Determination of Organic Hydroperoxide Using Indamine Dye Formation Catalyzed by Water-Soluble Iron-Porphyrin and Its Reaction Mechanism

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An assay method for determining organic hydroperoxides based on indamine dye formation using a water-soluble iron-(III) porphyrin as catalyst was developed. The assay was performed at pH 4.4 under anaerobic conditions and was applicable to flow-injection analysis. *t*-Butyl hydroperoxide was determined over the range of 1 to 40 nmol with $r=0.999$ and $SD(\%)=2.4$ ($n=5$ at 4.8 nmol). The mechanism of the catalytic reaction is discussed based on kinetic measurements, suggesting a general acid-base catalyzed reaction involving a heterolytic cleavage of the O-O bond.

Keywords Hydroperoxide, lipid peroxide, hemin, porphyrin, indamine dye, spectroscopic determination

The presence of activated oxygen and its derivatized species in biological systems has been focused on due to its medical importance. Unsaturated fatty acid peroxide (UFAPO) or lipid peroxide (LPO) is formed by radicals, such as hydroxy radical ($\cdot\text{OH}$) or superoxide (O_2^-), in biological systems. These peroxides affect the membrane and interfere with its functions; they are also suspected to be a potential cause of diseases¹, and to have roles in the aging process.²

Because of the uncertainty in determining LPO or UFAPO^{3,4}, the relation between various diseases and peroxides has not yet been clarified. An iodometric determination has traditionally been used in food and agricultural chemistries, and a small amount of peroxide has been detected using a thiobarbituric acid (TBA) method which was developed in clinical chemistry.⁵⁻⁷ However, it has been pointed out that the TBA method has a poor specificity to peroxide, and that the product formation was in the 10–20% range of the supposed amount.^{3,8} A method was recently introduced based on the formation of Methylene Blue by hydroperoxide catalyzed by hemoglobin.⁹ Other reported methods include an iodometric assay¹⁰, the formation of a Xylenol Orange-iron complex¹¹, an assay using cyclooxygenase¹²

and HPLC methods.¹³ However, a more reliable assay method for peroxide is still necessary in order to overcome the uncertainty of the commonly used TBA method.⁸

We previously developed a method for the determination of hydrogen peroxide using a water-soluble iron (III) porphyrin (hemin, FeTMPyPCL_3)¹⁴, which is now being applied to organic hydroperoxide with a slight modification. An indamine dye formation system using horse radish peroxidase (HRP) had been proposed for detecting hydrogen peroxide.¹⁵⁻¹⁸ Our previous system¹⁴ was used to specifically determine hydrogen peroxide using water-soluble porphyrin as a catalyst instead of HRP in an acidic media (pH 1–2). It was noted that when it was applied at low pH (1–2) only hydrogen peroxide reacted to form a dye; on the other hand, molecular oxygen and organic hydroperoxide developed color in a higher pH region (3–6).

In this report we describe our adoption of this reaction for determining organic hydroperoxide, *t*-butyl hydroperoxide (*t*-BuOOH) and cumene hydroperoxide (CuOOH)(Fig. 1). The reaction was performed in the 3–5 pH region and had to be carried out under anaerobic conditions in order to avoid oxidation by molecular oxygen.

Fig. 1 Coupling reaction forming indamine dye.

The study of the reaction mechanism was intended to confirm the stoichiometry of the reaction, which would assure the reproducibility of the reaction. Another purpose was to answer questions concerning the reaction mechanism. For instance, it was known that alkyl hydroperoxide reacted with oxyhemoglobin to form the alkoxy radical, which initiated additional reactions.¹⁹ In studying model-hemin systems, two mechanisms were proposed regarding cleavage of the O-O bond of hydroperoxide on hemin, namely heterolytic or homolytic. In the former case, the reaction involves an ionic process; in the latter case the reaction is a radical process. If the mechanism is heterolytic, the reaction stoichiometry would hold; and if it is homolytic, the alkoxy radical could be produced and then followed by a chain reaction.

Experimental

Materials and reagent solutions

Freshly distilled *N,N*-dimethylaniline (DMA)(Wako Pure Chemical Ind.) was dissolved in 0.1 mol dm⁻³ hydrochloric acid to be 2.06×10^{-2} mol dm⁻³ (Solution A).

3-Methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) (Wako Pure Chemical Ind.) was dissolved in deionized water to be 4.28×10^{-3} mol dm⁻³ (Solution B).

meso-Tetrakis(1-methyl-4-pyridyl)porphinatoiron(III) pentachloride (FeTMPyPCl₅) was made by inserting iron into *meso*-tetrakis(1-methyl-4-pyridyl)porphyrin (H₂TMPyP)(Dojindo Lab.). Iron insertion was performed following a method described in the literature²⁰, except for using *N,N*-dimethyl acetamide instead of *N,N*-dimethyl formamide as the solvent; it resulted in an easy separation of excess iron in the form of precipitated iron oxide. FeTMPyPCl₅ was purified by repeated recrystallization from hydrochloric acid (6 mol dm⁻³) while being evaporated at room temperature. The formed precipitate was dried in a desiccator over sodium hydroxide. FeTMPyPCl₅ was dissolved in deionized water and the concentration was adjusted to be 1.0×10^{-4} mol dm⁻³ (Solution F).

t-Butyl hydroperoxide (*t*-BuOOH)(Aldrich) and cumene hydroperoxide (CuOOH)(Aldrich) were assayed iodometrically according to a method described in the literature.²¹ An appropriate amount of hydroperoxide was dissolved in isopropyl alcohol containing 20% sodium iodide in a flask with a condenser; the mixture was then refluxed in a water bath for 10 min. After cooling in an ice-cold water bath, the liberated iodine was titrated by sodium thiosulfate. Hydroperoxide was diluted in a buffer solution (Solution H).

Phthalate buffer (0.05 mol dm⁻³) was used for the determination and kinetic measurements and phosphate buffer (0.05 mol dm⁻³) was also used for checking any pH effect on the reaction. Nitrogen gas was passed through Oxisorb (Messer Griesheim) in order to eliminate contaminated oxygen.

All of the other chemicals used were of reagent grade.

Apparatus

A Hitachi-220A spectrophotometer was used with an attached water circulator. Long-necked cuvettes with silicone rubber stoppers were used under anaerobic conditions. Bio-Rad Model EP-1 pumps and a Hitachi LC flow system attached to the spectrometer were used for a flow-injection analysis system (FIA). The pHs were checked with a Horiba H-7 LD pH meter.

General procedure

Into a glass cuvette of 1 cm length and a long neck with a silicon rubber stopper, 0.2 ml of Solution A (DMA), 0.2 ml of Solution B (MBTH), 0.5 ml of Solution F (FeTMPyPCl₅) and 2.0 ml of the buffer solution (pH 4.4) were charged; the mixed solution was then degassed by passing nitrogen gas through it. To the mixed solution 0.3 ml of degassed Solution H (*t*-BuOOH, CuOOH) was injected, and the solution was shaken; the absorbance at 590 nm was measured after 30 min.

For the flow-injection system shown in Fig. 2, degassed mixed solutions of A and F and Solution B were mixed and 30–100 µl sample solutions of H were injected into the flow. The reaction coil was kept at 30°C. The absorbance was monitored by a detector at 590 nm after 6 min; within that period 80–90% of the production was reached with good reproducibility.

Fig. 2 Flow system used in measuring organic hydroperoxide.

Kinetic measurements

Into a cuvette (1 cm in length with a long neck and a silicon rubber stopper) containing an MBTH solution, and buffered solutions (pH 4.4) of DMA and FeTMPyCl₅, 10–100 μ l of the degassed *t*-BuOOH solution was injected to start the reaction. The reaction conditions are indicated in the figure captions. The reaction rates were determined by following the increase in the absorbance at 590 nm.

Results and Discussion

Formation of indamine dye

In this system indamine dye was formed by the oxidative coupling between MBTH and DMA (Fig. 1). The reaction was initially developed for the determination of aromatic amines and imino heteroaromatic compounds²², and was then applied later for the determination of hydrogen peroxide using peroxidase as a catalyst and the activity of peroxidase itself.^{15–18}

Our previous method¹⁴ using synthetic hemin, FeTMPyCl₅, has been expanded for the determination of organic hydroperoxide using water-soluble iron(III) porphyrin; it was shown to be a useful method for determining lipid peroxide. In the 3–5 pH region any examined ordinary enzymes would be less active, and would not influence the determination. The resulting color of the dye was stable and its visible spectrum was the same as in the case of hydrogen peroxide.¹⁴ In a preliminary experiment, the molecular extinction coefficient of indamine dye was determined to be 3.87×10^4 .

The pH dependency of the reaction was checked by following the time course of the color formation, as

Fig. 3 Time course of the color formation with *t*-BuOOH at various pH (25°C). The final concentrations of the reagents were 1.45×10^{-5} mol dm⁻³ for FeTMPyCl₅, 1.24×10^{-3} mol dm⁻³ for MBTH, 5.97×10^{-3} mol dm⁻³ for DMA and 2.89×10^{-5} mol dm⁻³ for *t*-BuOOH. The concentration of phthalate buffer was 7.4×10^{-2} mol dm⁻³.

shown in Fig. 3. When examined in detail, it was clearly observed that the rate of the reaction increased at higher pH until 4–5, but slowed beyond pH 5–6. At pH 6–8, the reaction quickly stopped after the initial immediate increase. A similar phenomenon had been observed in the case of hydrogen peroxide¹⁴, and the reduction of hemin was observed in both cases of hydroperoxide and hydrogen peroxide at pH 6–8. From a detailed examination using a phthalate buffer the optimum pH for the assay was determined to be 4.4. As molecular oxygen reacted to increase dye formation at the pH studied¹⁴, solution degassing was necessary by passing nitrogen gas. Due to a spontaneous formation of dye at pH 4.4, a blank test was required.

The reaction was checked at 10, 20, 30 and 40°C. Although the rate increased at higher temperatures, a slight decomposition of hemin occurred at 40°C. The experiments described below were performed at room temperature (25°C) and 30°C for FIA.

Reproducibility and calibration curve

Similarly, as described in a previous paper¹⁴, the spectra taken after the reaction indicated that the catalyst hemin remained unchanged in the 1–5 pH region, clarifying the reproducibility of the reaction. A plot of the absorbance against the concentration of *t*-BuOOH and CuOOH in Fig. 4 shows a bending, which suggests that at higher concentrations the hydroperoxide, itself, reacted as a substrate, as had been described in the literature.^{23–30} On the other hand, the linearity was observed at low concentrations using the FIA method. Over the 1–40 nmol range of injection a linearity of the calibration curve was obtained by using *t*-BuOOH, as

Fig. 4 Plot of *t*-BuOOH and CuOOH (25°C). The final concentrations of the reagents were 1.18×10^{-5} mol dm⁻³ for FeTMPyCl₅, 2.0×10^{-3} mol dm⁻³ for MBTH and 4.9×10^{-3} mol dm⁻³ for DMA. The concentration of phthalate buffer was 3.8×10^{-2} mol dm⁻³.

shown in part in Fig. 5. The correlation factor was 0.999 and SD(%) was 2.4 ($n=5$) when 4.8 nmol was injected. As for CuOOH, the observed linear relation showed $r=0.998$.

Effect of foreign materials on the determination

The effect of materials which were supposed to be present in samples was examined in the FIA system, the results of which are given in Table 1. No severe interference was observed, except for the following. The presence of such reducing reagents as ascorbic acid, glutathione or iron(II) sulfate reduced the hydroperoxide competing with dye formation. Iron(III) and copper(II) ions which caused a positive effect on the formation of dye could be masked by adding EDTA (ethylenediaminetetraacetic acid), as indicated in Table 1. In case of FeCl₃, the addition of EDTA of about 10^{-3} mol dm⁻³ was enough, but in the CuCl₂ case 10^{-2} mol dm⁻³ of EDTA was necessary. There were some cases in which spontaneous dye formation occurred without any oxidant or catalyst, though its cause was not clear at this stage. The addition of EDTA or citric acid was found to be effective for reducing the spontaneous formation of dye.

Kinetic measurements and the rate equation with *t*-BuOOH

Generally, in biological systems oxygen is thought to convert to become a radical species that reacts rather rapidly with biological materials. To clarify the

Fig. 5 Calibration curve with *t*-BuOOH in FIA (30°C). Thirty microliters of a *t*-BuOOH solution was injected. The flow rate was 0.3 ml min⁻¹. The concentrations of the reagents in the flow were 1.7×10^{-5} mol dm⁻³ for FeTMPyPCl₃, 1.7×10^{-3} mol dm⁻³ for MBTH, 7.0×10^{-3} mol dm⁻³ for DMA. The concentration of phthalate buffer was 2.8×10^{-2} mol dm⁻³.

Table 1 Effect of foreign materials^a

Compound	Difference ^b , %	Compound	Difference ^b , %
NH ₄ Cl	+0.25	EDTA	-19.3 ^f
KCN	+6.61	Glycine	-1.53
KI	+4.70	L-Proline	0.0
NaN ₃	-5.94	L-Phenylalanine	+2.30
NaBr	+0.25	Imidazole	+2.53
CaCl ₂	-1.06	Bilirubin	-0.48 ^g
NiCl ₂	+1.05	Trichloroacetic acid	+4.92
MgSO ₄	-7.42	1%	+11.7
Zn(CH ₃ COO) ₂	+3.47	3%	+9.84
Co(NO ₃) ₂	+2.30	Levelose	+4.04
FeSO ₄	-91.8	L-α-Alanine	+2.53
CuCl ₂	— ^c	Acetic acid	+5.57
+EDTA (10 ⁻²)	-12.2	Ascorbic acid	— ^h
+EDTA (10 ⁻³)	+106	Pottasium citrate	-23.8
+EDTA (10 ⁻⁴)	+168	Urea	+4.56
FeCl ₃	+34.1 ^c	Glucose	+3.80 ^d
+EDTA (10 ⁻²)	-11.5		+6.07 ^e
+EDTA (10 ⁻³)	-16.9	Gluthathione	— ^{h,i}
+EDTA (10 ⁻⁴)	+13.6	Albumin, human	-1.77 ^j

a. Concentrations of *t*-BuOOH and foreign materials were 2.4×10^{-4} and 2.4×10^{-3} mol dm⁻³ respectively, unless otherwise noted (100 μl injected). b. Differences in percentage were those between numbers with and without foreign materials. c. Fe(III) and Cu(II) ions reacted without hydroperoxide. d. 1.8×10^{-2} mol dm⁻³. e. 1.8×10^{-3} mol dm⁻³. f. 1.0×10^{-2} mol dm⁻³. g. 0.5 mg dl⁻¹. h. Ascorbic acid and glutathione reduced hydroperoxide quickly. i. 50 mg dl⁻¹. j. Because of the low solubility of albumin the supernatant was added.

mechanism for the formation of activated species or an intermediate is important since, if during the reaction hydroxy or alkoxy radicals are formed, it would react quickly to cause other reactions. If these radicals are not formed, the stoichiometry of the reaction (Fig. 1) holds in this case. A kinetical study would answer this question.

Typical kinetic results with *t*-BuOOH are shown in Fig. 6. The reaction was revealed to be pseudo-first order when the reaction was plotted as $\log(A_\infty - A_t)$ against time. The initial rates were obtained from the time courses of the reactions. Figures 7, 8 and 9 show linear relations between the initial rates at pH 4.4 and the concentrations of the reactants and the catalyst, *t*-BuOOH, DMA and FeTMPyPCl₅, respectively. On

the other hand, the initial reaction rate was revealed to be independent of the concentration of MBTH. The intercepts at zero concentration indicated the "spontaneous" reaction rates.

The kinetic results thus showed that the catalyzed reaction was third order regarding with the concentrations of FeTMPyPCl₅, DMA and *t*-BuOOH, and that the rate equation (Eq. (1)) for the initial stage of the reaction can be written

Fig. 6 Time course of the reaction at various amounts of *t*-BuOOH (25°C). Concentration of reagents: 1.45×10^{-5} mol dm⁻³ for FeTMPyPCl₅, 5.97×10^{-3} mol dm⁻³ for DMA and 1.24×10^{-3} mol dm⁻³ for MBTH. Concentration of *t*-BuOOH ($\times 10^{-5}$ mol dm⁻³): 1: 11.6, 2: 5.78, 3: 2.89, 4: 1.45, 5: 0.72, 6: 0.29. Concentration of phthalate buffer was 2.1×10^{-2} mol dm⁻³.

Fig. 7 Kinetic plot of the reaction for *t*-BuOOH (25°C). The concentrations of the reagents were the same as in Fig. 6.

Fig. 8 Kinetic plot of the reaction for DMA (25°C). The concentrations of the reagents were the same as in Fig. 6 except for DMA and $[t\text{-BuOOH}] = 2.89 \times 10^{-5}$ mol dm⁻³.

Fig. 9 Kinetic plot of the reaction for FeTMPyPCl₅ (25°C). The concentrations of the reagents were the same as in Fig. 6 except for FeTMPyPCl₅ and $[t\text{-BuOOH}] = 2.89 \times 10^{-5}$ mol dm⁻³.

$$\left(\frac{d[\text{Dye}]}{dt}\right)_0 = k_{\text{obs}}[\text{FeTMPyP}(\text{Cl}_5)]_0 \times [\text{DMA}]_0 [\text{t-BuOOH}]_0 \quad (1)$$

k_{obs} was estimated to be $7.51 \times 10^4 \text{ mol}^{-2} \text{ dm}^6 \text{ s}^{-1}$.

Mechanism of the reaction at pH 4.4

Under the present anaerobic conditions, even when hydroperoxide was added to the hemin solution without MBTH and DMA, no hemin-hydroperoxide adduct was observed and no degradation of hemin was indicated by the UV-vis spectra.

As mentioned before, two mechanisms for the cleavage of the O-O bond of hydroperoxide coordinated on iron have been proposed. One was the heterolytic or ionic cleavage of the O-O bond (Eq. (3)), resulting in the iron(V)-oxygen intermediate (1) and alcohol (2); the other was a homolytic or radical cleavage (Eq. (4)) resulting in the iron(IV) oxygen intermediate (3) and alkoxy radical (4). These mechanisms were proposed by different authors;²³⁻³⁰ others suggested that the cleavage depended on the conditions where it occurred.³¹

Fe^+ denotes $\text{FeTMPyP}(\text{X})$; under these conditions the fifth ligand X is H_2O ^{29,35}, which is omitted in the equations for simplicity.

Because the reaction was not dependent on the concentration of MBTH, an actual dye formation took place after the step which formed an intermediate, "an activated oxygen" species, and which was rate-determining. The linear dependency on the DMA concentration indicated that DMA not only reacted to form the indamine dye, but also played the role of a general acid-base catalyst in the rate-determining step that cleaved the O-O bond; DMA was protonated at the examined pH region ($\text{p}K_a=5.15$ ³²). Supporting this, the addition of *N-t*-butyl- α -phenylnitron (radical trapping reagent) to the reaction mixture did not affect the reaction (data not shown).

The reaction mechanism in the present case could thus be explained as shown below. Hydroperoxide coordinated on iron(III) of hemin and heterolytic O-O bond

cleavage was followed to form active species:

where $k_{\text{obs}}=K \cdot k_1$.

The homolytic cleavage of the O-O bond to form an alkoxy radical was thus excluded under the present reaction conditions. If an alkoxy radical is formed according to Eq. (4), the radical would react so as to result in a chain decomposition of hydroperoxide. This, however, was not the case.

The other interesting feature was that the reduction of hemin occurred in the 6–8 pH region (data not shown). The reduction of hemin by MBTH was accelerated by adding *t*-BuOOH. This suggested that the different mechanism was dominant in the system which reduced hemin.³³ The reduction of hemin and the formation of rearranged or decomposed compounds of *t*-BuOOH were reported regarding the Cytochrome P-450 system.³⁴

Since the present determination of organic hydroperoxide was performed in the 4–5 pH region, the existing enzymes, if any, in the sample solution would be less active. In the proposed assay method the handling of samples was direct and easy resulting in good reproducibility. Although hydrogen peroxide caused color formation, it could be determined separately at pH 1–2, as previously described.¹⁴ Because the catalyzed reaction by water-soluble iron porphyrin was less specific to the kinds of peroxides, this method would be useful for fatty acid peroxide in cases in which actual enzyme systems, such as HRP or hemoglobin, might cause a problem regarding the specificity. This method would be arranged for an automatic assay in the field of clinical chemistry. Application of this method to the determination of lipid peroxide in biochemical systems is underway.

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