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References to a journal :

1. Yano S, Yanagawa H, Nishioka Y, Mukaida N, Matsushima K, Sone S : T kelper 2 cytokines differently regulate monocyte chemoattractant protein-1 production by human peripheral blood monocytes and alveolar macrophages. J Immunol 157 : 2660-2665, 1996

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2. Kinney JM, Tucker HN : Energy Metabolism. Tissue determinants and cellular corollaries. Raven Press, New York, 1992

Reference to a chapter in a book :

3. Takaue Y, Kawano Y, Kuroda Y : Mobilization of peripheral blood stem cells for autografts. In : Levit DJ, Mertelsmann R, eds. Hematopoietic Stem Cells. Biology and Therapeutic Application. Marcel Dekker Inc, New York, 1995, pp.611-639

Thioacetalization of Aldehydes Using a Flavin Catalyst and Blue LED Irradiation

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Department of Chemical Science and Technology, Tokushima University

Abstract

We developed a new method for thioacetalization of aldehydes that could be induced by visible light irradiation in the presence of a flavin organocatalyst under aerobic conditions. Several control experiments suggested that the reaction could be catalyzed by a flavin-based acidic complex generated *in situ* during the visible light irradiation.

Introduction

Flavin molecules, such as riboflavin and its derivatives, are known to be efficient photoredox catalysts for visible-light induced organic reactions including oxidations of benzyl alcohols and benzyl amines, sequential induction of isomerization and cyclization of cinnamic acids, and oxidative chlorination of arenes. In addition, they have been used as photosensitizing catalysts for oxidation of sulfides that singlet oxygen participates, isomerization of olefins, and [2+2] cycloadditions of dienes. These catalyses are based on either high oxidizing ability or energy transfer ability of excited flavins under continuous visible light irradiation, originally inspired by the functions of natural flavoenzymes such as photolyase and luciferase. In other words, the application of flavin molecules as catalysts has been limited to either redox or photosensitizing reaction systems so far. Herein, we report a novel use of neutral flavin molecules as a catalyst under visible light irradiation for thioacetalization of aldehydes, which generally requires expensive and potentially toxic Lewis acid catalysts.

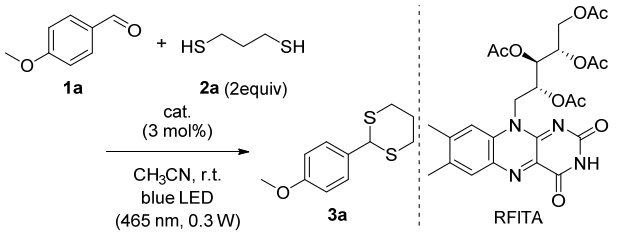
Experimental

In a 30 mL test-tube, a mixture of *p*-anisaldehyde (**1a**, 100 mg, 0.74 mmol), 1,3-propanedithiol (**2a**, 159 mg, 1.47 mmol), riboflavin tetraacetate (RFlTA, 11.9 mg, 21.9 μmol), and acetonitrile (3.5 mL) was stirred at room temperature under irradiation of visible light (blue LED, 465 nm, 100 mA, 0.3 W, 1 cm distance) for 0.5 h, and then stirred under non-irradiation of visible light for 9.5 h. The progress of the reaction was monitored by thin layer chromatography. After the reaction was completed, the reaction mixture was diluted with diethyl ether (35 mL) and washed with water (5 mL×3), 2N NaOH (3 mL×5), and water (5 mL×3). The organic phases were dried over $MgSO_4$ and evaporated under reduced pressure to give 2-(4-methoxyphenyl)-1,3-dithiane (**3a**) as a colorless solid (141 mg, 85%).

Result and Discussion

During our research on the development of flavin catalysis,[1] we serendipitously found that **1a** could smoothly react with **2a** in the presence of RFlTA (3 mol%) under blue LED light irradiation (465 nm) in air at room temperature to afford **3a** in quantitative yield within 6 hours (Table 1, entry 1). Importantly, no desired reaction occurred in the absence of either RFlTA, light, or oxygen, which indicated that all of them are essential for the reaction (entries 2–4). Although increasing the partial pressure of O_2 could make the reaction more efficient (entry 5), reaction conditions under air were used for further studies, because they are efficient enough. To understand the effectiveness of RFlTA, other organic dyes including 3-methyllumiflavin (MLFl), 1,3-dimethylalloxazine (DMA), eosin Y (EY), and methylene blue (MB) as

Table 1. Flavin-catalyzed thioacetalization of **1a**



entry	cat. (3 mol%)	visible light	atmosphere	time (h)	yield[a] (%)
1	RFlTA	on	air	6	99
2	–	on	air	6	0
3	RFlTA	off	air	6	0
4	RFlTA	on	N_2	6	0
5	RFlTA	on	O_2	3	100
6	RFlTA	on	air	1	52
7	MLFl	on	air	1	30
8	DMA	on	air	1	15
9	EY[b]	on	air	1	0
10	MB	on	air	1	0
11	$Ir(ppy)_3$[c]	on	air	1	0

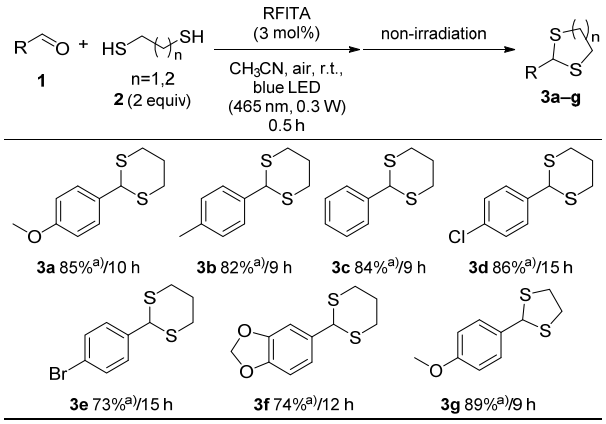
a) Determined by ^{1}H NMR spectroscopy using 1,4-dioxane as an internal standard.
b) In CH_3CN/DMF (6/1) with green LED light (525 nm).
c) In DMF.

well as a transition metal complex, $Ir(ppy)_3$, which can be excited by absorbing visible light, were also tested as photocatalyst for the present reaction. Interestingly, the flavin type molecules RFlTA, MLFl, and DMA were specifically effective, while the non-flavin type dyes EY, MB, and $Ir(ppy)_3$ showed no catalytic activity (entries 6–11). Particularly effective was RFlTA, probably because of its higher reduction potential compared to that of MLFl in their ground state (RFlTA: E_{red} = –725 mV, DMA: E_{red} = –809 mV, vs Ag/AgCl) and its higher absorption efficiency compared to that of DMA at 465 nm (RFlTA: $\lambda_{abs} \approx$ 450 nm, DMA: $\lambda_{abs} \approx$ 400 nm).

To gain insights into the mechanism of this new flavin catalysis, some control experiments were carried out using **1** and **2** as substrates and RFlTA as a catalyst. Since the quantum yield of the reaction was determined to be 7.8 by chemical actinometry with $K_3[Fe(C_2O_4)_3]$, an intermittent irradiation experiment was then performed to clarify whether the reaction proceeded via radical propagation mechanism or not, in which irradiation (ON) /non-irradiation (OFF) of visible light was switched every 1 hour, started from the ON state. There was no difference in overall reaction profiles between the reaction irradiated continuously and that irradiated intermittently, showing that visible light irradiation only required to initiate the reaction that may further proceed. Nevertheless, the addition of galvinoxyl free radical did not prevent the reaction at all, while it was completely suppressed by the addition of triethylamine with a change of color of the reaction mixture from yellowish brown to fluorescence yellow that is the characteristic color of RFlTA. These results suggested that the present reaction could be catalyzed by an acidic complex consisting of RFlTA, thiol, and O_2, plausibly like [RFlTA$^{\bullet-}$·RS$^{\bullet}$·O_2·H$^+$], generated *in situ* reversibly under visible light irradiation, which is a new type of flavin catalysis that can unusually promote non-redox reactions.

Finally, we evaluated the scope of the thio-acetalization and reacted a range of different aldehydes and dithiols in the presence of 3 mol% of RFlTA, which was irradiated for initial 30 minutes and then continued under non-irradiation conditions (Table 2). Various aromatic aldehydes with both electron-donating and electron-withdrawing

Table 2. Flavin-catalyzed thioacetalization of aldehydes



a) Isolated yield

substituents reacted smoothly with **2a** to give the corresponding 1,3-dithiane **3a**–**3e** in high yields. Remarkably, piperonal bearing an acetal functional group that are typically acid-labile could be tolerated to afford **3f** in good yield. Moreover, the use of 1,2-ethanedithiol (**2b**) instead of **2a** allowed for the formation of 1,3-dithiolane such as **3g**.

Conclusion

We developed flavin-catalyzed thioacetalization of aldehydes that could be induced by visible light irradiation under aerobic conditions.

Reference

[1] Imada, Y.; Naota, T. *Chem. Rec.* **2007**, *7*, 354–361.

Flavin Catalysts with Photoredox Activity Under Blue LED Irradiation

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Department of Chemical Sciences and Technology, Tokushima University

Abstract

We found that flavins have catalytic activity for reductive quenching-based photoredox reactions. Flavin derivative RFTA can catalyze α-oxyamination of aldehydes with TEMPO in the presence of secondary amine cocatalysts under blue LED irradiation, which previously required the transition metal-based photoredox catalyst. In addition, flavin–amine integrated catalysts, which have flavin and secondary amine moieties, can function both as the photoredox catalyst and the secondary amine catalyst.

Introduction

Flavins such as riboflavin are simple photoactive organic molecules that can be excited by absorbing visible light (Figure 1b), which are involved in DNA repair systems as a photolyase in nature.[1] However, there are few reactions using flavin as a photocatalyst so far.[2] On the other hand, transition metal complexes such as $Ru(bpy)_3^{2+}$ have become the most promising catalysts to carry out visible-light driven photoredox reactions in organic synthesis,[3] though they are expensive and potentially toxic (Figure 1a). In recent years, a variety of organic dyes, which are typically inexpensive and less toxic, have also been utilized as metal-free photoredox catalysts.[4]

Herein, we present the development of flavin catalysts for reductive quenching-based photoredox reactions. We found that α-oxyamination of aldehydes with TEMPO was successfully catalyzed by flavins in the presence of secondary amine cocatalysts under blue LED irradiation. In addition, flavins having secondary amino group could function both as the photoredox catalyst and the secondary amine catalyst.

Experimental

A 5φ NMR tube was charged with TEMPO (100 μmol), photoredox catalyst (1 μmol), 1,3,5-trimethoxy-benzene (17 μmol) as NMR internal standard, and CH_3CN (0.50 mL). 3-Phenylpropanal (**1**, 50 μmol) and morpholine (5 μmol) were added to the mixture, which was then deaerated by N_2 bubbling and irradiated by LED visible light (465 nm, 1.1 W, I_F=350 mA, 1 cm distance) at room temperature for a defined reaction time. The reaction yield was determined by ^1H NMR spectrospopy of the crude mixture.

Results and Discussion

We have chosen α-oxyamination of aldehydes as a model reaction, which previously required the

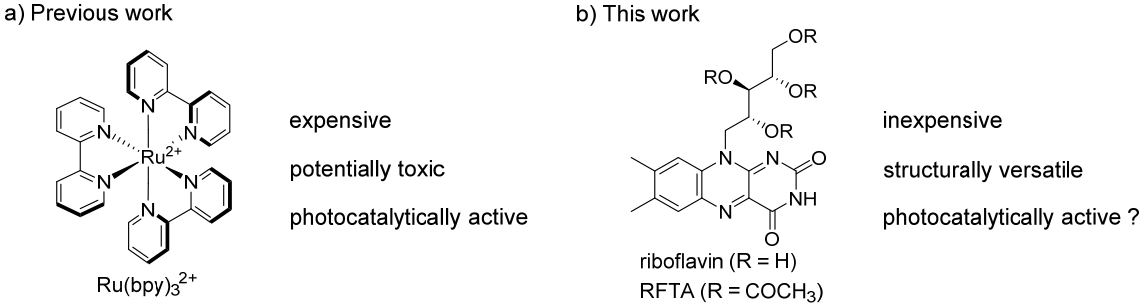
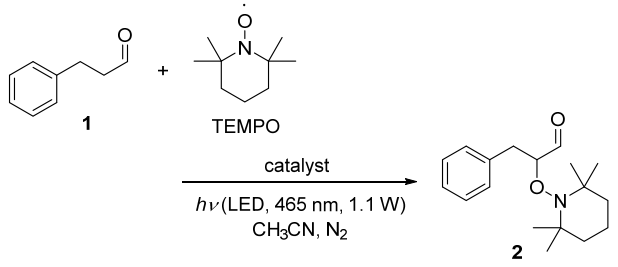


Figure 1. Conventional transition metal-based photoredox catalyst (a) and flavins (b).

transition-metal catalyst,[5] and started our investigation by exploring whether riboflavin derivative RFTA can promote the α-oxyamination as a photoredox catalyst. Under the above conditions, aldehyde **1** was fully consumed within 6 hours and the desired α-oxyaminated product **2** was obtained in 80% yield (Table 1, entry 1). Typical transition metal-based photoredox catalyst, $Ru(bpy)_3(PF_6)_2$, was much less active under the same conditions (entry 2). It should be noted that the lack of any one of flavin, morpholine, and LED light did not allow for the photoredox reaction, which suggested one electron transfer mechanism similar to that of previous transition metal-based catalysis.

Table 1. α-Oxyamination of 3-phenylpropanal



entry	catalyst (mol%)	time/h	conv.[a]/%	yield[a]/%
1	RFTA (2)–morpholine (5)	6	100	80
2	$Ru(bpy)_3(PF_6)_2$ (2)–morpholine (5)	6	48	37
3[b]	**3** (2)	15	58	25
4[b]	**4** (2)	15	84	67
5[b]	**5** (2)	15	95	75

[a] determined by ^1H NMR measurement with internal standard

[b] in the presence of Et_3N (2 mol%)

To further extend the versatility of flavin photoredox catalysts, we explored whether flavins bearing a secondary amino group could catalyze the α-oxyamination without adding external secondary amine cocatalyst. We prepared flavin–amine integrated molecules **3**–**5** from riboflavin (Figure 2), which showed similar excitation spectra to riboflavin, and evaluated their catalytic activity for the α-oxyamination. Reactions were performed with **1** and TEMPO (2 equiv) in the presence of flavin–amine integrated molecules (2 mol%) and Et_3N (2 mol%) in CH_3CN under N_2 atmosphere and blue LED irradiation, in which Et_3N was added to liberate a free secondary amine moiety *in situ*. As expected, flavin–amine integrated molecules functioned both as the photoredox catalyst and the secondary amine catalyst. In particular, **4** and **5** having relatively long spacers between flavin and secondary amine moieties showed higher catalytic activities than **3** (entries 3–5). Stable conformations of **3**–**5** predicted by DFT calculation (B3LYP/6-31G*) showed that the flavin ring and the secondary amino group could be spatially close to each other in **4** and **5** because of the flexible spacers, which wolud be a key point of the design of efficient flavin–amine integrated molecules.

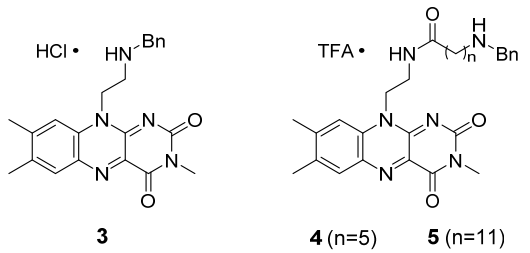


Figure 2. Flavin–amine integrated molecules

Conclusion

We found that riboflavin derivatives RFTA and FAs could be used as efficient photoredox catalysts for α-oxyamination of aldehydes under visible light irradiation.

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Synergistic sterilization effect on cabbage by simultaneous treatment of UVA-LED and sodium hypochlorite with effective residual chlorine degradation

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Abstract

To develop a new food surface sterilization device, sterilization effect of ultraviolet A (UVA) light-emitting diodes (LED) and/or sodium hypochlorite (NaClO) was investigated. UVA-LED and/or NaClO were treated on *E.coli* in the small device. Simultaneous treatment of UVA-LED and NaClO produced synergistic sterilization effect. In the same device, concentration of free and total residual chlorine were significantly decreased by 30 minutes UVA-LED irradiation. Next, sterilization device was developed as large amount of capacity. UVA-LED and/or NaClO were treated on 400 gram of shredded cabbage with *E.coli*. Simultaneous treatment of UVA-LED and NaClO produced synergistic sterilization effect as same in the small device. In this study, new food surface sterilization system was developed, simultaneous treatment of UVA-LED and NaClO produced synergistic sterilization effect and chlorine degradation was achieved so it can be a new and effective sterilization method.

1. Introduction

People have more interest in food safety and quality today however we have no end of foodborne diseases incident. In 2011, massive foodborne disease outbreak of Shiga-toxin producing O104:H4 serotype *Escherichia coli* (STEC) caused by fresh spouts consumption caused near 4000 cases including 54 deaths in Germany [(1)]. Bagged spinach caused an outbreak of *Eshrichia coli* O157:H7 in the United States 205 cases including 3 deaths in 2006[(2)].

Ready-to-eat or ready-to-cook foods consumption are increasing in recent years but these foods can be contaminated in their manufacturing processes. New system of sterilization and preserve for food is needed to meet the requirements of control bacterial growth during production process to the consumers.

Sodium hypochlorite (NaClO) is one of the common sterilization treatments for water and food because of its powerful oxidation effect and cost-effectiveness. However there are some chlorine-resistant microorganisms such as *Cryptosporidium parvum oocyst* or *Gialdia lamblia* have caused a severe waterborne infection [(3)]. Also, there is an anxiety of health and

environment effect by disinfection byproducts such as trihalomethan[4] .NaClO treatment has limitation of removing bacteria from shredded cabbage because sufficient amount of NaClO does not penetrate into the cut edges[5]. Moreover NaClO does not have enough ability on the lettuce surface because of stomata and biofilms formation [6].

Ultraviolet (UV) is a promising sterilization method of water or food which does not use any chemical addition. UV disinfection systems with mercury lamp mainly using UVC wavelength (<280nm) are widely installed because deoxyribonucleic acid (DNA) has the maximum absorption around 260 nm and UVC can achieve effective sterilization. However UVC induce damages not only harmful microorganism but also human skin or eyes and caused carcinogenic effects. We previously developed small capacity sterilization devices for water or food surface using UVA light-emitting diode (UVA-LED, at the peak wavelength was 365nm), and these devices have enough sterilization ability against various kinds of bacteria[7].In addition, inactivation of *Cryptosporidium* oocysts was achieved by UVA-LED irradiation[8].However these sterilization devices need comparatively long processing time. This study sought to make short processing time and increase the sterilization effect by using UVA-LED irradiation and NaClO simultaneously. Sterilization effect of simultaneous use of UVA-LED and low concentration of NaClO on bacterial solution or shredded cabbage were examined.

2. Materials and Methods

2.1. UVA-LED irradiation devices

Tow size of UVA-LED irradiation devices were used in this study. The one was small capacity device, 2L volume of box with nine UVA-LEDs (NCSU033E, Nichia Corp. Japan) were connected in series to a direct-current power supply (PAS40-9, Kikusui Electronics Corp. Japan). The distance from the UVA-LEDs to the surface of the *E.coli* solution was fixed at 50 mm. The other one was scale-up device which capacity was 50L, consisted of 4 units and total 200 UVA-LEDs (NCSU033E, Nichia Corp. Japan) in Fig.1, were connected in series to a direct-current power supply (Manufactured by SUN Electronics Industry Ltd. Japan). One unit included 50 UVA-LEDs and these were placed on the top, right, left and back side of refrigerator. Cylindrically-structured glass container was placed on the rotatable roller which UVA-LED irradiation could irradiate evenly. The UV fluence was measured by an accumulated UV meter (UIT-250, Ushio Inc. Japan). The values were 121 Wm^{-2} at the small device and 102 W m^{-2} at the inside of glass container in the refrigerator.

2.2. Bacterial strain and culture condition

Escherichia coli DH5α (Takara Bio Inc., Japan) was cultured in Luria-Bertani (LB) broth (1% tryptone, 1% NaCl, 0·5% yeast extract) at 37° C for 18 h aerobically. Cells were collected by centrifugation (5000g, 10min, 4° C), washed three times with phosphate-buffered saline (PBS) and suspended in PBS at 10^9 CFU ml^{-1}, 4ml was placed in 40 mm plastic petri dish (As One Corp.Japan) for the small device to examine solution sterilization effect. *E.coli* was suspended in PBS at 10^6 CFU ml^{-1}, 500ml was mixed with 400g of shredded cabbage to examine surface sterilization effect in glass container.

Figure 1. Sterilization device using 200 of UVA-LEDs. 54L of refrigerator and power supply.

2.3. Sodium hypochlorite treatment Sodium hypochlorite (NaClO, Wako Pure Chemical Industries, Ltd. Japan) was adjusted finally 6 ± 0.5 mg l^{-1} for the sterilization experiment or 200 ± 5.0 mg l^{-1} for the chlorine degradation measurement by diluting with PBS. The concentrations of free and total chlorine were measured by DPD method using potable residual chlorine analyzer (HI95734, HANNA Instruments Ltd. Japan) after diluting with PBS appropriately. 2.4. Cabbage Cabbage was purchased at a local supermarket in the morning of each experiment day and kept in the 4° C until experiments. The outer and core leaves were removed, the remaining leaves were shredded into 5 mm width by ethanol sterilized knife and cutting board then 400g of shredded cabbage were placed in a glass container. Shredded cabbage were premixed with *E.coli* solution and incubated 60 minutes then sterilization treatment (UVA-LED irradiation, NaClO and simultaneous use of UVA-LED and NaClO) were performed. 2.5. Determination of sterilization effect After sterilization treatment, bacterial solution were diluted appropriately then spread on LB agar (1% tryptone, 1% NaCl, 0·5% yeast extract, 1·5% agar) and incubated at 37° C for 18 h. After incubation the numbers of bacterial colonies were counted and log survival ratios were calculated using the following equation:

Log survival ratio = $\log_{10}(Nt/N0)$

Nt is the colony count of the sample with sterilizationd, and $N0$ is the colony count of the sample without sterilization.

The synergy value was of simultaneous treatment of UVA-LED irradiation and NaClO was calculated by the following equation[9]:

Synergy ($\log_{10}$) =Log survival ratio [simultaneous use of UVA-LED irradiation and NaClO treatment − (UVA-LED irradiation + NaClO treatment)]

3. Results

3.1. Sterilization effect UVA-LED or/and NaClO of on *E.coli*

The sterilization effect of UVA-LED irradiation, NaClO treatment and simultaneous use of UVA-LED irradiation and NaClO treatment on *E.coli* were shown in Fig.2. The survival ratios ($\log_{10}$) after UVA-LED irradiation was -0.27 ± 0.16, NaClO treatment was -1.06 ± 0.21 and simultaneous use of UVA-LED irradiation and NaClO treatment was -3.36 ± 0.47 on *E.coli* solution. Synergistic sterilization effect was indicated by simultaneous use of UVA-LED irradiation and NaClO treatment on *E.coli*. 3.2. Change in chlorine concentration by UVA-LED irradiation

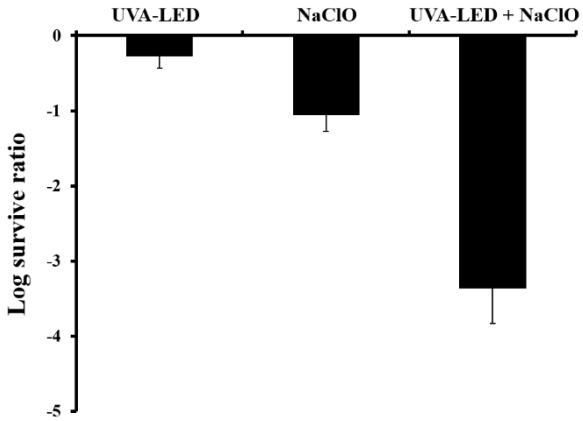

Figure 2. Sterilization effect on *E.coli* in a small device.
Values are shown as means ± SD (n=4, n=number of independent replicates)

To investigate how chlorine concentration were changed by UVA-LED irradiation, Free and Total chlorine concentration were measured. Free chlorine concentration was decreased significantly by 30 minutes UVA-LED irradiation and less than 15 mg l^{-1} by 120 minutes. Control sample was kept in the dark condition and Free chlorine concentration remained initial level even after 120 minutes. (Fig.3 (A)). Total chlorine concentration was also decreased significantly by 30 minutes and finally less than 15 mg l^{-1} by 120 minutes UVA-LED irradiation. Control sample was kept in the dark Total chlorine concentration also remained initial level even after 120 minutes (Fig3 (B)).

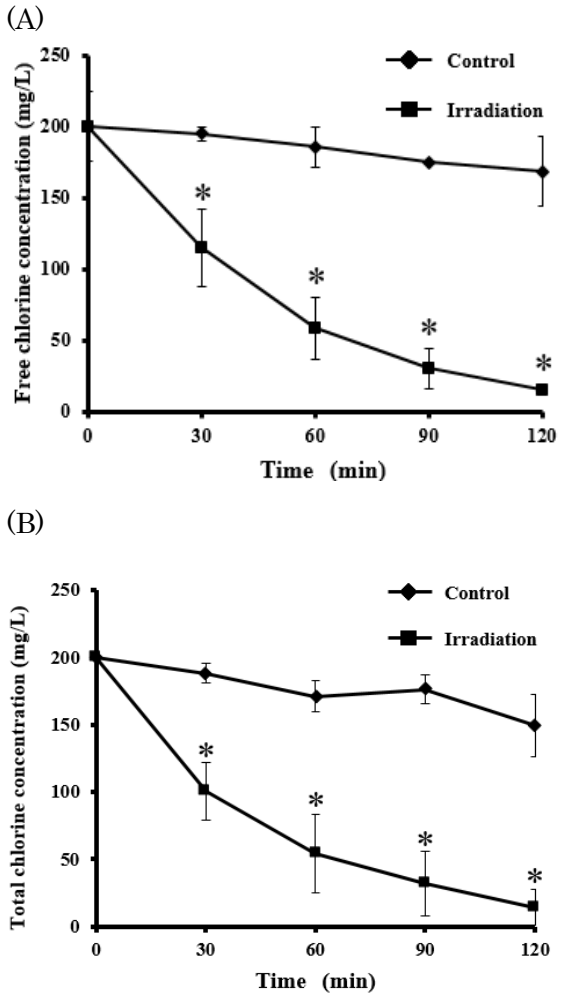

Figure 3. Time dependent change in levels of chlorine by UVA-LED irradiation. (A) Free chlorine concentration. (B) Total chorine concentration. (*) P＜ 0.05 versus control.

3.3. Sterilization effect of UVA-LED irradiation or/and NaClO treatment on shredded cabbage surface

The sterilization effect of UVA-LED irradiation, NaClO treatment and simultaneous use of UVA-LED irradiation and NaClO treatment on shredded cabbage with *E.coli* were shown in Fig.4. The survival ratios (log_{10}) after UVA-LED irradiation was -0.99 ± 0.01, NaClO treatment was -0.30 ± 0.05 and simultaneous use of UVA-LED irradiation and NaClO treatment was -4.13 ± 0.81. These results indicated that simultaneous use of UVA-LED irradiation and NaClO treatment produced significant synergistic sterilization effect on shredded cabbage with *E.coli* as well as *E.coli* solution.

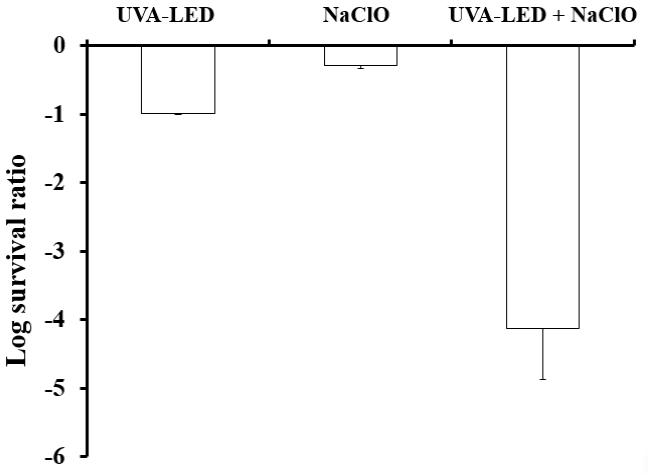

Figure 4. Sterilization effect of UVA-LED irradiation or/and NaClO treatment on shredded cabbage surface. Values are shown as means ± SD (n=4, n=number of independent replicates)

4. Discussion

UVA-LED and NaClO used simultaneously and produced synergistic sterilization effect both on *E.coli* in a small scale device and shredded cabbage with *E.coli* in a large scale device. Furthermore, Free and Total chlorine concentration were significantly decreased by UVA-LED irradiation. The synergistic sterilization effect may depend on increasing of Reactive Oxygen Species (ROS) production level. Because UVA-LED irradiation generated ROS predominantly singlet oxygen ($^1O_2$) and it suppressed Raw 264.7 cell viability [10]. UVA-LED irradiation increased Dissolved Oxygen (DO) (data not shown) suggested by next equations:

$2NaClO \rightarrow 2NaCl + O_2\uparrow$

DO increase suggested that ROS especially $^1O_2$ production also increased caused by simultaneous use of UVA-LED irradiation and NaClO treatment. $^1O_2$ has an extremely short lifetime (<1μs) and high reactivity[11] so that $^1O_2$ was turned out to be DO. In conclusion simultaneous use of UVA-LED irradiation and NaClO is expected to be a new and effective sterilization and preserve method for food.

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Irradiation of ultraviolet A light emitting diode (UVA-LED)suppresses cell growth and induces oxidative stress in *Chlorella* sp. MK201.

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Abstract

Ultraviolet (UV) irradiation is an increasingly used method of water disinfection. UV rays can be classified by wavelength into UVA (320-400 nm), UVB (280-320 nm), and UVC (< 280 nm). We previously developed UVA sterilization equipment with a UVA-light emitting diode (LED). In recent year, nutrient over-enrichment (eutrophication) of water systems has been a global issue. Eutrophication can affect human health by enhancing the growth of algal blooms which can release off-flavor or toxins. The aim of this study was to determine the effect of UVA-LED irradiation to a green microalga, *Chlorella* sp. MK201. UVA-LED irradiation at doses of 200 to 600 kJ/m^2 inhibited cell growth of *Chlorella* sp.. In addition, UVA-LED irradiation decrease significantly decreased chlorophylls-dependent fluorescent intensity in *Chlorella* sp. Content of intracellular chlorophyll a rather than chlorophyll b was decreased by UVA-LED irradiation. Intracellular oxidative protein and genomic DNA fragmentation were significantly increased by UVA-LED irradiation. Furthermore treatment with catalase, an enzyme which can catalyze hydrogen peroxide, recovered the inhibited cell growth by UVA-LED irradiation. From these results, UVA-LED irradiation could inhibit cell growth of *Chlorella* sp. MK201, and the effect might be depended irradiation-induced reactive oxygen species.

1. Introduction

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can live and grow in harsh conditions due to their unicellular and multicellular structures. One of prokaryotic microalgae is cyanobacteria, a gram negative photosynthetic bacteria. Green algae, such as *Chlorella* species, is belong to eukaryotic microalgae [1,2]. *Chlorella* sp. is a unicellular microalgae and can grow in fresh water. It has been living on earth and its genetic integrity has remained constant since 2.5 billion years ago (pre-Cambrian period) [3]. In recently, the abundant and diversity of green algae are increased in fresh water environments, such as lakes, ponds, streams, and wetlands, where they may form nuisance blooms under over nutrient conditions [4,5]. The algal blooms cause the taste-and-odor problem, discoloration, or scum

formation in drinking water [6]. It is also necessary to prevent algal blooms because it causes various problems such as aquatic ecosystem destruction, filter clogging in water purification plants, and toxicity by blue-green algae after the rainy season [7]. To prevent these problems from algal bloom, algacides, herbicides, and other biocides are widely used. But these treatments are environmentally hostile processes because these chemicals can steep and infiltrate into the surrounding area, and finally contaminate the ground water. Consequently, these treatments have been heavily criticized; as a result, some regulations require that chemical treatments be replaced by new environmentally friendly processes [8].

Ultraviolet (UV) irradiation is one of suitable alternative methods instead of chemical treatments. UV rays can be classified by wavelength into UVA (320-400 nm), UVB (280-320 nm) and UVC (< 280 nm). UVC disinfection systems with low-pressure mercury lamps have been installed and widely used as an effective sterilization method for drinking and wastewater [9]. Because deoxyribonucleic acid (DNA) has maximum absorption around the UVC wavelength, its irradiation can induce thymine dimers, such ascyclobutane pyrimidine dimers (CPDs), into the genomic DNA of an organism [10,11]. However some bacteria has DNA repair systems for thymine dimmers, such as photolyase and the SOS response, which impart tolerance to the effects of UVC on bacterial DNA [12,13].

We originally developed an UVA disinfection lamp system constructed comes with light-emitting diode (UVA-LED) [14]. This system could disinfect some enteropathogenic bacteria such as *Escherichia coli* or *V. parahaemolyticus* [13,14]. Some reports and our data have been shown that UVA irradiation induces cellular membrane damages and delays growth indirectly by increasing levels of reactive oxygen species, including superoxide anion radicals ($O2^{\bullet-}$), hydroxyl radicals ($OH^{\bullet}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$) [9-13]. Furthermore we reported recently that the recovery system to DNA damage by UVC irradiation was not by UVA-LED irradiation [13]. But the effect of UVA-LED irradiation to microalgae remains still unclear. Aim of this study was to determine effects of UVA-LED irradiation to cell growth, photosynthetic pigments, and protein oxidation in a green microalga, *Chlorella* sp. MK201.

2. Materials and Methods

2.1. Algal strain and cell preparation.

An algal strain *Chlorella* sp. MK201 was used in the present study [15]. It was routinely cultured with sterilized BG-11 medium (Gibco, Waltham, MA, USA) in a temperature controlled culture room at 22 ± 2 □ under cool white fluorescent light (18W). Before UVA-LED irradiation, algal suspension was washed by three times washing with BG-11 medium and adjusted the cell concentration at 8.0×10^6 cells/mL.

2.2. UVA-LED irradiation

UVA-LED irradiation system was originally developed. The device had six individual UVA-LED (NC4U134A, Nichia, Tokushima, Japan) connected in series to single power source. The peak wavelength of the diode was 385 nm. The averaged intensity was 336 mW/m^2 by a constant-current power supply (0.1 A), which was measured by an accumulated UV meter (UIT-205, Ushio, Tokyo, Japan). Fluences of UVA-LED irradiation were 100-600 kJ/m^2 (5-30 min irradiation). 200 μl of *Chlorella* sp. MK201 suspension were placed in 96-well plate and exposed from lower

to bottom of plate.

2.3. Fluorescence microscopy and measurement of absorption and fluorescence spectrum

After UVA-LED irradiation, algal suspension was transfer onto cell-counting hemocytometer and observed under a fluorescence microscopy (BX50, OLYMPUS) with U-MWU2UV filter (330-385nm excitation). Absorption and fluorescence spectrum of algal suspension after UVA-LED irradiation was measured by a micro plate reader Spectra Max i3 (Molecular Devices, Sunnyvale, CA, USA).

2.4. Measurement of chlorophylls in *Chlorella* sp

Chlorophylls were extracted from *Chlorella* sp. MK201 by ethanol. The samples were analyzed by Agilent liquid chromatography-time-of-flight mass spectrometry (LC-MS) system 6200 with ZORBAX Eclipse Plus C18 column (Agilent technologies, Santa Clara, CA) at a flow rate of 1 mL/min using water-methanol (40:60) as initial mobile phase. After sample injection, the percentage of methanol was increased to 0:100 at 10 to 30 min and continued for 20 min. Equipped ion source of MS in this study was Agilent jetstream electrospray ionization source (Dual AJS ESI). ESI source were operated in positive mode: spray voltages of 3500V for capillary entrance and 500V for nozzle, Nitrogen sheath gas temperature (350°C) at a flow rate of 12 L/min, Nitrogen drying gas was 150°C at a flow rate of 10 L/min, Nitrogen nebulizer at 55 psig. Extracted ion chromatogram (EIC) of chlorophylls and integral analysis of peak area were performed by Agilent MassHunter software (Agilent technologies).

2.5. Protein oxidation

To analyze the overall oxidative stress on protein level, we used OxyBlot kit (Millipore, Billerica, MA, USA) for immunodetection of these carbonyl groups, which is a hallmark of the oxidation status of proteins, following manufactures instructions [16].

2.6. Growth curve of *Chlorella* sp. and catalase treatment

After UVA-LED irradiation, algal suspension was diluted fifteen-times with BG-11 medium and started culturing at 22 ± 2 ºC under cool white fluorescent light, for 24 days. In the experiment of catalase treatment, 100 U/mL catalase from bovine liver (WAKO, Tokyo, Japan) was added into algal cell suspension for 30 min before UVA-LED irradiation. After irradiation at a dose of 200 kJ/m^2, catalase was removed by three-times washing with BG-11 and algal suspensions were cultured for 14 days. Because cell concentration of *Chlorella* sp. MK201 had positive correlation to optical density at 685 nm ($R = 0.99$, Supplemental figure S1), it was measured every 24 hour for monitoring cell growth by Spectra Max i3.

2.7. Genomic DNA fragmentation

Genomic DNA was purified from UVA-LED irradiated *Chlorella* sp. MK201 by using a phenol-free method as Singh *et al.* reported [17]. Agarose gel electrophoresis images were captured by a CCD camera system (Gel Doc EZ, Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Statistical analysis

Statistical analysis of differences was performed using ANOVA with Bonferroni's multiple comparison tests. Student's t-tests were used for paired data when appropriate. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of UVA-LED irradiation to fluorescent intensity of *Chlorella* sp. MK201.

Microalgaes have some photosynthetic pigments such as chlorophylls, which have fluorescent intensity. To determine the effect of UVA-LED on these intensities, we irradiated UVA-LED to *Chlorella* sp. MK201 and observed that under a fluorescence microscopy with 330-385 nm excitation wavelength. Under the light fields, UVA-LED irradiation at the doses of 200 to 600 kJ/m^2 decreased green-colored microalgaes in a dose dependent manner (Figure 1, top panels). In addition, those irradiations significantly decreased the number of fluorescent cells and increased that of non-fluorescent cells (Figure 1). Next to determine which fluorescent pigments were decreased by UVA-LED irradiation, we measured absorption spectrum of irradiated *Chlorella* sp.. *Chlorella* sp. had two peak of absorption wavelength (440 and 685 nm), which significantly decreased by UVA-LED irradiation in a dose-dependent manner (Figs. 2a and 2b). Because that absorption spectrum was closely similar with chlorophyll a, we measured fluorescent intensity of 435 nm excitation, which is a one of major fluorescence excitation wavelength of chlorophyll a [18]. UVA-LED irradiation decreased significantly fluorescent intensity of 685 nm emission wavelength in *Chlorella* sp, in a dose dependent manner (Figs. 2c and 2d). From these results, UVA-LED could decrease intracellular chlorophyll a in *Chlorella* sp. MK201.

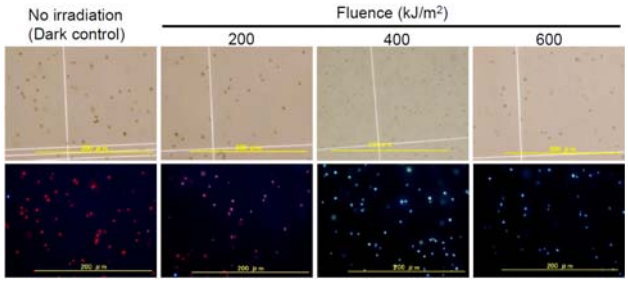

Figure 1. Microscopic images of *Chlorella* sp. MK201 after UVA irradiation. Top panels; bright field images, bottom panels; fluorescent images.

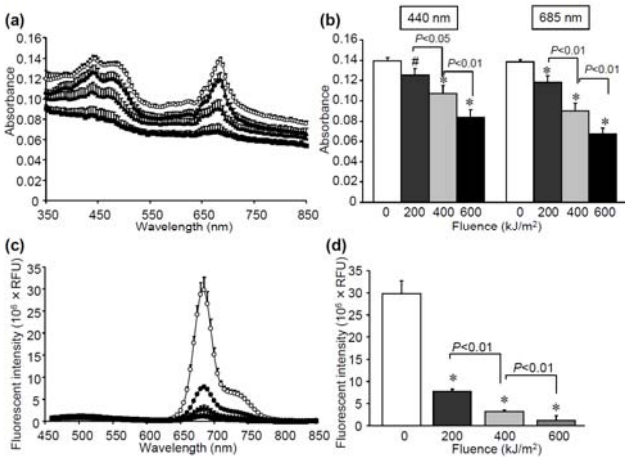

Figure 2. Absorbance and fluorescence intensities of *Chlorella* sp. MK201 after UVA irradiation. (a) Absorbance spectrum of No-irradiation (○), or irradiation with 200 (●), 400 (■), or 600 (▲) kJ/m^2. (b) Absorbance at 440 or 685 nm. (c) Fluorescent spectrum of No-irradiation (○), or irradiation with 200 (●), 400 (■), or 600 (▲) kJ/m^2. (d) Fluorescence intensity at 685 nm excitation. Fluorescence intensities were measured by 435 nm emission. Values are shown as means ± SD (n = 3, n = number of independent replicates). ($^{\#}$) $P < 0.05$, or (*) $P < 0.01$ versus versus no-irradiation.

3.2. Effect of UVA-LED irradiation to chlorophylls in *Chlorella* sp. MK201.

Chlorella sp. has mainly two subtypes of chlorophylls, chlorophyll a and b. To determine the effect of UVA-LED irradiation to chlorophylls, we extracted those from UVA-LED irradiated *Chlorella* sp. and measured ion counts of accurate mass of those by LC-MS. UVA-LED irradiation at 200 kJ/m^2 decreased ion counts of precursor and fragment ion mass of chlorophyll a, m/z = 893.54 (M+H) and m/z = 871.57

(M+H-Mg), respectively, but did not change those of chlorophyll b, m/z = 907.52 (M+H) and m/z = 929.50 (M+Na), respectively (Figure 3). Over 400 kJ/m^2 irradiation decreased significantly those of both chlorophyll a and b. In addition, *Chlorella* sp. had higher ion counts of chlorophyll a than those of chlorophyll b. These results were suggested UVA-LED irradiation-induced decrease of fluorescent intensity in *Chlorella* sp. MK201 depended on degradation of chlorophyll a *Chlorella* sp. MK201.

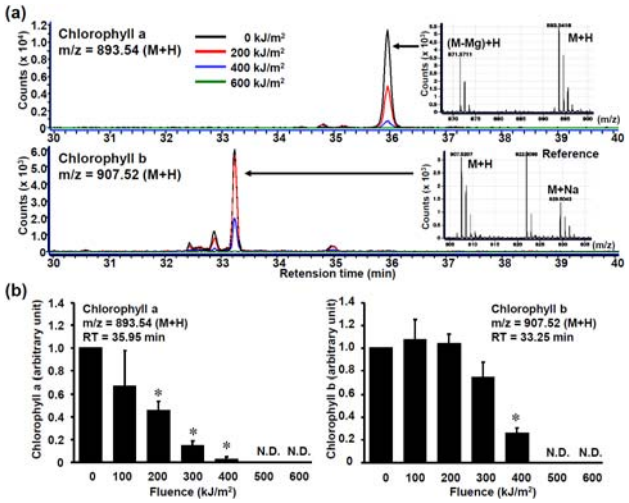

Figure 3. Measurement of chlorophylls by liquid chromatography-mass spectrometry. (a) Extracted- ion chromatogram of chlorophyll a (m/z = 893.54, M+H) and chlorophyll b (m/z = 907.52, M+H). (b) Relative change of ion counts of chlorophyll a (left panel) and b (right panel) after UVA irradiation. Values are shown as means ± SD (n = 4, n = number of independent replicates). (*) $P < 0.01$ versus versus no-irradiation. N.D.; not-detected.

3.3. Intracellular protein oxidation by UVA irradiation

Our previous studies reported that bactericidal effects of UVA-LED irradiation to enteropathogenic bacteria, such as *Escherichia coli* and *Vibrio parahaemolyticus*, were depended on up-regulation of intracellular reactive oxygen species (ROS) [10]. Next to check whether UVA-LED irradiation induced ROS in *Chlorella* sp. MK201, we measured protein oxidation by Oxyblot™ analysis. UVA-LED irradiation at the dose of 200 kJ/m^2 increased low molecular of oxidative protein (Figure 4). Those of 400 and 600 kJ/m^2 increased significantly protein oxidation. From these results, UVA-LED irradiation could increase intracellular ROS in *Chlorella* sp.

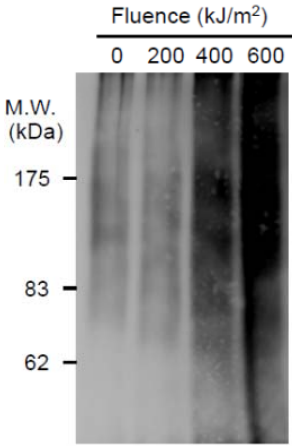

Figure 4. Protein oxidation in *Chlorella* sp. MK201 by UVA irradiation. The image showed visualized oxidized protein by westernblot analysis. M.W., molecular weight.

3.4. Growth curve of *Chlorella* sp. MK201. and catalase treatment.

To check the effect of UVA-LED irradiation to cell growth, we monitored cell concentration every per day for 24 days during culturing irradiated *Chlorella* sp. MK201.. UVA-LED irradiation inhibited cell growth in a dose dependent manner (Figure 5a). Previous study demonstrated that bactericidal effect by UVA-LED irradiation was suppressed by catalase [10], an enzyme which can catalyze hydrogen peroxide. Immediate after UVA-LED irradiation at a dose of 200 kJ/m^2, cell concentration was not different between with and without catalase treatment (data not shown). After culturing 14 days, it with catalase treatment was higher than without treatment, but significantly lower than with no irradiation controls (Figure 5b). These results suggested that UVA-LED irradiation inhibited cell growth of *Chlorella* sp. MK201. and these

depended on some of irradiation-induced ROS.

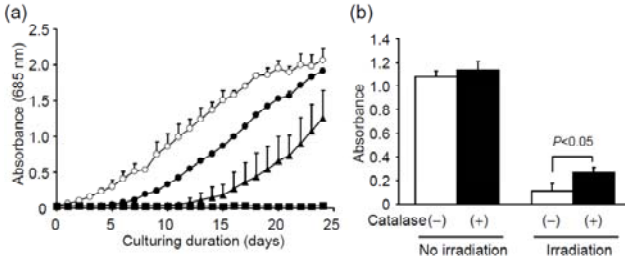

Figure 5. Growth curve of *Chlorella* sp. MK201 after UVA irradiation and effect of catalase treatment. (a) Growth curve after UVA irradiation. (○) No-irradiation. (●) 200 kJ/m^2. (■) 400 kJ/m^2. (▲) 600 kJ/m^2. (b) Effect of catalase treatment to *Chlorella* sp. MK201. Cells were irradiated by UVA-LED at a dose of 200 kJ/m^2 and then cultured for 14 days. Values are shown as means ± SD (n = 3-4, n = number of independent replicates). (*) $P < 0.01$ versus versus no-irradiation.

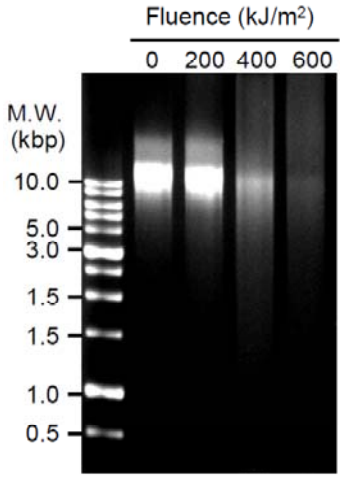

Figure 6. DNA degradation in *Chlorella* sp. MK201 by UVA irradiation. The image showed agarose gel electrophoresis of purified DNA from UVA-irradiated cells.

3.5. Genomic DNA fragmentation of *Chlorella* sp. MK201. by UVA-LED irradiation

Irradiation at a dose of 600 kJ/m^2 inhibited completely cell growth of *Chlorella* sp. (Figure 6a). To check the effect of UVA-LED irradiation to cell death, we purified genomic DNA from irradiated measured *Chlorella* sp. MK201. and measured DNA fragmentation by agarose gel electrophoresis. Irradiation at doses of 400 and 600 kJ/m^2 increased DNA fragmentation (Figure 6). From these results, high fluence UVA-LED irradiation could induced cell death.

4. Discussion

This study showed that UVA-LED irradiation decreased chlorophyll a, a most important photosynthetic pigments, in *Chlorella* sp.. Photosynthesis is one of the most sensitive metabolic processes for cell growth of algae. Over the past few decades, some reports demonstrated the effect of irradiation at other UV wavelength to algae. Bordeie *et al.* reported that UVC irradiation decreased drastically chlorophyll content in a filamentous green alga [19]. In addition, Prasad *et al.* found that UVB irradiation decreased cell survival and growth *via* down-regulation of photosynthetic activity of algae [20]. These effects were depended on decrease of some photosynthetic pigments including chlorophyll a [20]. From these reports, degradation of chlorophyll a by UVA-LED irradiation might be associated with inhibition of cell growth in this study.

Photosystem (PS)-II is well-known as a critical component of the photosynthetic activity in algae and plats. PS-II consists a complex of Mn_4CaO_5 inorganic metal cluster, some photosynthesis pigment and protein [21]. Photoinhibition is a protective phenomenon against excessive light illumination by suppression of photosynthesis [21]. To avoid some photodamages by excess energy illumination, the complex of PS II is inactivated in green algae and cyanobacteria [22, 23]. D1 subunit (psbA) protein, a protein of PSII complex, had important roles in recovering and maintain of PS II activity from photoinhibition [24]. Nishiyama *et al*. reported that that oxidative stress (ROS) inhibited repair of inactivated PS II, which was depended on suppression of the D1 protein recycling [25]. In this study,

UVA-LED irradiation induced protein oxidation in *Chlorella* sp. MK201 (Figure 4). From these reports and our results, inhibition of cell growth by UVA-LED irradiation might be depended on ROS-induced suppression of recovery of PS-II activity.

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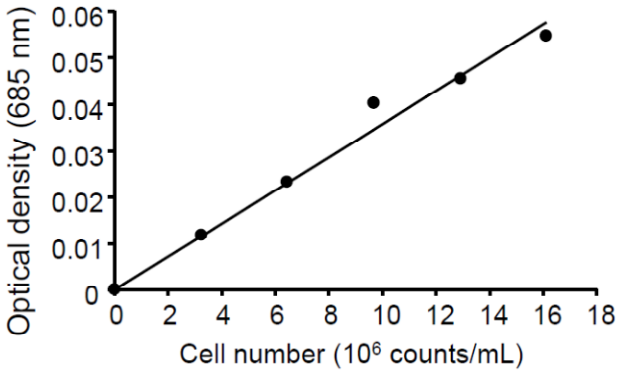
Suppelemental figure



S1. Relationship between cell number and optical density at 685 nm.