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Generation of a novel fluorescent product, monochlorofluorescein from dichlorofluorescin by photo-irradiation†

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Abstract

Dichlorofluorescin (DCFH), a widely used fluorescent probe for reactive oxygen species (ROS) was decomposed completely and generated two distinct fluorescent products by photo-irradiation at 254 nm for 30 min. In the previous study, we had shown that one was dichlorofluorescein (DCF), a well known oxidized product of DCFH. In this study we investigated the other product and identified it as monochlorofluorescein (MCF) by ^{1}H-NMR and fast atom bombardment/mass spectrum (FAB/MS) analyses. MCF was generated by photo-irradiation, but not by ROS. On the other hand, DCF was produced by both photo-irradiation and ROS. MCF showed similar fluorescent emission spectrum to DCF, however, its fluorescence intensity was more than that of DCF. The kinetic study suggested that MCF was not generated from DCF but from monochlorofluorescin, which might be generated from DCFH by photo-irradiation.

Keywords: *Dichlorofluorescin, monochlorofluorescein, photochemical-reaction, reactive oxygen species*

Introduction

Dichlorofluorescin (DCFH) is widely used for the quantitative determination of reactive oxygen species (ROS) [1–4]. DCFH is converted to dichlorofluorescein (DCF) after reaction with ROS, especially hydrogen peroxide and peroxidase [1–4]. Because DCFH is non-fluorescent while DCF is fluorescent, the determination of fluorescent intensity of DCFH samples reflects the amounts of ROS those reacted with DCFH [1–4]. However, DCFH was converted to fluorescent compounds by photo-irradiation [5–6]. Photo-irradiation is inevitable to determine the fluorescence of DCF for its excitation. We have reported that photo-irradiation to DCFH produced two fluorescent products, one was DCF and the other was unknown compound [6]. Here using fast atom bombardment/mass spectrum (FAB/MS) and ^{1}H-NMR analyses we identified that the unknown product is monochlorofluorescein (MCF). We also investigated how MCF is produced from DCFH by photo-irradiation.

Materials and methods

Materials

DCFH-diacetate (DCFH-DA) was obtained from Molecular Probes (Eugene, OR, USA). DCF, esterase, methanol-*d*4 (CD_3OD) and 2,2′-azobis

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(2-amidinopropane) dihydrochloride (AAPH) were obtained from Sigma Aldrich (Tokyo, Japan). *N*,*N*′-bis(2-hydroperoxy-2-methoxyethyl)-1,4,5,8-naphthalenetetracarboxylic-diimide (NP-III) was prepared according to Matsugo et al. [7]. Peroxidase and xanthine oxidase were purchased from TOYOBO (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively.

Preparation of DCFH

DCFH was prepared from DCFH-DA by two different ways. DCFH-DA was dissolved in dimethyl sulfoxide (DMSO) and diluted with phosphate buffered saline (pH 7.4). DCFH-DA was incubated at 37°C for 10 min with esterase, which cleaves DA from DCFH-DA [1–3]. DCFH was also prepared with NaOH according to LeBel et al. [8] DCFH-DA was incubated with 0.02 M NaOH for 30 min at 25°C. After that the pH was adjusted to 7.4 with Na-phosphate buffer.

Photo-irradiation of DCFH

Photo-irradiation was done with MINI TRANS-ILLUMINATOR Model NTM-10 or UV TRANS-ILLMINATOR (Funakoshi, Tokyo, Japan). DCFH, 100 μM in phosphate buffered saline containing 2% DMSO (pH 7.4) was irradiated at 254 nm for 30 min at a distance of 10 cm at room temperature. According to the manufacture's data the doses applied to samples were 14–16 J/cm^2. To investigate the time course of the photo-reaction, we photo-irradiated DCFH for the indicated time as shown in Figure 8.

HPLC separation of photo-irradiated DCFH

DCFH, DCF or photo-irradiated DCFH were applied to COSMOSIL 379–71 (4.6 × 150 mm, Nacalai Tesque) equilibrated with 1:5/acetonitrile: 0.1 M Na-phosphate (pH 7.4), then eluted with the same buffer and monitored the absorbance at 254 nm.

Purification and collection of unknown product

HPLC analysis revealed two peaks (P1 and P2, Figure 1B) in photo-irradiated DCFH samples. Because the second peak (P2) showed the same elution time as that of DCF, we collected the first peak (P1) for further analyses [6].

^{1}H-NMR and FAB/MS analyses

Photo-irradiated DCFH or fractionated P1 were concentrated with a rotary evaporator and then their pH were adjusted to 2.5 with HCl. After that the aqueous phase was removed with ethyl acetate. Ethyl acetate was then evaporated, and residual DMSO was removed with diethyl ether. After evaporating the solvent the precipitates were dissolved with CD_3OD. The dissolved samples were used for ^{1}H-NMR and FAB/MS analyses. ^{1}H-NMR spectra were recorded at 400.13 MHz and 25°C using Bruker Advance 400SB spectrometer (Bruker, Tsukuba, Japan). Tetramethylsilane was used as an internal standard. FAB/MS spectra were recorded on JEOL HX-110 spectrometer (JEOL, Tokyo, Japan) by the positive-FAB ionization method with 3-nitrobenzyl alcohol as matrix.

Fluorescent spectrum analyses

DCF and the purified P1 were dissolved in ethanol and their pH was adjusted to 12. The fluorescence emission spectra were obtained with excitation at 506 nm. Respective compounds had absorption peaks at 506 nm. FP-750 (JASCO Tokyo, Japan) was used for fluorescent spectrum analyses.

DCFH oxidation with ROS

DCFH at 100 μM was incubated with 100 μM xanthine and 0.5 u/ml xanthine oxidase, 100 μM hydrogen peroxide alone, 100 μM hydrogen peroxide and 100 μM $FeCl_2$, 100 μM hydrogen peroxide and (16 u/ml) peroxidase or 100 μM AAPH at room temperature for 30 min. DCFH (100 μM) was also mixed with 300 μM NP-III, then irradiated with 365 nm light for 30 min. The mixtures were subjected to HPLC analyses.

Results and discussion

DCFH was eluted at 48.9 min in the present HPLC condition (Figure 1A). After photo-irradiation at 254 nm for 30 min, DCFH was disappeared completely while two new peaks (P1 and P2) were appeared (Figure 1B,C). P2 was eluted at 7.46 min, that was almost the same as DCF (Figure 1D). These findings were in good agreement with our previous data. The data indicated that photo-irradiation to DCFH generated two fluorescent compounds and that one compound showed the same elution time as DCF [6]. To identify P1 we collected P1 and subjected it for FAB/MS and ^{1}H-NMR analyses.

FAB/MS spectrum of P1 showed two signals at m/z 154.1 and 367.1 (Figure 2A). Because the molecular weight of 3-nitrobenzyl alcohol, which was used as a matrix, is 153.1 [9], we analyzed the spectrum from m/z 364 to 371. We found two major signals at m/z 367.1 and 369.1, and the ratio of 367/369 signals was about 3 (Figure 2B). Chlorine atom is present as 75% ^{35}Cl and 25% ^{37}Cl. Thus we speculated that P1 had one chlorine atom. The molecular weight of P1 was calculated as 367.6 from the FAB/MS spectrum. Because FAB/MS adds one hydrogen ion to the molecules the molecular weight of P1 would be 366.6.

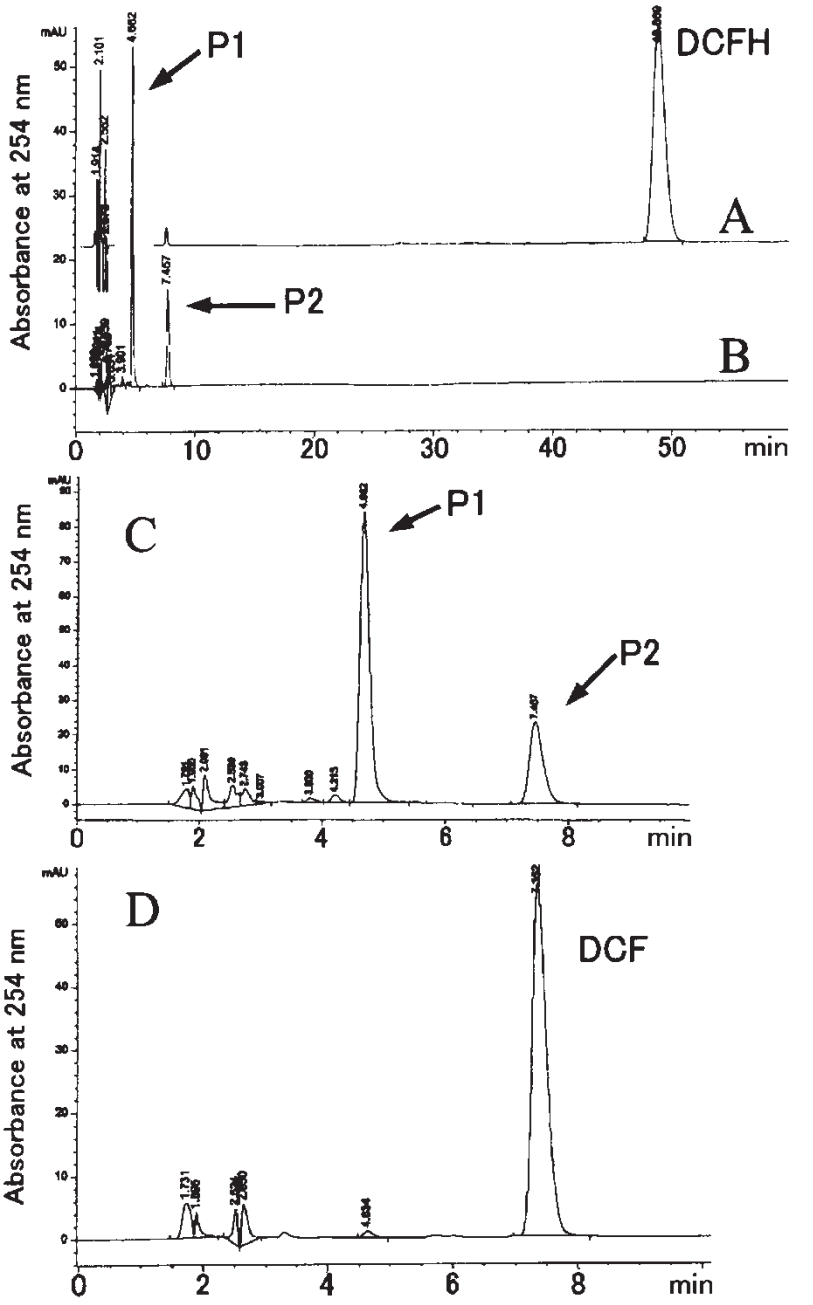

Figure 1. HPLC analyses of DCFH, DCF and photo-irradiated DCFH. DCFH (A) was injected into HPLC system and analysed as described in Materials and methods. DCFH, photo-irradiated at 254 nm for 30 min (B, C), was injected into HPLC and analysed as described in DCFH. DCFH was completely disappeared while new peaks, P1 and P2, were appeared. DCF (D) was injected into HPLC and analyzed as described in DCFH. Figure 2C and D showed chromatograms from 0 to 10 min

Molecular weight of DCF is 401.2 [10], which is 34.6 more than that of P1. We considered that one chlorine atom of DCF was replaced with a hydrogen atom in P1. From these results we considered P1 as MCF and we listed the chemical structure of MCF in Figure 3A. When we analyzed photo-irradiated DCFH, unfractionated DCFH, we found two major signal complexes. One was appeared from m/z 367 to 369, the other one was from m/z 401 to 405. Because DCF has two chlorine atoms, its signal should be at 401, 403 and 405, and the ratio of 401/403/405 signals should be 9/6/1. The signal from 401 to 405, which we obtained from FAB/MS (Figure 2C), was in good agreement with the theoretical signal of DCF, and the molecular weight was calculated to be 402 from the spectrum. The signal from 367 to 369 was the same as that of P1 (Figure 2B,C).

From HPLC profiles and FAB/MS spectra we concluded that photo-irradiation to DCFH generated DCF, and we considered that it also generated MCF.

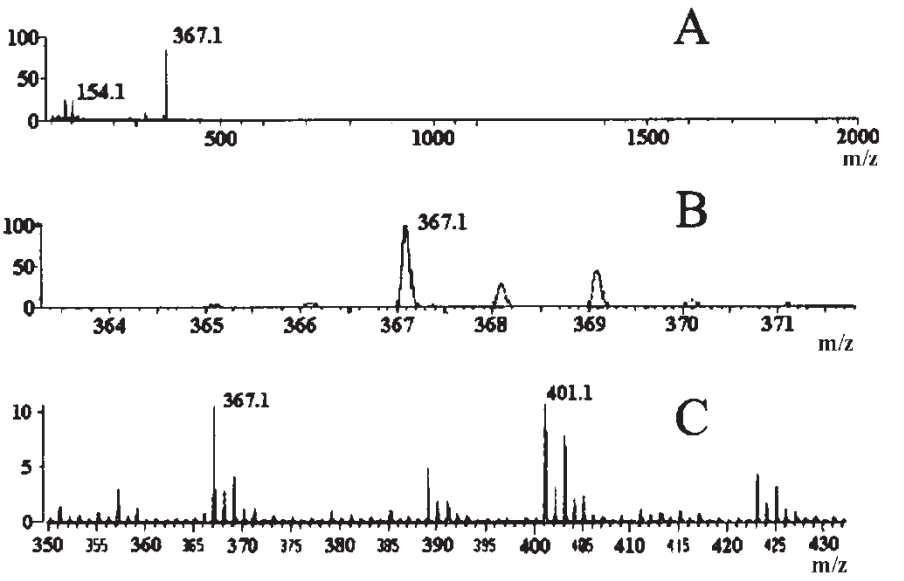

Figure 2. FAB/MS spectra of P1 and unfractionated photo-irradiated DCFH. Figure 2A depicted FAB/MS of P1 from m/z 100 to 2000. Figure 2B depicted FAB/MS of P1 from m/z 364 to 371. Figure 2C depicted FAB/MS of unfractionated photo-irradiated DCFH from m/z 350 to 430. FAB/MS spectra were measured on a JEOL HX-110 spectrometer by the positive-FAB ionization method with 3-nitrobenzyl alcohol as matrix.

We then analyzed ^{1}H-NMR spectra of P1 and DCF. The obtained ^{1}H-NMR spectrum of DCF (Figure 4B) was exactly the same as that of DCF listed in the database [11]. When we compared the ^{1}H-NMR spectrum of P1 with that of DCF, we found that the signals from 8.1 to 7.2 ppm was the same as those of DCF (Figure 4A,B). The finding indicates that P1 had the same benzene moiety as DCF. In contrast, signals from 6.9 to 6.5 ppm were different from those of DCF, indicating xanthene moiety of P1 was different from that of DCF. The finding also indicates that xanthene moiety of DCF was symmetric, while that of P1 was not. The hydrogen signals of P1 were assigned as shown in Table I. Due to low resolution of our NMR system small errors may be present in coupling constants. With FAB/MS and ^{1}H-NMR spectra we have concluded P1 as MCF (Figure 3A).

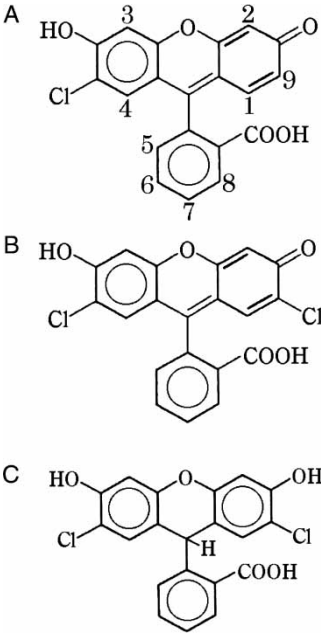

Figure 3. Putative and conclusive structure of P1. A, putative structure of P1 was speculated from FAB/MS spectrum as MCF, and the structure was confirmed by ^{1}H-NMR spectrum. B, structure of DCF. C, structure of DCFH.

We then compared the fluorescent spectra of DCF and MCF (Figure 5). MCF spectrum was similar to that of DCF, however, MCF showed a fluorescent peak at 524 nm while DCF at 530 nm. We used the same concentration of MCF and DCF for fluorescent spectrum analyses. As shown in Figure 5 MCF was three to four times more fluorescent than DCF. These results were in good agreement with our previous report that photo-irradiation to DCFH produced two fluorescent compounds [6].

Because esterase, which was used to prepare DCFH from DCFH-DA, might contain impurities, we also prepared DCFH by alkaline hydrolysis. Both DCFH preparations generated MCF and DCF similarly by photo-irradiation (Figure 6), confirming that photo-irradiation, but not impurities, generated MCF.

We investigated whether MCF was generated when DCFH was reacted with ROS. As shown in Figure 7 none of ROS so far tested generated MCF. ROS generated only DCF when reacted with DCFH (Figure 7A). NP-III is a photo-sensitizer and generates hydroxyl radical after photo-irradiation at 365 nm [7,12]. Photo-irradiation at 365 nm generated both MCF and DCF in the absence of NP-III.

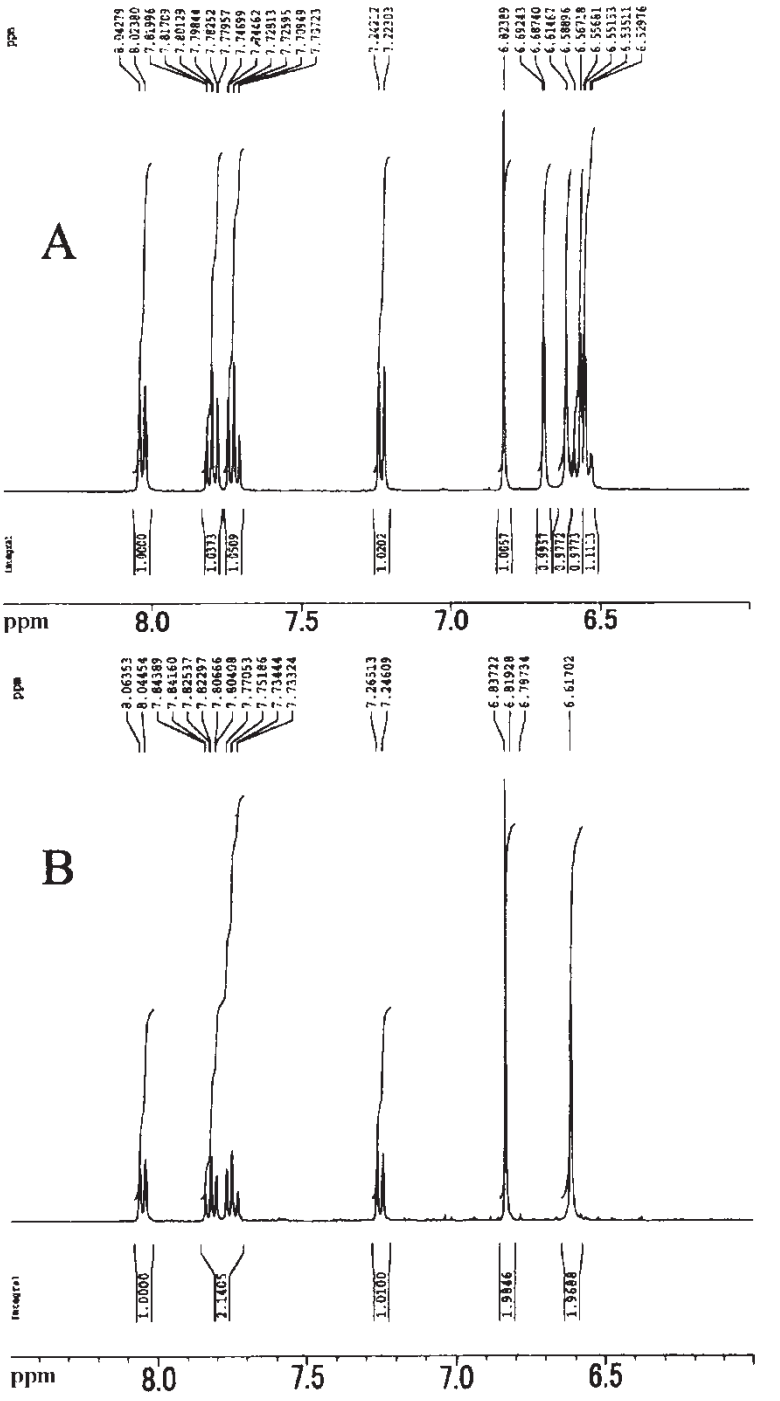

Figure 4. ^{1}H-NMR spectra of P1 and DCF. Figure 4A depicted ^{1}H-NMR spectrum obtained from P1. Figure 4A depicted ^{1}H-NMR spectrum from 6.5 to 8.0 ppm. Figure 4B depicted ^{1}H-NMR spectrum of DCF from 6.5 to 8.0 ppm. ^{1}H-NMR was measured at 400.13 MHz and 25°C using Bruker Advance 400SB spectrometer as described in Materials and methods.

However, in the presence of NP-III photo-irradiation at 365 nm generated DCF only, suggesting that the generation of hydroxyl radical from NP-III was faster than the generation of MCF (Figure 7B). It is interesting that the generation of MCF was considerably less when DCFH was irradiated with 365 nm than irradiated with 254 nm (Figures 6 and 7B). Irradiation to DCFH at 500 nm brought a similar result to that at 365 nm (data not shown). The findings that wavelengths of irradiated light had effects on the generation efficiency of MCF and DCF were in good agreement with our previous observation [6].

We then investigated the time course of the photoreaction. As shown in Figure 8 MCF and DCF were generated simultaneously. After reaching a plateau the amounts of MCF and DCF were not changed, indicating that MCF was not generated from DCF.

Table I. Assignments of ^{1}H-NMR signals of P1.

Assignment	Shift (ppm)
1	6.58 (d, $J = 8.7$ Hz)
2	6.69 (d, $J = 2.0$ Hz)
3	6.61 (s)
4	6.82 (s)
5	7.23 (d, $J = 7.6$ Hz)
6	7.73 (d,d,d, $J = 7.5$, 7.5, 0.9 Hz)
7	7.80 (d,d,d, $J = 7.5$, 7.5, 1.2 Hz)
8	8.03 (d, $J = 7.6$ Hz)
9	6.54 (d,d, $J = 8.7$, 2.1 Hz)

The numbers under assignment indicate the number of hydrogen atom in Figure 3A.

The results obtained from irradiation study together with ROS, different wavelengths and time course study, we propose a mechanism for the formation of MCF as described below. DCFH may photo-isomerize to its ketone form upon photo-irradiation. Dehydrochlorination from the ketone form is easily taken place because this reaction is a kind of rearomatization [13]. The mechanism suggests that MCF is produced without any intervention of ROS. Photo-irradiation was reported to release chlorine from chlorinated compounds [14,15]. To clarify the exact mechanism of MCF generation, further studies are required. Understanding the mechanism may help

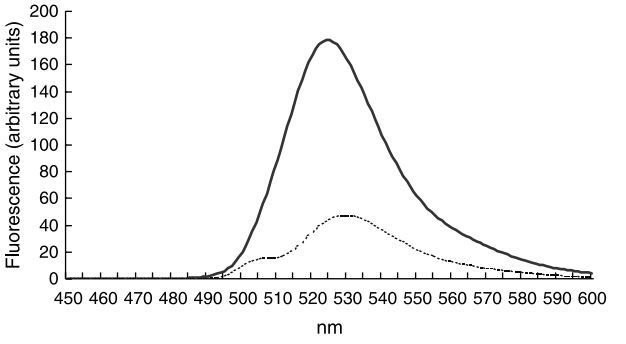

Figure 5. Fluorescent spectra of MCF and DCF. The same concentration of MCF (solid line) and DCF (dotted line) at 15 μM were excited at 506 nm and the emission spectra were recorded as described in Materials and methods.

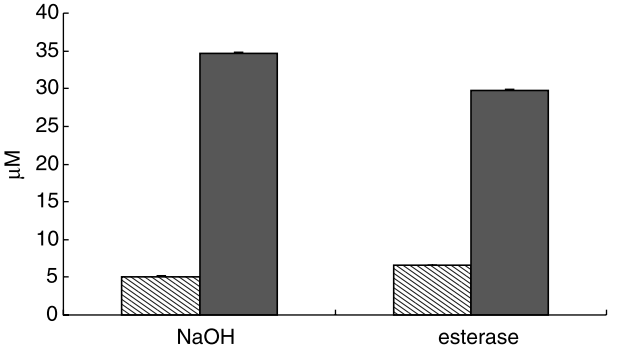

Figure 6. Generation of DCF and MCF by photo-irradiation to different DCFH samples. DCFH were prepared from DCFH-DA with esterase or with NaOH as described in Materials and methods. Then the DCFH samples were photo-irradiated at 254 nm for 30 min. After that the samples were injected into HPLC and analysed as described in Materials and methods. Solid bars represent MCF and hatched bars represent DCF. Bars represent the average of two independent experiments and dashes represent the range.

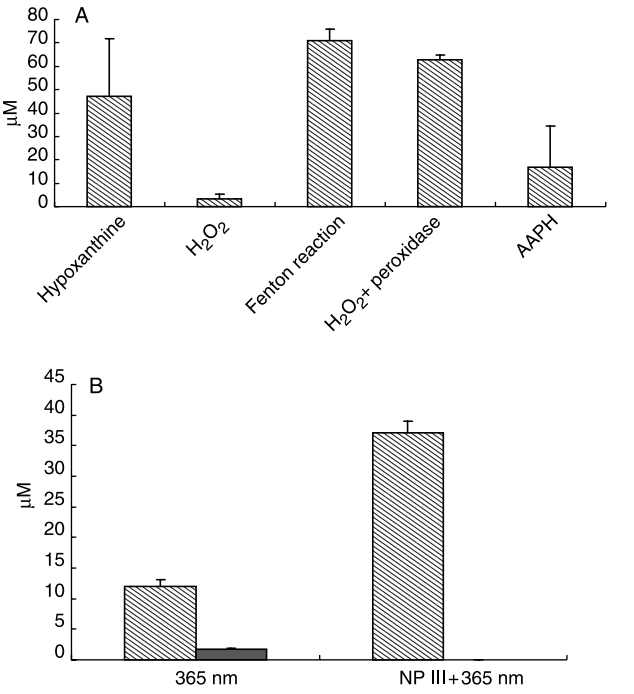

Figure 7. Generation of DCF and MCF by ROS. DCFH were incubated with various ROS at room temperature for 30 min (A), then the reaction mixtures were analysed by HPLC as described in Materials and methods. DCFH were incubated with or without NP-III, then irradiated at 365 nm for 30 min (B). After that the reaction mixtures were analyzed by HPLC as described in Materials and methods. Solid bars represent MCF and hatched bars represent DCF. Bars represent the average of 5 independent experiments and dashes represent standard deviation.

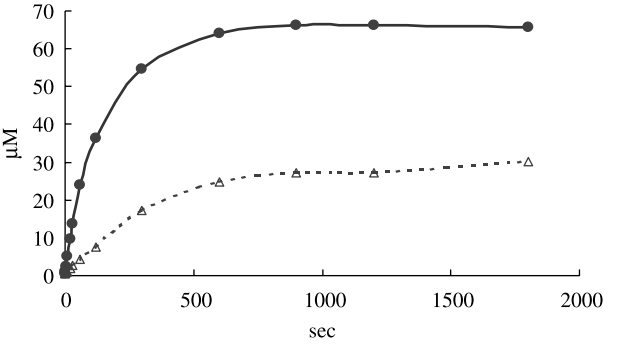

Figure 8. Time course of the photo-reaction of DCFH irradiated at 254 nm. DCFH was irradiated at 254 nm, then parts of the reaction mixture were taken and analyzed by HPLC. Dotted line and bold line indicate DCF and MCF, respectively. Lines represent the average of five independent experiments.

to explain the photo-reaction of various molecules especially the biological molecules and the mechanisms underlying the photo-aging, and may also help to find out markers for photo-aging.

Many reports had described that DCFH is changed to fluorescent product(s) after reaction with ROS and other molecules or under certain conditions [6,16–18]. However, all reports considered that DCFH was converted to DCF, and no reports had identified what was/were generated. This is the first report to describe the generation of MCF from DCFH by photo-irradiation.

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