Fluorophotometric Determination of Hydrogen Peroxide and Other Reactive Oxygen Species with Fluorescein Hydrazide (FH) and Its Crystal Structure

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Methods for the fluorophotometric determination of hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) were proposed by using the fluorescence reaction between H_2O_2 or other ROS and fluorescein hydrazide (FH). In the determination of H_2O_2, the calibration curve exhibited linearity over the H_2O_2 concentration range of 2.1—460 ng ml^{-1} at an emission wavelength of 527 nm with an excitation of 460 nm and with the relative standard deviations (n=6) of 4.06%, 1.78%, and 2.21% for 3.1 ng ml^{-1}, 30.8 ng ml^{-1}, and for 308 ng ml^{-1} of H_2O_2, respectively. The detection limit for H_2O_2 was 0.7 ng ml^{-1} due to three blank determinations (ρ=3). The calibration curves for ROS-related compounds were also constructed under the optimum conditions. This method was successfully applied in the assay of H_2O_2 in human urine. In addition, we performed the characterization of FH, and interesting information was obtained with regard to the relationship between the chemical structure and fluorescence.

Key words hydrogen peroxide; reactive oxygen species; fluorescein hydrazide; human urine; characterization

Reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radical ($\cdot$OH), nitric oxide (NO), peroxynitrite ($ONOO^-$), and peroxide (R–OOH) affect the living body and cause various types of diseases such as cancer,[1] cardiovascular disorders,[2] and neurodegenerative diseases.[3,4] On the other hand, it is accepted that some ROS control disinfection in cells and work as a biofactor for signal transmission of insulin.[5] In addition, H_2O_2 is a major by-product of ROS in living organisms and a common marker for oxidative stress in the field of clinical inspection.[6—8] The development of methods for the determination of these substances is significant in the fields of clinical and biological studies. Several methods for the determination of the spectrophotometry,[9—11] fluorophotometry,[12—18] and chemiluminescence [19,20] of H_2O_2 and other ROS have already been reported. These methods focus on the determination of individual ROS. However, it appears that most of ROS have only minute life times under biological environments. Therefore, we considered that it is more practicable to measure the total rather than individual ROS for a clinical purpose. Hence, we developed a handy and highly sensitivity method for the determination of ROS, including H_2O_2. We have already developed methods for the fluorometric determination of cobalt(II) and H_2O_2 with fluorescein hydrazide (FH),[21] however, the method adopted for the determination of H_2O_2 exhibited remarkably poor reproducibility. In this study, method to obtain a more reproducible and sensitive spectrofluorimetric determination of H_2O_2 and other ROS were examined. Furthermore, the applicability of the method for measuring H_2O_2 in urine samples was examined. Moreover, because an excellent crystal of FH was obtained, structural analysis by X-ray diffractometry was performed. As a result, this FH crystal was confirmed to have a five-membered spirolactam structure. The relationship between fluorescence and the chemical structure was studied; the fluorescein, which was the strong fluorescent substance that caused the spirolactam formation by acting as the hydrazine conductor, became a colorless, non-fluorescent substance.

Experimental

Synthesis of Fluorescein Hydrazide (FH) A modification of the procedure of Baeyer and Akita.[22,23] for the synthesis of fluoescein and FH was employed. Resorcinol 2 mol and phthalic anhydride 1 mol were combined with methanesulfonic acid. The mixture was heated and dissolved in 5% sodium hydroxide solution. The solution was poured into 30% acetic acid; the resulting fluorescein, a yellow precipitate, was collected with a yield of 92.5%. Subsequently, fluorescein was added to a solution of excess 98% anhydrous hydrazine and heated at 80 °C in a warm water bath for 3 h. Upon cooling to room temperature and with the addition of strong hydrochloric acid into the solution, the light yellow substance was deposited. Then, the substance obtained was dissolved in ethanol and treated with active carbon. After removal of solvent, the residual was further purified by recrystallization in ethanol, acetic acid and acetonitrile, providing pure FH as a white board-shaped crystal with a yield of 89%. The structure of FH was verified by MS, NMR, and X-ray crystallography. MS (EI$^+$): m/z 346 (M$^+$). ^{1}H-NMR (500 MHz, DMSO) δ: 4.37 (2H, s), δ: 6.39 (2H, d, J=8.5 Hz), δ: 6.45 (2H, dd, J=8.5, 2.5 Hz), δ: 6.59 (2H, d, J=2.2 Hz), δ: 6.99 (1H, m), δ: 7.49 (2H, m), δ: 7.77 (1H, m), δ: 9.80 (2H, s).

Apparatus The fluorescence measurements were performed by using a Hitachi model F-2500 spectrofluorophotometer equipped with an Usio 150 W Xenon lamp and 10×10 mm quartz cells. A Horiba model F-22 pH meter equipped with a glass combined electrode was used for all the pH measurements.

Reagent and Solutions An FH solution (1.0×10^{-4} M) was prepared by dissolving FH (highly purified by recrystallization) in ethanol. A cobalt(II) solution (1.0×10^{-4} M) was prepared by dissolving cobalt(II) chloride, 6-hydrate (Kishida Chemical Co., Ltd.). A 0.2 M Na_2CO_3/$NaHCO_3$ buffer (pH 9.6) was used for pH adjustments. A 1.0% dodecyltrimethylammonium chloride (DTAC) solution was prepared as a cationic surfactant by dissolving DTAC (Kishida Chemical Co., Ltd.) in water. A stock solution of 1.0×10^{-2} M H_2O_2 was prepared by the dilution of 30% H_2O_2 solution (Kishida Chemical Co., Ltd.) and corrected by permanganometry, and the working solution was prepared by the suitable dilution of this stock solution, as required. Superoxide (O_2^-) was added as solid KO_2. Hydroxyl radicals ($\cdot$OH) were generated by the reaction of Fe^{2+} with H_2O_2. Nitric oxide (NO) was delivered using 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminoethyl)-3-methyl-1-triazene (NOC7) (Dojindo Chemical Co.). A standard solution of NOC7 was freshly prepared in 0.1 M NaOH solution. Peroxynitrite ($ONOO^-$) was prepared by using peroxynitrite solution (Dojindo Chemical Co.). *tert*-Butyl hydroperoxide (TBHP) was delivered from 70% aqueous solution.

Standard Procedure for the Determination of H_2O_2 De-ionized water was used throughout the experiment. All the materials and reagents were of

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the analytical grade and were used without further purification. An H_2O_2 sample (2.1—460 ng) was placed in a 10 ml calibrate flask, and then 2.5 ml of 1.0% DTAC solution, 2.0 ml of the Na_2CO_3/$NaHCO_3$ buffer solution (pH 9.6), 1.0 ml of 1.0×10^{-4} M cobalt(II) solution, and 0.4 ml of a 1.0×10^{-4} M FH solution were added. The solution was diluted to 10 ml with water, transferred into a test tube, mixed well, and maintained at 80 °C for 20 min. After cooling at room temperature in water for 5 min, the difference between the relative fluorescence intensities {RFI=(A−B)/B} of solution A and reagent blank (solution B), which were prepared under the same conditions, was measured by the excitation/emission wavelength at 460/527 nm.

Results and Discussion

Spectral Properties FH is a colorless, non-fluorescent spirolactam hydrazide. Figure 1 shows the absorption spectra of fluorescein, FH and its reaction solution with H_2O_2 in a 10 ml calibrate flask, 2.5 ml of 1.0% DTAC solution, 2.0 ml of the Na_2CO_3/$NaHCO_3$ buffer solution (pH 9.6), and 1.0 ml of 1.0×10^{-4} M cobalt(II) solution. It can be observed that FH is not absorbed in the visible region (curve C); the molar absorptivity of FH at the characteristic absorption (494 nm, curve A) of fluorescein is only 4.0×10^{-3} M^{-1} cm^{-1}. This is attributed to its closed spirolactam form. However, upon reaction with H_2O_2, the green color (curve B), which indicates fluorescein, is noticeably restored. When H_2O_2 was introduced into a solution of FH, a fluorescence emission identical to that of fluorescein was observed with the maximum fluorescence emission at 527 nm. Further, the fluorescence development reaction was significantly facilitated when it was carried out in the presence of cobalt(II) and cationic surfactants. In order to explore the possible reaction products, the fluorescence emission spectra of the reaction solution were compared with that of fluorescein (Fig. 2, G). Figure 2 shows the fluorescence emission spectra of the mixture of FH with cobalt(II) and DTAC as blank solution (Fig. 2, A), and obtained after H_2O_2 was added to the blank solution as a sample solution (Fig. 2, B—F). The excitation and emission spectra of the sample solution were observed at pH 9.6. The results indicate that these spectra are identical and both have the same values of the maximum emission wavelengths. From the above results, it was assumed that FH was oxidized by H_2O_2 to yield the highly fluorescent product, fluorescein.

Optimization of Reaction Variables The effect of pH on the fluorescence investigated by using various buffer solutions and pH such as 0.2 M Na_2CO_3/$NaHCO_3$, 0.05 M borax/0.1 M sodium hydroxide, 0.2 M NH_3/NH_4Cl and, 0.2 M HCl/tris(hydroxymethyl)aminomethane. The maximum, constant value of RFI was observed in the at pH range 9.6—9.8 with 0.2 M Na_2CO_3/$NaHCO_3$ buffer solution; thus, 2.0 ml of the buffer solution (pH 9.6) was used for the pH adjustments in the final volume of 10 ml.

An addition of various surfactants in the coloring or fluorescence reactions between various reagents and metal ions has already offered many advantages in comparison to the absence of surfactants.[24—29] Accordingly, in order to develop the fluorescence and enhance the sensitivity, we examined the effects of different surfactants: Cationic [DTAC, cetyltrimethyl ammonium chloride (CTAC), stearyltrimethyl ammonium chloride (STAC), cetylpyridinium chloride (CPC), cetylpyridinium bromide (CPB), benzyldimethyltetradecyl ammonium chloride (Zephiramine)], Anionic [sodium dodecyl sulfate (SDS), di-(2-ethylhexyl) sodium sulfosuccinate (AOT)], Nonionic [polyoxyethylene sorbitan monolaurate (Tween 20), Triton X-405, polyvinylpyrrolidone (PVP), polyvinylalchol (PVA $n=500$)], and Amphoteric [Swanol AM-301]. The maximum, constant value of RFI was obtained over the range of 1.0—2.5 ml of 1.0% DTAC solution in a final volume of 10 ml.

The metal ions reacted catalytically when small amounts of the metal ions coexisted in various redox reactions. The various metal ions were tested for the assay of H_2O_2. Cobalt(II) is superior to the various metal ions tested: cobalt(II), zinc(II), iron(II), nickel(II), copper(II), palladium(II), manganese(II), platinum(II), iron(III), aluminum(III), gold(III), yttrium(III), lanthanum(III), rhodium(III), terbium(III), zirconium(IV), tin(IV), tantalum(V), molybdenum(VI), and tungsten(VI). The maximum, constant value of RFI was obtained in the fluorescence reaction with 1.0×10^{-5} M cobalt(II) in the final concentration. In this examination condition, Cu(II) was not reacted with FH, though it was reported that FH provided fluorescent responses to Cu(II) itself.[30,31] The effect of concentration of FH was examined by varying the amounts of the FH solution, while maintaining a fixed final concentration of H_2O_2 (ng ml^{-1}). The maximum, almost constant value of RFI was obtained by

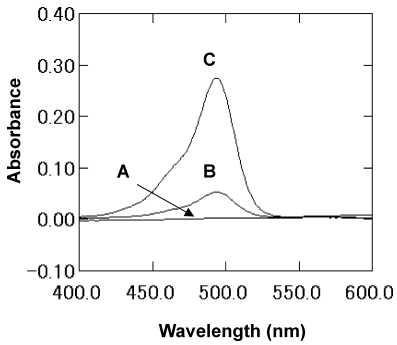

Fig. 1. Absorption Spectra of the Probe FH and Its Reaction Solution with H_2O_2 as well as Fluorescein in the Standard Procedure Solution against the Corresponding Reagent Blank

(A) 4×10^{-6} M FH; (B) reaction solution of 4×10^{-6} M FH with H_2O_2 (300 ng ml^{-1}); and (C) 4×10^{-6} M fluorescein.

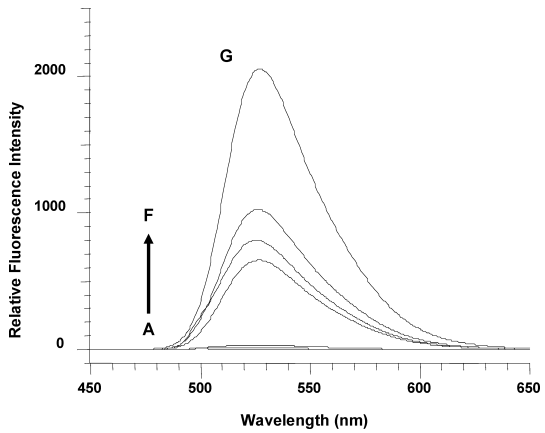

Fig. 2. Fluorescence Emission Spectra of FH in the Absence and Presence of H_2O_2 with a Standard Solution

(A) 4×10^{-6} M FH; (B)—(F) reaction solution of 4×10^{-6} M FH with H_2O_2 (3.0, 30, 300, 3000, 30000 ng ml^{-1}, respectively); (G) 4×10^{-6} M fluorescein. The conditions were 2.5 ml of 1.0% DTAC solution, 2.0 ml of the Na_2CO_3/$NaHCO_3$ buffer solution (pH 9.6), and 1.0 ml of 1.0×10^{-4} M cobalt(II) solution. The excitation and emission values were selected as 494 nm and 527 nm, respectively. Slit band: excitation/emission=5.0/5.0 nm.

using the final concentration of FH at 4.0×10^{-6} M for the determination of H_2O_2.

The fluorescence development in this reaction system did not occur instantaneously at room temperature. Thus, the effects of the incubation temperature and time were investigated by heating for 10—60 min at 50, 60, 70, and 80 °C. The maximum, constant value of RFI was obtained at 80 °C for 15—40 min, followed by cooling in water for 5 min. The RFI value remained constant for at least 2 h after the solution was cooled to room temperature.

Calibration Curve and Reproducibility A calibration curve for H_2O_2 was constructed by the standard procedure. A good linear relationship and wide dynamic range were observed over 2.1—460 ng ml^{-1} of H_2O_2. The limit of detection, defined as (3.3×S.D. of blank)/(slope of analytical calibration), was 0.7 ng ml^{-1}. The correlation coefficient was 0.999. The relative standard deviations (n=6) were 4.06% for 3.1 ng ml^{-1} of H_2O_2 1.78% for 30.8 ng ml^{-1} of H_2O_2, and 2.21% for 308 ng ml^{-1} of H_2O_2. The sensitivity of the proposed method is almost doubled and the reproducibility obtained is excellent. When the other ROS, superoxide (O_2^-), hydroxyl radical (·OH), nitric oxide (NO), peroxynitrite ($ONOO^-$), and peroxide (R–OOH) are examined, an excellent result is obtained, as shown in Table 1.

Interference from Foreign Substances For the assessment of the advantages of our method in environmental and clinical assays, the influences of various foreign substances on the determination of H_2O_2 were examined. Inorganic ions such as magnesium(II), zinc(II), calcium(II), molybdenum(VI), sodium, chloride, fluoride, potassium, bromide, cyanide, phosphate, ammonium, nitrate, and sulfate did not noticeably affect the accuracy of the determination of H_2O_2, even when these ions were present in excessively large amounts as compared to that of H_2O_2. Among foreign metal ions, the presence of copper(II), nickel(II), iron(II), and iron(III) resulted in a slight increase or decrease in determination of H_2O_2. The presence of organic substances such as ascorbic acid, caffeine, glucose, HSA, urea, uric acid, creatinine, and alanine caused considerably less interference. The results are summarized in Table 2.

Application The proposed method was used for the determination of H_2O_2 in human urine. Spot human urine samples were collected from healthy human volunteers and assayed immediately. Preliminary treatments were not conducted to remove co-existing substances and the urine was merely diluted 100 times. Neat standards were prepared under the same conditions. The average recovery of H_2O_2 from samples was 96.7%, with RSD values of less than 5.8%. A *T*-test demonstrated that the results obtained by the present method were not different from those obtained by the comparison procedure, the Cayman Clinical H_2O_2 Assay Kit (the Wolff's FOX Assays), at a 95% confidence level: the calculated t=0.584 was lower than the critical t value (p=0.05, n=6) of 2.447. These results are given in Table 3.

Characterization of FH Xanthene dyes such as fluorescein have been commonly used in various fields for a long time. Though, the relationship between a chemical structure and fluorescence remains almost unknown. Recently, it was reported that the lacton form of fluorescein is non or almost no fluorescent, while its carboxyl form is fluorescent.[32] However, there are not too a lot of examples of the crystal structure where these ideas can be proven. The factors responsible for this are considered to be the difficulty in the crystallization and the logical design of a fluorescent group. This time, we were able to obtain the crystal of FH from the ethanol–acetonitrile solution after purifying it several times. Further the structure was confirmed by MS, NMR, and X-ray crystallography. As shown in Table 4 and Fig. 3, FH was

Table 1. Determination of ROS

Sample	Concentration range (ng ml^{-1})	RSD (%)	DL (=3.3 σ/slope)
H_2O_2	2.1—460	1.8	0.7
O_2^-	1.2—120	5.2	9.2
·OH	1.7—170	2.9	2.1
NO	6.0—300	3.5	1.2
$ONOO^-$	5.6—580	3.8	1.3
R–OOH	30—900	5.3	10.2

Conditions: H_2O_2, 30.8 ng; FH, 4.0×10^{-6} M; DTAC, 0.25%; Co(II), 1×10^{-5} M; pH, 9.6; excitation/emission, 460/527 nm; correlation coefficient is all 0.999.

Table 2. Effect of Foreign Substances on the Determination of H_2O_2

Substances	Added as	Added ng ml^{-1}	Added Mole ratio	RFI (S−B)/B	Recovery %
None	—	—	—	9.3	100
Cu(II)	Nitrate	6.4×10^3	1	9.8	105.4
Ni(II)	Nitrate	2.9×10^2	1/10	11.6	124.4
Mg(II)	Chloride	2.4×10^3	100	9.3	100
Zn(II)	Chloride	6.5×10^3	100	9.2	98.4
Ca(II)	Chloride	1.1×10^4	10	9.7	104
Fe(II)	Sulfate	1.4×10^4	5	10.2	110
Fe(III)	Alum	11	1/5	9.1	98.1
Mo(VI)	Sodium	9.6×10^3	100	9.3	100
NaCl	—	2.9×10^3	50	10.1	109
NaF	—	4.3×10^3	100	9.3	100
KBr	—	1.2×10^4	100	9.3	100
KCN	—	6.5×10^3	100	8.5	91.3
Na_2HPO_4	—	1.4×10^3	10	9.3	100
NH_4Cl	—	5.4×10^2	10	9.2	99
KNO_3	—	1.0×10^4	100	9.3	100
K_2SO_4	—	1.7×10^4	100	9.3	100
NH_3	—	1.7×10^3	1000	9.3	100
Ascorbic acid	—	1.8×10^3	10	8.3	89.2
Caffeine	—	1.9×10^4	100	10.8	116.1
Glucose	—	1.8×10^5	1000	9.3	100
HSA	—	10	—	9.8	105.4
Urea	—	6.0×10^3	100	9.3	100
Uric acid	—	1.7×10^5	100	9.3	100
Creatinine	—	6.6×10^4	50	10.6	114
Alanine	—	8.9×10^4	1000	9.3	100

H_2O_2, 30.8 ng ml^{-1}; FH, 4.0×10^{-6} M; DTAC, 0.25%; Co(II), 1.0×10^{-5} M; pH, 9.6; excitation/emission, 460/527 nm.

Table 3. Determination of H_2O_2 in Human Urine

Sample	Found: Present method	Found: Other method[a)]	RSD[b)] (%)	Recovery[c)] (%)
A	6.3	6.1	3.1	103.3
B	19.3	10.9	2.2	92.2
C	4.8	2.9	5.8	95.3
D	8.6	10.1	4.3	96.1

The calculated t=0.584 was lower than the critical t value (p=0.05, n=6) of 2.447. *a*) Other method; The Cayman Chemical Hydrogen Peroxide Assay Kit. *b*) Average of 5 determinations. *c*) H_2O_2 taken; 30.8 ng ml^{-1}. FH, 4.0×10^{-6} M; DTAC, 0.25%; Co(II), 1×10^{-5} M; pH, 9.6; excitation/emission, 460/527 nm.

Table 4. Crystal and Experimental Data for FH

Formula	$C_{20}H_{14}N_2O_4$, CH_3CN, 0.5(CH_3OH)
Formula weight	403.41
Crystal system	Monoclinic
Space group	$P2_1/n$
A	9.854(1) Å
B	8.643(1) Å
C	23.298(3) Å
β	91.687(2)°
V	1983.5(4) Å^3
Z	4
T	120(2) K
Crystal size	0.35×0.35×0.05 mm^3
D_x	1.351 g cm^{-3}
F(000)	844
μ(Mo$K\alpha$)	0.096 mm^{-1}
No. of reflections (obs)	21332
R_{int}	0.0275
θ_{max}	27.10°
No. of reflections (refine)	4361
No. of reflections ($I>2\sigma(I)$)	3478
No. of parameters	296
R	0.0549
wR	0.16
Goodness of fit	0.973
$(\Delta/\sigma)_{max}$	0.008
Fraction for θ_{max}	0.998
$\Delta\rho_{max}$	0.918 e Å^{-3}
$\Delta\rho_{min}$	−0.310 e Å^{-3}

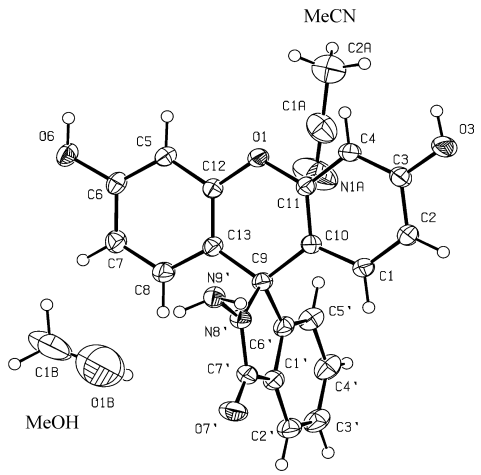

Fig. 3. OTEP of FH

formed with the benzene ring through hydrazine for a xanthene scaffold to have a plane structure and a spirolactam round was formed with a connection that was almost right angled. We found that hydrazation fluorescein would force this platform to adopt a closed, colorless, and non-fluorescent spirolactam form. Oxidation with H_2O_2 and other ROS would subsequently produce the open, colored, and fluorescent fluorescein product.

Conclusion

We have described the synthesis, properties, application, and characterization of fluorescein-hydrazide, a new class of fluorescent probes for detecting ROS. A simple, highly sensitive, and large dynamic range fluorometric method for the determination of H_2O_2, was established with FH and cobalt(II) in a DTAC micellar medium. This procedure is based on a redox reaction among H_2O_2, cobalt(II), and FH. The proposed method is more sensitive and reproducible than the previous method. On the occasion of characterization of FH, the xanthene scaffold and benzene ring have two-facedness and an almost formed spirolactam round form at right angles to each other. As a result, it hardly probable that the total fluorescence may have disappeared because an electronic conjugated system of the xanthene scaffold that was a fluorescent characteristic of fluorescein collapsed by the formation of a spirolactam ring. Although further investigations are necessary for the elucidation of this reaction mechanism, the developed procedure is suitable for the analysis of ROS in clinical studies. In conclusion, the improved method offers many advantages for H_2O_2 determination. First, the method is remarkably improved with respect to reproducibility and sensitivity in comparison with the former method. Second, there is a remarkable improvement in the influences of foreign substances by this method. Finally, the new method is superior to the former method with regard to the wide dynamic range of the calibration curve.

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