Non-reductive Scavenging of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) by Peroxyradical: A Useful Method for Quantitative Analysis of Peroxyradical

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A stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) has long been used as a convenient method for the antioxidant assay of biological materials such as cysteine, glutathione, ascorbic acid, tocopherol and polyhydroxy aromatic compounds (hydroquinone, pyrogallol, *etc*). In this study, non-reductive scavenging of DPPH was investigated by electron spin resonance (ESR) analyses for the purpose of developing a useful method for quantitative determination of peroxyradical. Since DPPH was degraded in the presence of peroxyradical derived from UV-irradiated benzoylperoxide and the peroxyradical-induced degradation of DPPH was inhibited by the addition of a spin trapping agent 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), it is concluded that DPPH is non-reductively scavenged by peroxyradical. Therefore, it is suggested that DPPH could be a useful agent for the quantitative measurement of peroxyradical.

Key words 1,1-diphenyl-2-picrylhydrazyl (DPPH); non-reductive scavenging; peroxyradical; electron spin resonance (ESR)

For quantitative investigations of hydrogen-radical donation, stable radicals have the advantage that their concentrations are readily and directly measurable. Among them a stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH)[1] was investigated as a reactive hydrogen acceptor[2] and further found to be useful for the antioxidant determination.[3] Since then DPPH has been mainly used to examine radical scavenging activity of antioxidative vitamins and polyhydroxy aromatic compounds[4,5] based on the reactions in Fig. 1. Besides DPPH-scavenging by reductive hydrogen transfer between various donors and DPPH, non-reductive DPPH-decomposing and -scavenging were found to be induced by reacting with tertiary hydroperoxides[6] and by radical-binding at the *para* position in the phenyl rings of DPPH,[7] respectively.

However, nowadays non-reductive DPPH-scavenging has hardly applied for biochemical and physicochemical purposes. It has been reported that peroxyradicals are unique among reactive oxygen species implicated in the production of DNA damage because they possess an extremely long half-life (order of seconds) and are predicted to have a relatively greater chemical selectivity in its reactions as compared with other radical intermediates.[8] Several methods have developed for the determination of peroxyradicals such

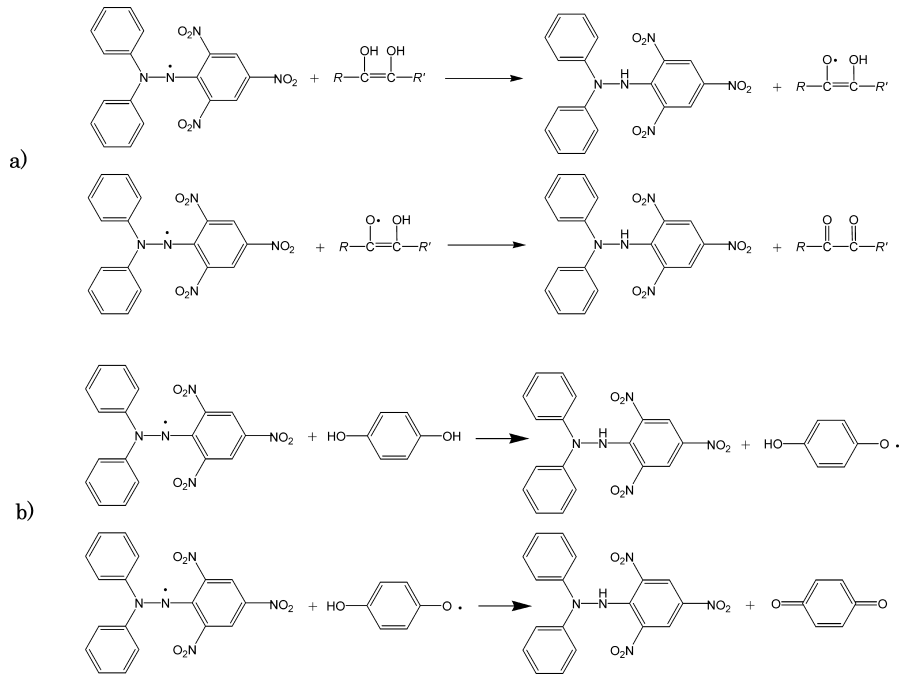

Fig. 1. The Reaction Scheme between DPPH and the Conjugated Group of Ascorbic Acid (a) or Hydroquinone (b)

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as the Peroxy Radical Amplification (PERCA) technique that is based on the amplified conversion into NO_2 of the peroxyradicals entering the reactor,[9] and the bioassay system that could determine the viability of *Staphylococcus aureus* by peroxyradical-induced cytotoxicity.[10] However, simple and direct methods for quantitative determination of peroxyradicals have not been reported so far. We here present a new quantitative method for measuring peroxyradical, which is one of the reactive oxygen species causing cellular damage,[11—14] by a non-reductive DPPH-scavenging reaction.

Experimental

Materials Benzoylperoxide, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and DPPH were purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.), Labotec Co., Ltd. (Tokyo, Japan) and Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), respectively. All the other reagents used were of analytical grade.

ESR Analysis Measurement conditions of ESR throughout the experiment were as follows; field sweep: 330.70—340.70 mT, field modulation: 100 kHz, 0.07 mT, amplitude: 2000, sweep time: 2 min, time constant: 0.03 s, microwave freq.: 9.427 GHz, microwave power: 4 mW.

Peroxyradical Formation from Benzoylperoxide Benzoylperoxide (0.156 mM) ethanol solution containing a spin trapping agent DMPO (4.4 M) in a flat quartz cell was exposed to UV radiation at 254 nm at 4 W by UVGL-25 compact UV lamp (UVP Inc., Upland, CA, U.S.A.), and the cell was immediately placed in a ESR spectrometer (JES-FA100, JEOL, Tokyo, Japan). Then ESR-spectrum was measured by coupling to spin trapping with DMPO. As the next step, radiation time-dependent peroxyradical formation was checked as follows; 4.00 mM benzoylperoxide ethanol solution containing 4.45 M DMPO in a flat quartz cell was exposed to UV radiation at 254 nm at 4 W for 1 to 5 min at a distance of 95 mm, and the cell was immediately placed in a ESR spectrometer. Then ESR-spectra were measured. The quantitative determination for peroxyradical was performed by being compared with the signal intensity of Mn^{2+} as an external standard that was normalized in 5 μM TEMPOL solution.

Non-reductive DPPH-Scavenging by Peroxyradical Before examining the radical-dependent DPPH-scavenging, the degradation effect of UV-radiation on DPPH was firstly checked. DPPH (0.1 mM) ethanol solution in the flat quartz cell was exposed to UV-radiation at 254 nm at 4 W for 1 to 5 min at different distances from the UV-source. UV-intensities measured by a digital UV meter (UVC-254, Mother Tool Co., Ltd., Nagano, Japan) at the different distances were as follows; 0.788 mW/cm^2 at 15 mm, 0.500 mW/cm^2 at 35 mm, 0.344 mW/cm^2 at 55 mm, 0.244 mW/cm^2 at 75 mm and 0.184 mW/cm^2 at 95 mm. Then ESR-spectra were measured for a quantitative analysis of DPPH by the signal intensity of DPPH as previously reported.[5] As the next step, non-reductive DPPH-scavenging by peroxyradical was further examined. A mixture of DPPH (0.1 mM) and different concentrations of benzoylperoxide dissolved in ethanol was added to the flat quartz cell. Following the exposure of the cell to UV radiation at 254 nm at 4 W for 15 to 60 s at 95 mm from the UV-source, ESR-spectra were measured for quantitative analysis of DPPH.

Inhibitory Effect of DMPO on DPPH-Scavenging by Peroxyradical To further confirm non-reductive DPPH-scavenging by peroxyradical, inhibitory effect of a radical trapping agent DMPO was examined. The reaction mixture containing different concentrations of DMPO, DPPH (0.1 mM) and benzoylperoxide (0.1 mM) dissolved in ethanol was added to the flat quartz cell and ESR-spectra were measured for quantitative analysis of DPPH.

Results and Discussion

Peroxyradical Formation from Benzoylperoxide Peroxyradical formation by the exposure of benzoylperoxide to UV radiation was examined. After the exposure, ESR analysis was performed using DMPO as a spin trapping agent. Figure 2 shows the representative ESR spectrum of a resultant spin adduct, showing that the adduct is derived from peroxyradical and DMPO since the hyperfine coupling constants obtained from the spectrum are as follows; $a_N = 1.4$ mT, $a_H = 0.8$ mT and $a_{H'} = 0.2$ mT which coincide with those of

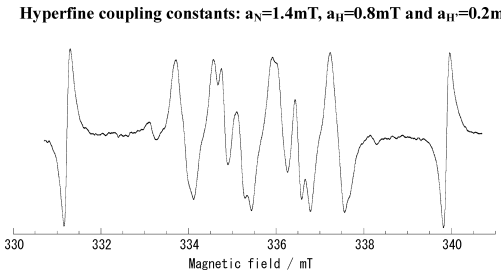

Fig. 2. The ESR Spectrum of Spin Adduct of DMPO and Peroxyradical

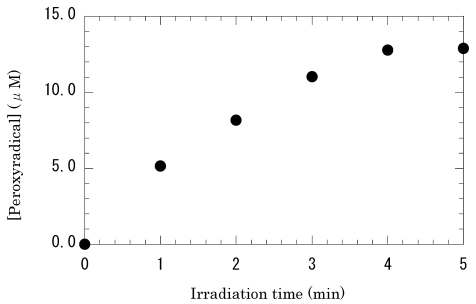

Fig. 3. The Irradiation Time Effect of Peroxyradical Formation from Benzoylperoxide

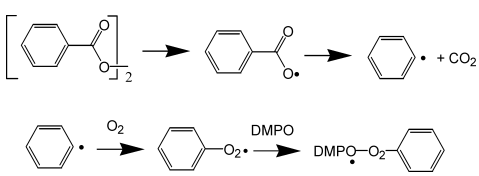

Fig. 4. The Proposed Reaction of Peroxyradical and DMPO

peroxyradical adduct reported in a previous study.[15] The hyperfine coupling constants are also confirmed by computer simulation spectrum. Although there is a possibility that phenyl radical or benzoyloxy radical reacts with DMPO, no ESR parameters specific for the adduct of phenyl radical and DMPO or the adduct of benzoyloxy radical and DPPH were obtained, indicating that DPPH reacts specifically with peroxyradical. Figure 3 shows peroxyradical formation by given time of UV radiation. Peroxyradical was formed from benzoylperoxide time-dependently by UV radiation.

Proposed reaction based on the analysis of the hyperfine coupling constants is summarized in Fig. 4.

Non-reductive DPPH-Scavenging by Peroxyradical Since the DPPH-scavenging activity of peroxyradical was evaluated by exposing UV to a mixture of DPPH and benzoylperoxide, effect of UV radiation on DPPH degradation was firstly checked. When UV was irradiated for 1 min, the distance of 75 mm or longer between the UV source and DPPH solution resulted in no degradation of DPPH (Fig. 5). Then the time effect of UV radiation on DPPH degradation was examined at the distance of 95 mm between the UV source and DPPH solution. The radiation for more than 1 min caused time-dependent degradation of DPPH (Fig. 6), indicating that the conditions of UV irradiation for 1 min at the 95 mm or longer distance exerted no degenerative effect on DPPH. Finally, the concentration effect of benzoylperoxide on DPPH-scavenging was examined under UV radiation for 15 to 60 s. Concentration-dependent degradation of

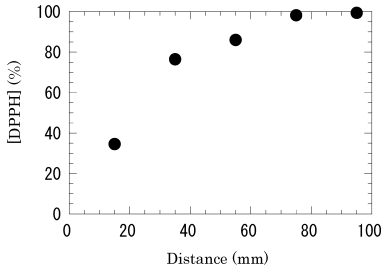

Fig. 5. The Distance Effect of UV Irradiation on DPPH Degradation

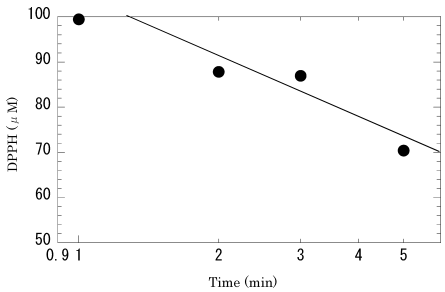

Fig. 6. The Time Effect of UV Irradiation on DPPH Degradation

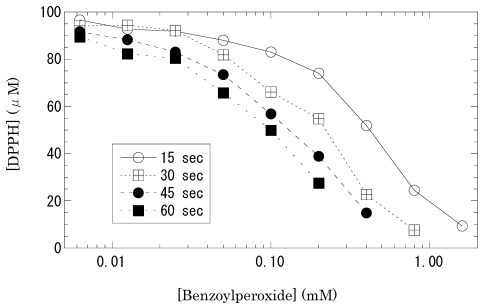

Fig. 7. The Concentration Effect of Benzoylperoxide on DPPH-Degradation under UV Radiation for 15 to 60 s

DPPH was observed (Fig. 7), indicating that an increase in the peroxyradical production is correlated well with DPPH-scavenging capacity. Based on the results obtained here, a proposed reaction of non-reductive DPPH-scavenging by peroxyradical is shown in Fig. 8.

Inhibitory Effect of DMPO on DPPH-Scavenging by Peroxyradical Non-reductive DPPH-scavenging by peroxyradical was further confirmed by examining the effect of DMPO on the DPPH-scavenging. As shown in Fig. 9, DPPH-scavenging by peroxyradical was inhibited by the addition of DMPO in a concentration-dependent manner, suggesting that peroxyradical formed by the exposure of benzoylperoxide to UV radiation was competitively trapped by DMPO since we have checked that DPPH is not reacts with DMPO (data not shown). These results demonstrate that a stable free radical DPPH is non-reductively scavenged by peroxyradical derived from UV-irradiated benzoylperoxide. In other words, DPPH could be a useful agent for the quantitative determination of peroxyradical. However, whether

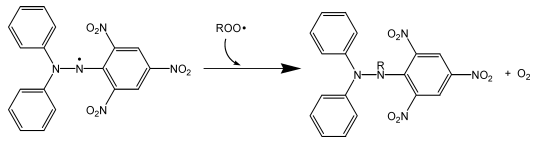

Fig. 8. The Reaction Scheme of Non-reductive DPPH-Scavenging by Peroxyradical

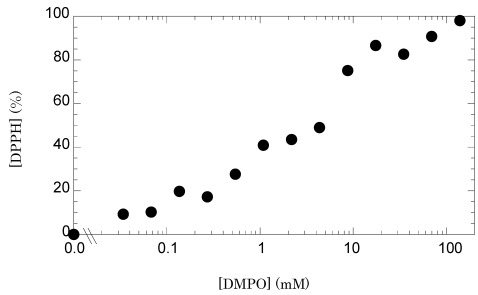

Fig. 9. The Inhibitory Effect of DMPO on DPPH-Scavenging by Peroxyradical

DPPH reacts specifically with peroxyradicals is not clear. In this assay system, the effect of hydroxyl radical on DPPH is completely negligible since ethanol used as a solvent is a potent scavenger for the radical. In the case of nitric oxide (NO), it was reported that NO does not react with DPPH.[16] Nevertheless, the possibility of the reaction between other radical species and DPPH is still remained. Therefore, in case, other assay(s) in combination with the DPPH method might be required or the effects of other radical species should be eliminated when reactive radical species and peroxyradicals are simultaneously determined.

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