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DETECTION OF HYDROXYL RADICAL IN INTACT CELLS OF *CHLORELLA VULGARIS*

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Using ESR with 5,5-dimethyl-1-pyrroline N-oxide (DMPO) as a spin-trapping reagent, we measured the levels of free radical species generated from living cells of *Chlorella vulgaris* var. *vulgails* (IAM C-534). To investigate the production of free radicals in the living *Chlorella vulgaris* cells, the influence of DMPO toward the intact cells of the *Chlorella vulgaris* using the O_2 evolution rate was first studied as a guide. Since the O_2 evolution rate was not changed by DMPO, it was judged that DMPO has no toxicity toward the intact cells of *Chlorella vulgaris*.

Only hydroxyl radicals (·OH) were detected as the DMPO-OH adduct in the suspension of intact cells of *Chlorella vulgaris* irradiated with visible light. Moreover, since production of ·OH was inhibited by some hydroxyl radical scavengers such as KI and ethanol, production of ·OH was proved to be due to hydroxyl radicals. It was also clear that the intensity of ·OH increased with increasing irradiation intensity of visible light. Therefore, it was suggested that ·OH might be one of the photoinhibition factors of the intact *Chlorella vulgaris* cells in severe light conditions.

KEY WORDS: Free radical, hydroxyl radical, spin trap, microalgae, visible light, photosynthetic efficiency.

INTRODUCTION

Oxygen radicals, namely superoxide anion radical (O_2^-) and hydroxyl radical (·OH), and other active oxygens such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) have been implicated as the initial toxic agents in pathology[1, 2] and plant physiology.[3, 4] ·OH is known as an extremely highly reactive oxygen species which reacts rapidly with biological components such as proteins, lipids, and nucleic acids, causing oxidative damages.[5]

The ESR spin-trapping technique is useful for detecting short-lived free radicals. Therefore, this technique has been useful for studying generation mechanisms of free radical active oxygens in chemical reactions and biological model systems.[6]

The detection of free radicals from a photosynthetic organ was previously investigated using chloroplast thylakoids with an EPR spectrometer,[7] but detection of free radicals from intact cells such as Chlorella and other microalgae using this technique are rarely reported.

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In this work, using the ESR spin trapping technique, we describe for the first time the detection of oxygen radicals in the intact cells of *Chlorella vulgaris*.

MATERIALS AND METHODS

Reagents

DMPO was purchased from Labotec Co. (Japan). All other reagents were of the highest grade commercially available.

Preparation of Algae

Chlorella vulugaris var. *vulgaris* (IAM C-534) was obtained from the culture collection of the Institute of Applied Microbiology, University of Tokyo.

The medium content was as follows; KNO_3, 5 g; KH_2PO_4, 1.25 g; K_2HPO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; NaCl, 1.8 g; $FeSO_4 \cdot 7H_2O$, 2.8 mg; trace-metal mixture A_5, 1 ml in 1 L of deionized water. Trace-metal mixture A_5 contained the following components: H_3BO_3, 2.86 g; $MnCl_2$, 1.81 g; $ZnSO_4 \cdot 7H_2O$, 0.22 g; $CuSO_4 \cdot 7H_2O$, 0.08 g; Na_2MoO_4, 0.021 g; concentrated H_2SO_4, 1 drop in 1 L of deionized water. The cultures were laterally illuminated by fluorescent lamps at a light intensity of 15,000 lux. The algal cultures were continuously sparged with air containing 5% CO_2 at a flow rate of 50 ml/min at 25°C. Turbidity of the *Chlorella vulgaris* were determined by OD_{680}.

Conditions of Radical Detection

The reaction mixtures were exposed to visible light at room temperature. Visible light irradiation was performed with the spot system (KTS-100, Kenko Co., Ltd., Japan) and its intensity was measured using an illuminometer (T-1M, Minolta Co., Ltd. Japan).

Instruments

Hydroxyl radical was analyzed using an ESR spectrometer, JEOL Model RE-3X, having an aqueous quartz flat cell (inner size 60 mm × 10 mm × 0.31 mm) with the spin trapping reagent 5,5-dimetyl-1-pyrroline N-oxide (DMPO).[8] The Mn^{2+} cation was used as an internal standard to calculate relative amounts from the ESR signal intensity. The *g* values of the peaks were 2.0334 and 1.9810 at the resonance frequency of 9450.0 MHz. The ESR conditions were as follows; microwave power 6 mW, modulation frequency: 100 kHz, modulation amplitude: 0.1 mT, response time: 0.03 sec, gain: × 200 ~ 600 and sweep time:2.5 mT/min.

O_2 Evolution Rate

The O_2 evolution rate in the *Chlorella vulgaris* suspension was measured using an O_2 electrode at 25°C.

RESULTS AND DISCUSSION

Effect of DMPO Toward Intact Cells of Chlorella vulgaris

In order to detect free radicals in intact cells of the *Chlorella vulgaris* which is undergoing photosynthesis, using a spin trapping reagent, we first evaluated the effect of DMPO toward intact cells of the *Chlorella vulgaris* using its photosynthetic O_2 evolution rate as a guide. Ninety mM to 448 mM of DMPO was added to each *Chlorella vulgaris* suspension at the same cell density, and the O_2 evolution rate after 20 min was compared to the control. Figure 1 shows that no change in the O_2 evolution rate was observed in all the tests. From these data, it was judged that the adopted 90 mM DMPO has no toxicity toward the intact cells of the *Chlorella vulgaris*. Therefore, DMPO was able to be used as a reagent for the detection of free radicals in the intact cells of the *Chlorella vulgaris* for ten minutes using the ESR spin trapping technique.

Detection of Free Radical

With 80,000 lux of visible light irradiated into the *Chlorella vulgaris* suspension, only DMPO-OH adducts, which are assigned as the hydroxyl radical (DMPO-OH; $aN = 1.49\,mT$, $aH = 1.49\,mT$) was observed (Figure 2(a)),[9] and its signal height gradually increased. However, DMPO-OH adducts are occasionally caused by an artifact.[8] We investigated whether the DMPO-OH adducts in the *Chlorella vulgaris* suspension are actually artifacts. Hydroxyl radical scavengers such as KI and ethanol were added to the *Chlorella vulgaris* suspension. When 25 mM KI was added to the suspension, ·OH formation in the suspension was completely supressed (Figure 2(b)). Moreover, when 860 mM ethanol was added to the suspension, the inhibition

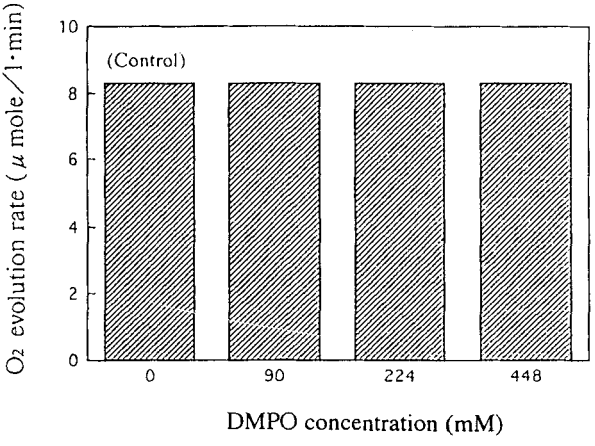

FIGURE 1 Effect of DMPO toward *Chlorella vulgaris* living cells. O_2 evolution rate of intact *Chlorella vulgaris* cell was measured with irradiating 17,000 lux of visible light in various concentrations of DMPO at 1.44 OD_{680} at 25°C. Data points represent the means of three replicates.

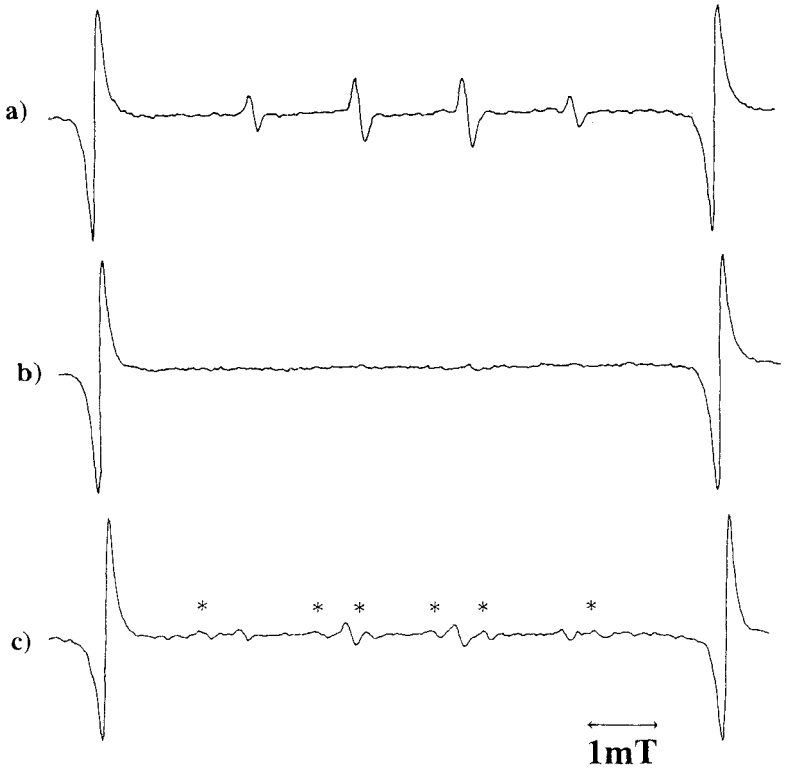

FIGURE 2 ESR spectra of the spin adducts of ·OH observed using the spin trap DMPO and the effect of ·OH scavenger such as KI and ethanol on the ·OH production from *Chlorella vulgaris* suspension using visible light irradiation. This chart indicates the *Chlorella vulgaris* suspension 6 min after irradiation was started by visible light. The signals on both sides are those of Mn^{2+}. (a) Control; (b) 25 mM KI; (c) 860 mM ethanol. The signal with "*" is the adduct of hydroxyl ethyl. Hyperfine splitting constants (mT) of DMPO-OCH_2CH_3 were aN equals 1.59 and aH equals 2.31.

of the DMPO-OH signal and the appearance of the DMPO-C_2H_4OH adduct, which is assigned as the ethanol radical (DMPO-C_2H_4OH; aN = 1.59 mT, aH = 2.31 mT) adduct, was observed (Figure 2(c)).[10] Hence, these results suggest that the *Chlorella vulgaris* suspension was the source of free hydroxyl radicals and produced the DMPO-OH adduct.

Production Site of Hydroxyl Radical in Chlorella vulgaris Suspension

To evaluate the source of the ·OH, the ·OH formation at various concentrations of *Chlorella vulgaris* was investigated. Figure 3 shows that increasing the *Chlorella vulgaris* concentration tends to increase the ·OH production. In addition, ·OH was scarcely observed in the supernatant of the *Chlorella vulgaris* suspension that was irradiated by visible light at 80,000 lux. From these data, it was clear that the source of the ·OH in the *Chlorella vulgaris* suspension is the *Chlorella vulgaris* living cell itself.

On the other hand, a large intensity of ·OH was detected in the supernatant of

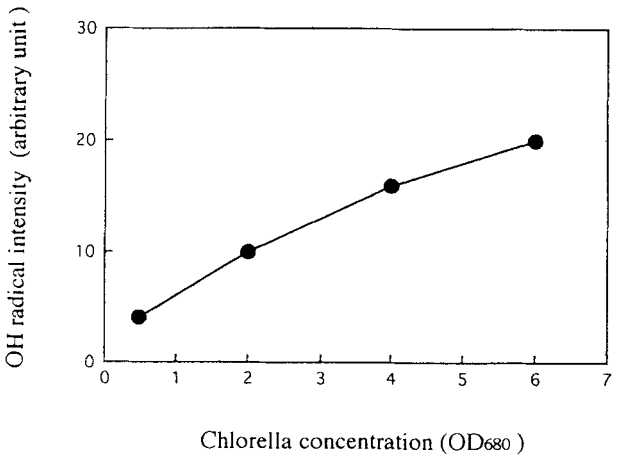

FIGURE 3 Effect of *Chlorella vulgaris* concentration on the ·OH production from *Chlorella vulgaris* solution by irradiation with visible light. Each *chlorella vulgaris* solution of various concentrations were irradiated with visible light at 80,000 lux.

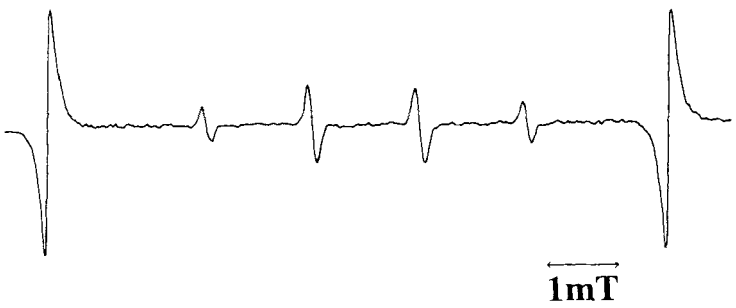

FIGURE 4 ESR spectra of spin adducts of ·OH observed using visible light irradiation from supernatant of *Chlorella vulgaris* homogenate prepared from the intact cells of *Chlorella vulgaris* at OD_{680} equal to 6.0.

the *Chlorella vulgaris* homogenate prepared from the intact cells of *Chlorella vulgaris* at OD_{680} equal to 6.0 with irradiated visible light (Figure 4). With respect to time, since the dissolved oxygen in the supernatant or the homogenate decreased with irradiated visible light (data not shown), the ·OH must come from the oxidizing process. In contrast, the dissolved oxygen did not decrease in the supernatant of the *Chlorella vulgaris* suspension shown in Figure 3 (data not shown). Hence, it is clear that the ·OH in the *Chlorella vulgaris* suspension comes from the intact cells of the *Chlorella vulgaris* without an oxidizing process.

In addition, it was thought that detected ·OH in the cells of *Chlorella vulgaris* irradiated with visible light must be come from the photosynthetic process. To clarify this point, the ·OH in the *Chlorella vulgaris* suspension was measured under dark conditions with DMPO after 80,000 lux of visible light was irradiated for

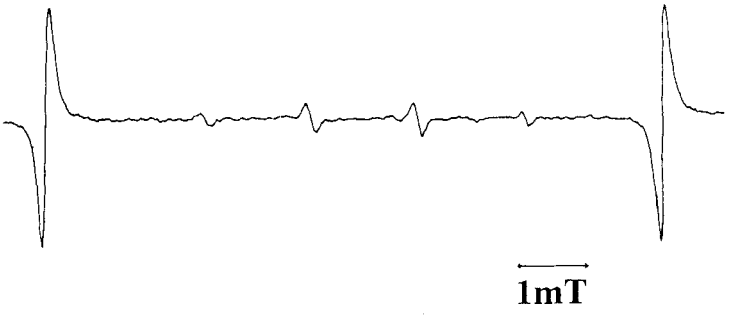

FIGURE 5 The ESR spectra of the spin adducts of ·OH observed under a dark condition. The ESR spectra of the *Chlorella vulgaris* solution was measured by ESR under a dark condition with DMPO after 80,000 lux of visible light was irradiated for 20 min without DMPO.

20 min without DMPO. Figure 5 shows that ·OH was observed under dark conditions with DMPO. This meant that the intact cells of *Chlorella vulgaris* start the photosynthetic process using irradiated visible light for 20 min without DMPO. Hence, these data suggest that the ·OH comes from the photosynthetic process.

Figure 6 shows the ·OH intensity under some conditions, (which are shown in Figures 2–5) where the ESR measuring condition is constant and all sample turbidities were adjusted to OD_{680} equals 6. It is clear that ·OH was easier to produce in bruised *Chlorella vulgaris* than in intact cells of *Chlorella vulgaris* and almost the same intensity was detected under continuous irradiation and discontinuous irradiation.

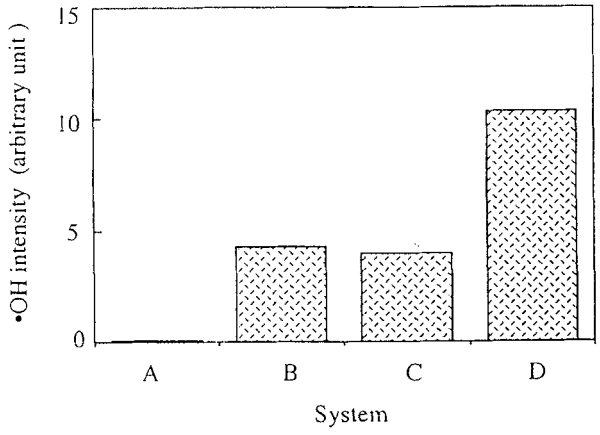

FIGURE 6 Comparison of the ·OH in various conditions. All the samples' turbidity were adjusted to OD_{680} equals 6 at 80,000 lux when ESR measuring condition became constant. A, supernatant of the *Chlorella vulgaris* suspension with visible light irradiation; B, continuous visible light irradiation to intact cells of the *Chlorella vulgaris*; C, discontinuous visible light irradiation to intact cells of the *Chlorella vulgaris*; D, visible light irradiation to supernatant of the homogenate from intact cells of the *Chlorella vulgaris*.

Change of Hydroxyl Radical Production in Chlorella vulgaris

To determine the factors which could increase ·OH formation, we studied the conditions of visible light irradiation to the intact cells of *Chlorella vulgaris* suspension. When irradiation of visible light toward the *Chlorella vulgaris* suspension was increased, ·OH production in the *Chlorella vulgaris* suspension was measured. As shown in Figure 7, the ·OH production increased with increasing light intensity.

·OH is known as an extremely reactive oxygen species.[11] Therefore, the detection of ·OH in the *Chlorella vulgaris* suspension suggests that some damage might be produced by free hydroxyl radicals.

One of the mechanisms of ·OH production with regard to light irradiation, however, was the known UV irradiation to H_2O_2. Since our experimental light was visible light, there must be a different mechanism of ·OH production in this system

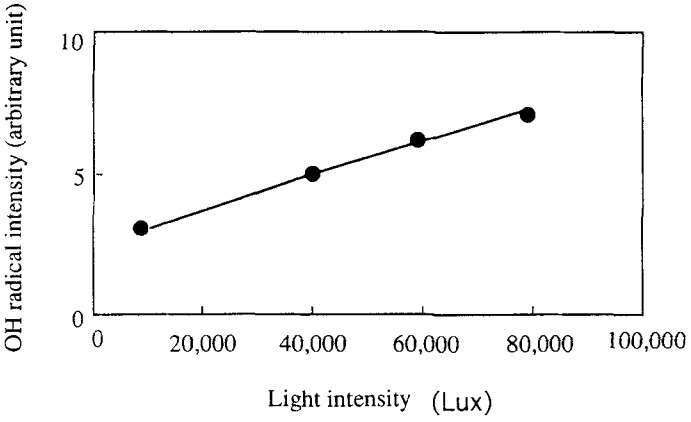

FIGURE 7 Relationship between the ·OH formation and light intensity in the *Chlorella vulgaris*.

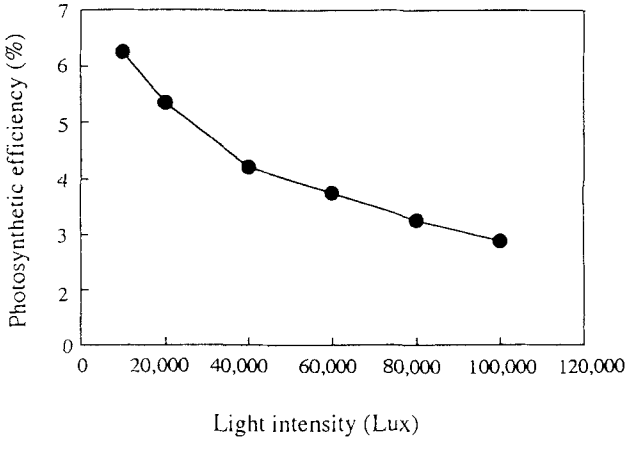

FIGURE 8 Relationship between light intensity and photosynthetic efficiency in the *Chlorella vulgaris*.

compared to the H_2O_2-UV system. In fact, if visible light was used to irradiate H_2O_2, ·OH was not detected under these conditions.

The ·OH production mechanism in *Chlorella vulgaris* is now being investigated.

Relationship between ·OH and Photosynthetic Efficiency

Since it was thought that the photosynthetic efficiency might decrease when light intensity of irradiation was increased, the photosynthetic efficiency of the *Chlorella vulgaris* was practically measured. As shown in Figure 8, its photosynthetic efficiency decreased with increasing visible light intensity. As previously mentioned, it was clear that ·OH increased with increasing visible light intensity in intact *Chlorella vulgaris* cells as shown in Figure 7.

Hydrogen peroxide, which is a kind of active oxygen, is also reported as a dangerous species toward carbon dioxide fixation sites in a chloroplast, including thiol enzymes such as fructose bisphoshatase, sedoheptulose bisphosphatase and ribulose phosphate kinase.[4] Futhermore, it was regarded that ·OH was the most active species of active oxygens. Hence, it is suggested that the ·OH in *Chlorella vulgaris* might be one cause of the decreasing photosynthetic efficiency under severe light intensity conditions.

Targeting points of ·OH for photosynthetic components is our research objective and as described here, ·OH was detected in intact cells of the *Chlorella vulgaris* for the first time. It is unclear whether ·OH can be detected in other microalgae at present. Therefore, the authors are now searching for ·OH in other microalgae, and results will be reported in the near future.

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