTION

Haematococcus pluvialis, a unicellular green alga, is usually found in temporary shallow pools of rain water on bumpy rocks (Droop, 1954; Czygan, 1970). It unavoidably faces environmental challenges during its life such as osmotic shock, drought and waterlogging, high and cold or freezing temperatures, nutrient deficiency, and radiation or ultraviolet light exposure (Domínguez-Bocanegra, 2004). However, *H. pluvialis* seems to have adapted to these ecological challenges physiologically by accumulating astaxanthin (Orosa, 2005) and morphologically by changing from motile to non-motile cells (cysts) (Kobayashi et al., 1991), in addition to changes in color from green to red via brown or other transitional colors (Kibayashi et al., 1997b).

Recent studies have focused on the possible photoprotection (Fan et al., 1998; Kobayashi et al., 1992; García-Malea, 2005) and molecular biology of astaxanthin biosynthesis (Linden, 1999; Grünewald et al., 2000). Oxidative stress was considered to be involved in the regulation of astaxanthin biosynthesis in *H. pluvialis* (McCond et al., 1969; Schroeder et al.,

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1995). Wang et al. (2003) have provided direct evidence of the antioxidative defense mechanisms in *H. pluvialis* by two-dimensional gel electrophoresis. More than 70 proteins involved in photosynthesis, mitochondrial respiratory and nitrogen assimilation were up-regulated or down-regulated after the onset of stress. Kobayashi et al. (2000) considered that astaxanthin plays an important role in the antioxidative processes against reactive oxygen species (ROS) in red *H. pluvialis* cells.

In recent decades, free radical balance theory has been widely used to explain tissue damage and defense mechanisms in animals, plants and microorganism during exposure to stressful environments (McCond, 1969; Asada, 1994; Ip et al., 2005). To further understand the antioxidant capacity and responsive sequence of astaxanthin and protective enzymes, we compared membrane lipid peroxidation, astaxanthin content and activities of ROS eliminating enzymes (superoxide dismutase [SOD], peroxidase [POD], catalase [CAT] and ascorbate peroxidase [APX]) in different cell types by incubating *H. pluvialis* with methyl viologen (MV) to generate excessive levels of superoxide anion radicals (O_2), as described by Kobayashi (Kobayashi et al., 1997a; Kobayashi, 2000)

2 MATERIALS AND METHODS

2.1 Algal culture and treatment

H. pluvialis was obtained from the Algal Collection Lab of Institute of Hydrobiology, Chinese Academy of Sciences. Modified MCM medium was composed of the following (in mg/L): KNO_3, 200; KH_2PO_4, 20; $NaHCO_3$, 450; $MgSO_4{\cdot}7H_2O$, 100; $CaCl_2{\cdot}6H_2O$, 80; V_{B12}, 4×10^{-3}; $Na_2EDTA{\cdot}2H_2O$, 3.36; $FeCl_3{\cdot}6H_2O$, 2.44; $ZnCl_2$, 4.1×10^{-3}; H_3BO_3, 61×10^{-3}; $CoCl_2{\cdot}6H_2O$, 5.1×10^{-3}; $CuSO_4{\cdot}5H_2O$, 6×10^{-3}; $MnCl_2{\cdot}4H_2O$, 4.1×10^{-3}; $(NH_4)_6MO_7O_{24}{\cdot}4H_2O$, 38×10^{-3} (Liu et al., 2002). N-limitation medium was prepared similar to the above, but lacked KNO_3. Fresh medium (100 ml) was added to each Erlenmeyer flask (200 ml) before autoclaving. The culture flasks were manually shaken several times a day.

H. pluvialis at the logarithmic growth stage were inoculated to 2×10^4 cells ml^{-1} under a laminar flow cabinet and transferred to a temperature-controlled culture room at 22°C. Low (50 μmol photons m^{-2} s^{-1}) and high (200 μmol photons m^{-2} s^{-1}) illumination was provided by white fluorescent lamps in a 12-h light-dark cycle. Light intensity was measured by a photometer placed on the surface of the culture (DM-100, Jiangsu China).

Different levels of astaxanthin in the cells were induced by culture in different conditions for 1 week. Green cells containing low levels of astaxanthin and brown cells with an intermediate level of astaxanthin were obtained by replacing the cultures with full nutrients and exposure to low and high illumination, respectively. Meanwhile, the astaxanthin-enriched red cells were obtained by placing the N-limitation culture under a high light intensity for 1 week. After 1 week of culture, the final cell concentrations of green, brown and red *Haematococcus* cells were 1.2×10^5, 1.0×10^5 and 7.0×10^4 cells ml^{-1}. The cell number was the average of three samples, and each sample was microscopically counted with a hemacytometer 20 times.

Methyl viologen (MV, superoxide anion radical generator) stock solution (10 mmol/L) was prepared in ethanol and was added to flasks at a final concentration of 10 μmol/L.

2.2 Chlorophyll, Astaxanthin and Malondialdehyde (MDA) determination

Algal pellets were repeatedly extracted by 80% acetone in dim light at 22±2°C with a tube-type glass mortar until colorless. The chlorophyll content was assayed by Arnon's method (Arnon, 1949). Carotenoids were measured and calculated according to the method of Davies (Davies, 1976). The average astaxanthin content in brown and red cells was about 30 and 65 pg $cell^{-1}$, respectively, based on the fact that over 90% of carotenoids were astaxanthin (data not shown). Green motile cells were considered to be astaxanthin-free because of the low carotenoid content in these cells (4 pg $cell^{-1}$) and the low proportion of astaxanthin in carotenoids.

MDA was measured using methods described by Hernandez et al. (Hernandez et al., 2002). To determine MDA content, algal samples were centrifuged and the algae pellets were homogenized with tube-type glass mortars in 5% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 *g* for 10 min. An aliquot (1.5 ml) of the supernatant was added to 2.5 ml of 0.5% thiobarbituric acid dissolved in 20% TCA. The mixture was heated at 90°C for 30 min in a shaking water bath, and then cooled to room temperature. Then, the mixture was centrifuged at 10 000 *g* for 5 min, and the absorbance of the supernatant was measured at 532 nm and 600 nm. The MDA concentration was calculated as the difference in absorbance at 600 nm and 532 nm (Hernandez et al., 2002).

2.3 Enzyme activity analysis

SOD activity was determined based on the reduction of nitroblue tetrazolium (NBT) to blue formazan, as described by Beauchamp (1971). Algal pellets of each cell type were collected by centrifugation at 2 000 *g* for 10 min and washed in 5 m$_{mol/L}$ phosphate buffer (pH 7.8). The cells were ground at 0°C in tube-type glass mortars and centrifuged (13 000 *g* for 15 min). The supernatant was used to determine SOD activity by measuring optical density at 560 nm with a spectrophotometer. The mixture for determining SOD activity contained 20 μmol/L flavine mononucleotide, 1.0 μmol/L EDTA, 26 mmol/L methionine, 0.75 m$_{mol/L}$ NBT and 50 mmol/L phosphate buffer (pH 7.8). The initial absorbance at 560 nm of the mixture was measured, followed by recording the absorbance after adding 0.5 ml of supernatant for 15 min at 25°C. A control, without cell extract, was prepared simultaneously. A unit of SOD activity was expressed as the amount that causes a 50% decrease in nitrite formation via hydroxylamine oxidation by O_2. The specific activity was expressed in units per cell.

Total POD activity was determined spectrophotometrically by measuring the oxidation of methyl catechol at 470 nm (Srivastava et al., 1973) and was expressed as units (μg of oxidized methyl catechol per min) per cell. The buffer used to determine POD activity was composed of the following: 1.0 ml of 0.2 mol/L acetic acid buffer (pH 5.0), 1.0 ml of 0.1% methyl catechol, 1.0 ml of 0.08% H_2O_2 and 1.0 ml of cell extract.

CAT activity was measured spectrophotometrically according to the method of Aebi (1974), by monitoring the decline in the absorbance at 240 nm, as H_2O_2 was consumed. The final reaction mixture contained 2.0 ml 50 mmol/L sodium phosphate buffer (pH 7.0), to which 1.0 ml of 0.1 mol/L H_2O_2 was added. The reaction was activated by adding 1.0 ml of algal extract to the reaction mixture. CAT activity was expressed as units (mmol of H_2O_2 decomposed per min) per cell.

To determine APX activity, 10 ml of algal culture was centrifuged and the pellets were homogenized in 100 mmol/L phosphate buffer (pH 7.0) containing 5 mmol/L ascorbate, and 5 mmol/L EDTA. APX activity was determined by adding 0.5 ml of algal extract to a 3.5 ml reaction mixture containing 50 mmol/L phosphate buffer (pH 7.0), 0.1 mol/L ascorbate and 0.3 mmol/L H_2O_2. The decrease in absorbance was measured at 290 nm for 3 min (Nakano et al., 1981).

Means and standard error in this article were calculated from triplicates samples. All measurements were subjected to analyses of variance to determine the last significant difference.

3 RESULTS AND DISCUSSION

3.1 The primary membrane lipid peroxidation in each type of *H. pluvialis* cell

The primary oxidative damage (membrane lipid peroxidation) was assessed by measuring the amount of MDA (Fig.1) in each type of *H. pluvialis* cell exposed to MV for 2 hours. The highest content of MDA was found in astaxanthin-deficient green cells indicating significant membrane-lipid damage, which is consistent with the fact that green cells in nature usually exist in favorable growth conditions rather than in stressful environments. Because the MDA content in green cells increased by 60% (P<0.01) after exposure to MV for 2 hours, these cells were vulnerable to ROS overdose and were easily damaged by environmental stress. By contrast, the lowest MDA level in astaxanthin-enriched red cells implies limited membrane lipid peroxidation in these cells, which was probably due to its strong defense system (antioxidant substrates or antioxidative enzymes) against ROS. Moreover, there was no increase in MDA levels in the red cells exposed to MV indicating that the internal defensive system against oxidation is quite effective. This appears to explain why *H. pluvialis* in natural stressful environments is dominated by red cells.

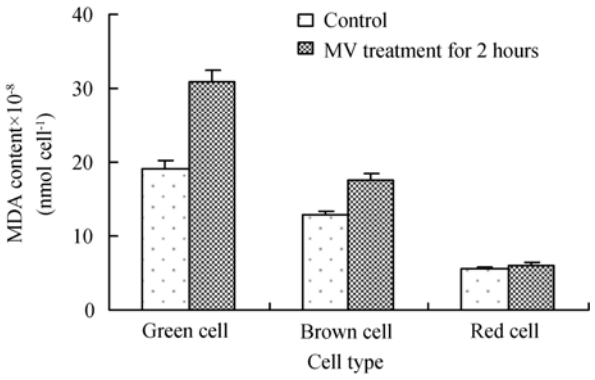

Fig.1 The extent of membrane-lipid peroxidation in each cell type of *Haematococcus pluvialis* after exposure to methyl viologen for 2 hours

3.2 Quick responses of enzyme activity in each cell type

It is commonly known that membrane lipid peroxidation is not only related to ROS generation, but is also dependent on ROS scavenging. Therefore,

to understand the antioxidative mechanism, the activities of four ROS scavenging enzymes (SOD, POD, CAT and APX) in each type of cell exposed to MV 2 hours were compared (Fig.2). SOD, the first line of defense against membrane lipid damages caused by ROS, reacted with O_2 directly by catalyzing the following reaction (Mittler, 2002): $2O_2+H^+ \rightarrow H_2O_2+O_2$. Therefore, a high SOD activity theoretically indicates low membrane lipid peroxidation

Fig.2a shows that it is the green cells rather than the red cells with the highest SOD activity. The antioxidative capacity of SOD in green cells was 1.8 times higher than that in red cells. This result clearly does not support the MDA data described in section 3.1. SOD activity showed a very positive and rapid response to the increase in reactive oxygen levels. After exposure to MV for 2 hours, SOD activity in the green cells and the brown cells increased by 61% (P<0.01) and 38% (P<0.05), respectively. Meanwhile, SOD activity in red cells did not change significantly. Therefore, in terms of SOD, the green cells had a greater antioxidative capacity than the brown cells, and the latter were showed greater capacity than the red cells. The SOD activity in each type of *H. pluvialis* clearly conflicts with the MDA results (Fig.1), and does not explain the natural ecological phenomena, i.e., red non-motile cells better tolerate stressful environments than green motile cells.

H_2O_2, a by-product of SOD, together with $ROOH^{\cdot}$, in cells attack DNA, proteins and lipids and causes cell damage if H_2O_2 is not removed in time. Fortunately, POD in *H. pluvialis* can catalyze H_2O_2 and $ROOH^{\cdot}$ into H_2O and R-OH to avoid cell damage. POD activity also showed a very positive quick response to the increase in ROS. POD activity in the different cell types varied greatly (ranging from 6×10^{-8} to 23×10^{-8} U cell^{-1} min^{-1}) but had a similar tendency to SOD (Fig.2b). POD activity was highest in green cells and increased significantly (about 1.6-fold of control, P<0.01) when the cells were exposed to MV. This result implies that ROS such as H_2O_2 and $ROOH^{\cdot}$ are effectively removed by POD. However, POD activity was lowest in red cells and exhibited a small change when the cells were exposed to MV, which indicates that the protective role of POD against ROS is relatively poor, and was even weaker than that in brown cells. Clearly, the results obtained for SOD and POD activity do not support the MDA results (Fig.1) either.

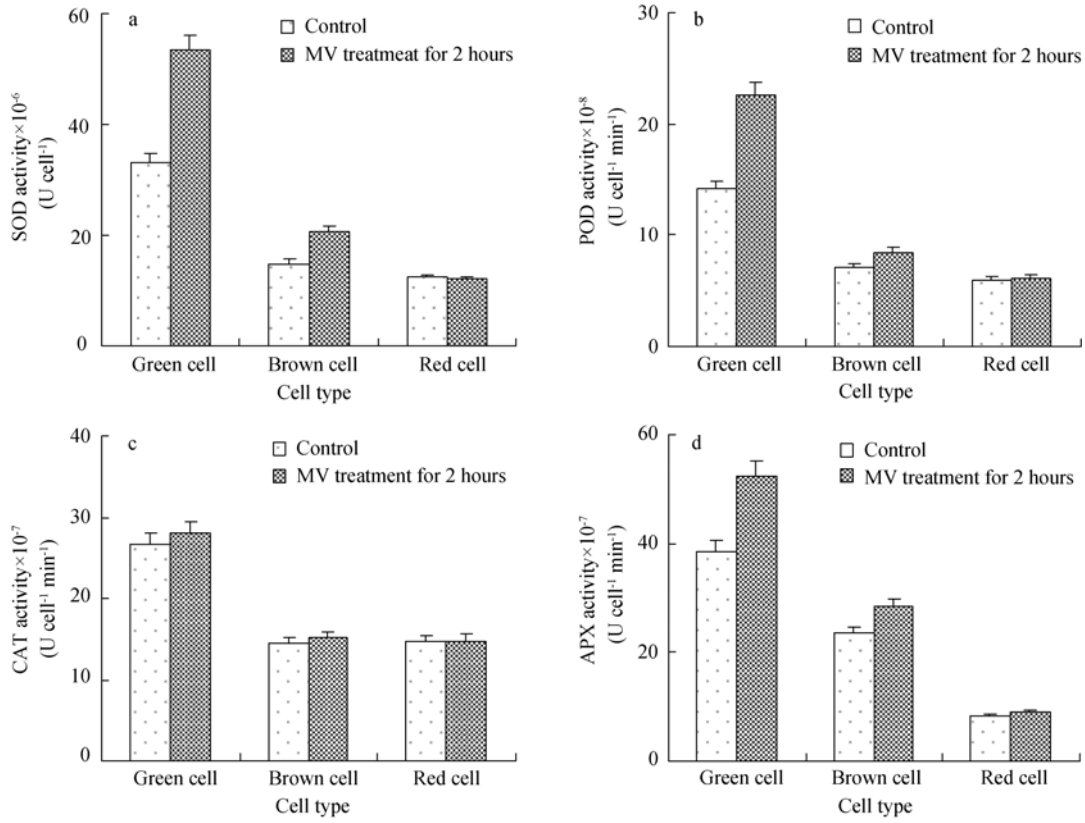

Fig.2 Effects of exposure to methyl viologen (10 μmol/L) for 2 hours on superoxide dismutase (a), peroxide (b), catalase (c) and ascorbate peroxidase (d) activities in each cell type of *Haematococcus pluvialis*

Nevertheless, the results of SOD and POD activity indicate that once excessive O_2^- is produced in the green and brown cells, that the antioxidative enzyme SOD is rapidly activated to deoxidize O_2^- into H_2O_2 and O_2, and that POD scavenges H_2O_2. SOD and POD showed a rapid response to the increase of ROS, commencing activity within 2 hours.

H_2O_2 can be converted to $^{\cdot}HO$ and impairs the electron transport chain in chloroplasts and mitochondria (Jiménez et al., 1997; Meneguzzo et al., 1998), lipid peroxidation (Quartacci et al., 1995; Navari-Izzo et al., 1996), protein denaturation (Baccio et al., 2004) and DNA damage (Conte et al., 1996). APX and CAT can effectively remedy those damages by quenching H_2O_2 in higher plants. The activities of CAT and APX in *H. pluvialis* are shown in Fig.2c and Fig.2d, respectively. Although CAT activity was higher in green cells than in brown and red cells, its variation was much less than that of SOD and POD. After cells were exposed to MV, there were no marked differences between any *H. pluvialis* cell types. Based on the results of CAT activity, we suggest that CAT is not an effective antioxidative enzyme in *H. pluvialis* during stress.

Changes in APX activity showed a similar tendency to those of SOD and POD in control cells and cells exposed to MV, although the variation in activity was relatively small. Compared with the changes in APX and CAT activities in green cells, the increase in APX activity (36%, $P<0.05$) was higher than that of CAT (5.0%) when the cells were exposed to MV. Therefore, it seems that APX rather than CAT plays the critical role in quenching H_2O_2 in green cells, which agreed with finding that APX is a critical enzyme with strong affinity for H_2O_2 in chloroplasts (Nakano et al., 1981). Comparing the enzyme activities, we found that the protective role of APX against ROS was more important than that of CAT, although it was less than that of SOD and POD in any *H. pluvialis* cell type.

The findings obtained with the four ROS scavenging enzymes strongly suggest that *H. pluvialis* has a protective enzyme system in which SOD and POD play a key role, followed by APX and CAT. Interestingly, comparison of the enzyme activities between the three different types of cells revealed that the green cells consistently showed greater defensive enzyme activity followed by brown cells and red cells. The enzyme activity results also suggest that the protective enzyme system in green cells was very efficient because of its high SOD, POD, CAT and APX activities; the SOD, POD, CAT and APX activities in red cells are only 37%, 42%, 55% and 22% of those in green cells, respectively.

Theoretically speaking, membrane lipid peroxidation in red cells should be increased after exposure to MV because of the low activities of SOD, POD, CAT and APX. However, the MDA content (Fig.1) was contrary to what was expected. This indicates that another defensive mechanism strongly affected the lipid peroxidation level in *H. pluvialis* red cells. We consider that astaxanthin, an antioxidative ketocarotenoid, plays a key protective role in *H. pluvialis* against ROS. Therefore, the dynamic physiological changes in astaxanthin and enzyme activity in each type of cell exposed to MV were studied.

3.3 Dynamic changes in SOD and POD activities

SOD activity increased greatly in green cells and reached a maximum within 24 hours of MV exposure due to the initial inducement of free radicals, and started to decrease afterwards and dropped by 66% of its original level 72 hours later (Fig.3). SOD activity in the brown cells showed a similar pattern to that in green cells, with a maximum variation (more than 3-fold of the original level) at 24 hours. However, the activity in red cells showed almost no changes, which indicates that no free radicals were induced, even after exposure to MV for 60 hours. Only at the end of the experiment period was a slight increase in SOD activity observed in the red cells (Fig.3).

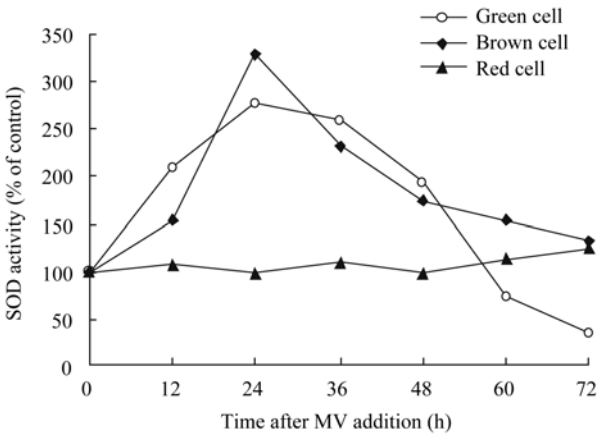

Fig.3 Dynamic changes in superoxide dismutase (SOD) activity in each cell type of *Haematococcus pluvialis* exposed to methyl viologen

Green cells were incubated in full MCM medium, brown cells were incubated under a 200-μmol photons m^{-2} s^{-1} light source, and red cells were incubated under a 200 μmol photons m^{-2} s^{-1} light source and in nitrogen-free medium

POD activity in green cells markedly increased within 24 hours of exposure to MV to reach a maximum (about 3.8-fold), and then decreased at the end of the experiment (Fig.4). POD activity in the brown cells showed a similar pattern to the green cells although its variation was less than that in green cells. The peak value in the brown cells was at 24–48 hours after exposure to MV, which lagged behind that of the green cells. In red cells, POD activity did not change significantly and was maintained at a lower level than that in the other cells during the entire experiment period.

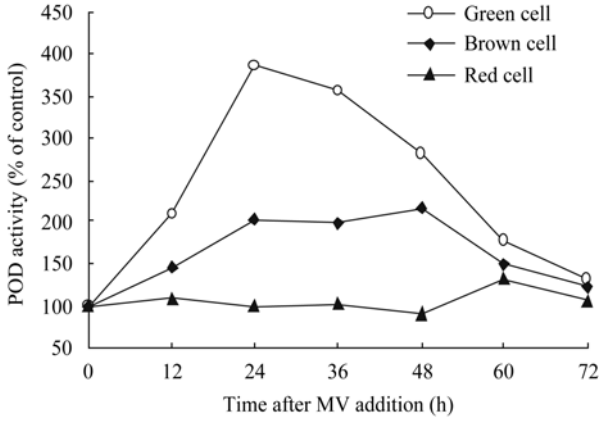

Fig.4 Dynamic changes in peroxide (POD) activity in each cell type of *Haematococcus pluvialis* after exposure to methyl viologen

Green cells were incubated in full MCM medium, brown cells were incubated under a 200 μmol photons m^{-2} s^{-1} light source, and red cells were incubated under a 200 μmol photons m^{-2} s^{-1} light source and in nitrogen-free medium

Chlorophyll content was then analyzed, on the assumption that the above results reflect a general protein behavior or direct antioxidation. The data showed that the chlorophyll content decreased from 45 pg chlorophyll $cell^{-1}$ to 4 pg chlorophyll $cell^{-1}$ in green cells during the 72 hour exposure to oxidative stress, and from 18 pg chlorophyll $cell^{-1}$ to 6 pg chlorophyll $cell^{-1}$ in brown cells. By contrast, in red cells the chlorophyll content remained low (around 10 pg $cell^{-1}$) throughout the experimental period. The changes in chlorophyll content, which are usually parallel with changes in protein synthesis, indicate that protein biosynthesis was inhibited in green and brown cells, but not in red cells. In terms of SOD and POD activity per μg of chlorophyll (Fig.5 and Fig.6, respectively), the green and brown cells showed a similar improvement rather than a decline compared with the initial value. The increase in SOD and POD activity per chlorophyll in green and brown cells was approximately 1–2 fold higher than that of SOD activity per cell (Fig.3) and POD activity per cell (Fig.4). Therefore, the changes of ROS scavenging enzymes in green and brown cells represent direct antioxidation rather than general protein behavior.

In addition, SOD peaks (Fig.3 and Fig.5) were relatively steeper and appeared earlier than the POD peaks (Fig.4 and Fig.6), which indicates that SOD responded to MV slightly faster than POD, and can be explained that SOD scavenges O_2^- first and generates H_2O_2, and POD is then initiated to eliminate H_2O_2.

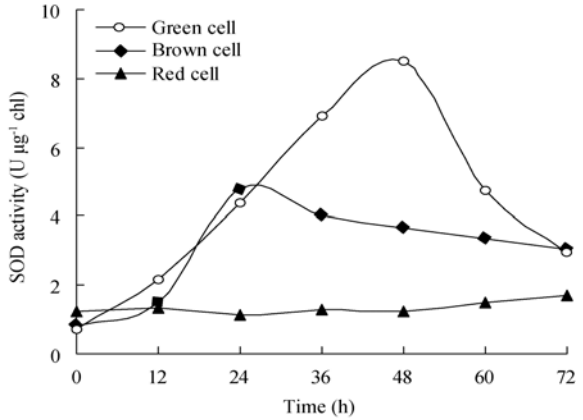

Fig.5 Dynamic changes of superoxide dismutase (SOD) activity per μg chlorophyll in each cell type of *Haematococcus pluvialis*

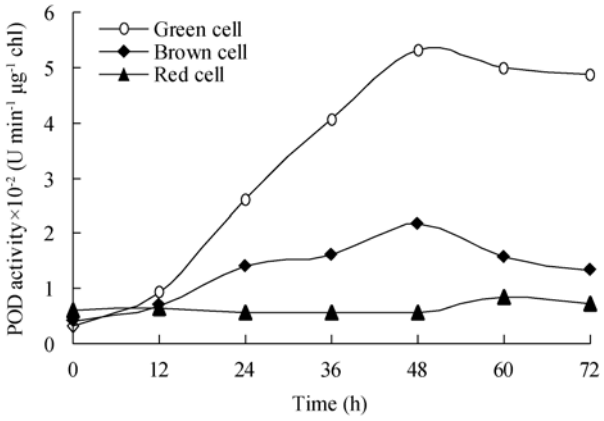

Fig.6 Dynamic changes in peroxide (POD) activity per μg chlorophyll in each cell type of *Haematococcus pluvialis*

Based on the SOD and POD activity per cell (Fig.3 and Fig.4) and per chlorophyll (Fig.5 and Fig.6) in addition to the constant level of chlorophyll in red cells during the experimental period, the mechanisms involved in ROS scavenging enzymes cannot be fully explained. Therefore, other ROS defensive mechanisms, distinct from those in green cells, may be present in red cells.

3.4 The relationship between changes in astaxanthin content and MDA content

The astaxanthin content in the red cells increased continuously to 73.6 pg cell^{-1} within 3 days after exposure to MV. Meanwhile, the astaxanthin content in brown cells only increased during the early stage and then decreased after 36 hours of MV exposure. The small amount of carotenoids (astaxanthin close to zero) in the green cells decreased to 50% of the initial value at the end of the experiment (Fig.7).

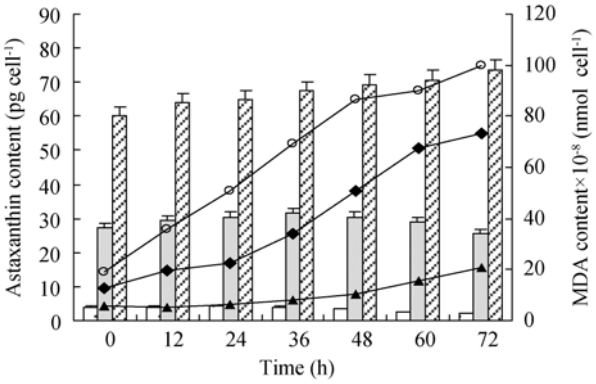

Fig.7 Relationship between changes in astaxanthin content and malondialdehyde content

Legend: Astaxanthin content (histogram), MDA content (line). White bars and circles (○) correspond to green cells, grey bars and squares (◆) correspond to brown cells, and hatched bars and triangles (▲) correspond to red cells

The MDA content increased rapidly in green and brown cells with ongoing exposure to MV exposure, and was more pronounced in green cells. By contrast, MDA content did not change much in red cells during the early stage, but increased slightly during the later stages and, at the end of experiment, the MDA content was only 21% and 28% of that in the green and brown cells, respectively (Fig.7). These results indicate a negative correlation between MDA content and astaxanthin content in *H. pluvialis* cells and that the production of the lipid peroxide (MDA) was inhibited by astaxanthin.

Excessive accumulation of ROS in plants exposed to different environmental stressors may be due to the low efficient elimination of absorbed light energy (Apel et al., 2004). During environmental stress, the Mehler reaction and photorespiration are enhanced inside the chloroplasts where most ROS are generated (Asada, 1999; Boussiba, 2000; Chen et al., 2004; Foyer et al., 2000; Ye et al., 2000). O_2^- might leak from the chloroplast to the cytoplasm and be quenched chemically (Boussiba, 2000). The detoxified oxygen molecules that may result from ROS reactions may provide a substrate to convert β-carotene to astaxanthin. Most astaxanthin accumulates in lipid globules in the cytoplasm at a certain distance from the membrane (Lang, 1968; Santos et al., 1984; Grünewald et al., 2001). Undoubtedly, the leaked O_2^- would attack the lipid membrane much earlier than the reaction with astaxanthin in the cytoplasm. Therefore, a question was raised regarding the mode for trapping ROS in astaxanthin-enriched red cells of *H. pluvialis*.

We suggest that an alternative mechanism is that some astaxanthin molecules do not accumulate in lipid globules in the cytoplasm but are inserted into the bi-layer membranes, as suggested in animal studies (Goto et al., 2001). The inserted astaxanthin molecules with a hydrophobic conjugated polyene chain are aligned parallel to the fatty acid tails and the hydrophilic terminal heads on both sides of the membrane surface. The conjugated polyene moiety and terminal ring moieties allow the astaxanthin molecules to act defensively in *H. pluvialis* and trap radicals on both the membrane surface and in the interior of the bilayer. Moreover, our microscopic results (data not shown) indicate that many green cells died and broke down after the addition of MV. By contrast, this situation did not occur in red cells. The red cells developed a darker color after MV treatment, probably due to a progressive increase in astaxanthin. These results also suggested that astaxanthin-rich red cells were more tolerant to excessive oxidative stress than astaxanthin-poor green cells.

The defensive mechanisms varied greatly between the three cell types and the protective role of astaxanthin was much stronger than that of antioxidative enzymes. In the astaxanthin-enriched red cells, astaxanthin quenched O_2^- before the activation of protective enzymes. Therefore, although there were no changes in the activity of protective enzymes (Fig.2), the MDA content did not increase and was sustained at a low level (Fig.1). With regard to the green cells, containing almost no astaxanthin, the enzymatic system played the lead defensive role. Therefore, after exposure to MV, the enzyme activities rapidly increased to improve the antioxidant capacity of the cells and inhibit ROS formation. However, because ROS levels constantly increased, the enhanced enzyme activities were unable to repair ROS-induced damage and the MDA content increased.

In conclusion, *H. pluvialis* possesses two distinct antioxidative mechanisms: the defensive enzyme system and the antioxidative ketocarotenoid

astaxanthin. However, the two systems worked differently in the different types of cells to protect the organism from ambient oxidative stress. Astaxanthin acted more quickly and powerfully against oxidation than enzymes. Only when astaxanthin is absent or at very low levels, such as in green cells, the scavenging of ROS is dependent on the antioxidative enzyme system. In this case, the antioxidative enzymes play the key role in scavenging ROS, as follow: first, SOD traps O_2^- and POD then quenches H_2O_2, a by-product of SOD activity, and APX and CAT scavenge the remaining ROS.

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