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Production mechanism of active species on the oxidative bromination following perhydrolase activity

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Hypobromous acid and molecular bromine have been described as the active species involved in the oxidative bromination using perhydrolase, which catalyzes the reaction from acetic acid and hydrogen peroxide to peracetic acid (AcOOH). However, the brominating activity of them in a chemical model system was lower than that of the active species produced by the spontaneous reaction between AcOOH and Br^-. Consequently, acetyl hypobromite (AcOBr) was suggested as new active species on the bromination by detection of the decarboxylation in the reaction between AcOOH and Br^- and the strong brominating power with some tolerance against H_2O_2. Its production mechanism was explained as the ionic reaction involving the protonated intermediate of AcOOH by kinetic analysis. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: active species; oxidative bromination; non-enzymatic mechanism; monochlorodimedone; peracetic acid; acetyl hypobromite; perhydrolase; kinetic analysis; ionic reaction; decarboxylation

INTRODUCTION

Brominations are especially important reactions for the preparation of synthetic intermediates that contribute to forming carbon–carbon bonds and changing the functional group. Chemical brominations are generally performed using electrophilic reagents such as *N*-bromosuccinimide and molecular bromine (Br_2) in organic solvent; however, they are poisonous sacrificial reagent. Accordingly, many researchers recently have focused on the oxidative halogenation, [1] which makes use of a brominating species produced form the reaction between Br^- and oxidant such as H_2O_2,[1] O_2,[2] DMSO,[3] oxone,[4] $(NH_4)S_2O_8$,[5] *t*BuOOH,[6] peracid,[7] $(NH_4)_2Ce(NO_3)_6$,[8] $NaIO_4$,[9] PPh_3O,[10] selectfluor, [11] and so on. This method employs the safety salt such as NaBr, KBr, NH_4Br, Bu_4NBr, or available HBr as a bromide source. Although the oxidative bromination under mild condition is often required as a catalyst such as metal ion and haloperoxidase, the enzyme realizes the aqueous bromination using low concentration of H_2O_2 at room temperature. Haloperoxidase is expected as an environmental harmony-type catalyst in the oxidative bromination. Because various enzymatic brominations of terpenes[12] have been confirmed using several haloperoxidases, many brominated natural products have been suggested as the product induced by the enzymes. [12,13]

Haloperoxidases are classified into four groups of the heme-dependent, [13] vanadium-dependent, [13,14] flavin-dependent, [13a–c] and metal-free types (which was renamed perhydrolase) [13,15] containing iron protorphyrin IV, vanadate (HVO_4^{2-}), $FADH_2$, and the Ser–His–Asp catalytic triad in the active site, respectively. It has been believed that the heme-dependent, vanadium-dependent, and flavin-dependent-type haloperoxidases catalyze direct production of hypobromous acid (HOBr). On the other hand, perhydrolase catalyze to produce percarboxylic acid (RCOOOH) from carboxylic acid (RCOOH) and H_2O_2. [16] The catalytic mechanism of perhydrolase was explained by esterification of RCOOH to the nucleophilic Ser residue and then perhydrolyzation of the ester using H_2O_2 (Fig. 1(a)).[16b,15,17] The oxidative bromination following perhydrolase activity is easily detected by using two synthetic substrates of monochlorodimedone (MCD, **1**) [18] and phenol red affording monobromomonochlorodimedone (MBMCD, **2**) and bromophenol blue, respectively, in the presence of Br^- (Fig. 1 (b)). A brominating species is produced at enzyme outside by the non-enzymatic reaction between Br^- and RCOOOH liberated from the active site in the enzyme. The non-enzymatic bromination involving the activity has been explained by using the active species such as HOBr (Eqns 1 and 2) [13b,d,15a] or Br_2 (Eqns 3 and 4).[16b,17a] However, evidence for the non-enzymatic step still remains unclear. This step should be contemplated for the application of perhydrolase. The study on the oxidative bromination following perhydrolase activity is simplified using RCOOOH instead of the enzyme in the model system of the bromination in principle. In this paper, the detailed investigation as to the oxidative bromination is described.

$$AcOOH + H^+ + Br^- \rightarrow AcOH + HOBr + H_2O \quad (1)$$

$$MCD + HOBr \rightarrow MBMCD + H_2O \quad (2)$$

$$AcOOH + 2H^+ + Br^- \rightarrow AcOH + 1/2\ Br_2 + H_2O \quad (3)$$

$$MCD + Br_2 \rightarrow MBMCD + H^+ + Br^- \quad (4)$$

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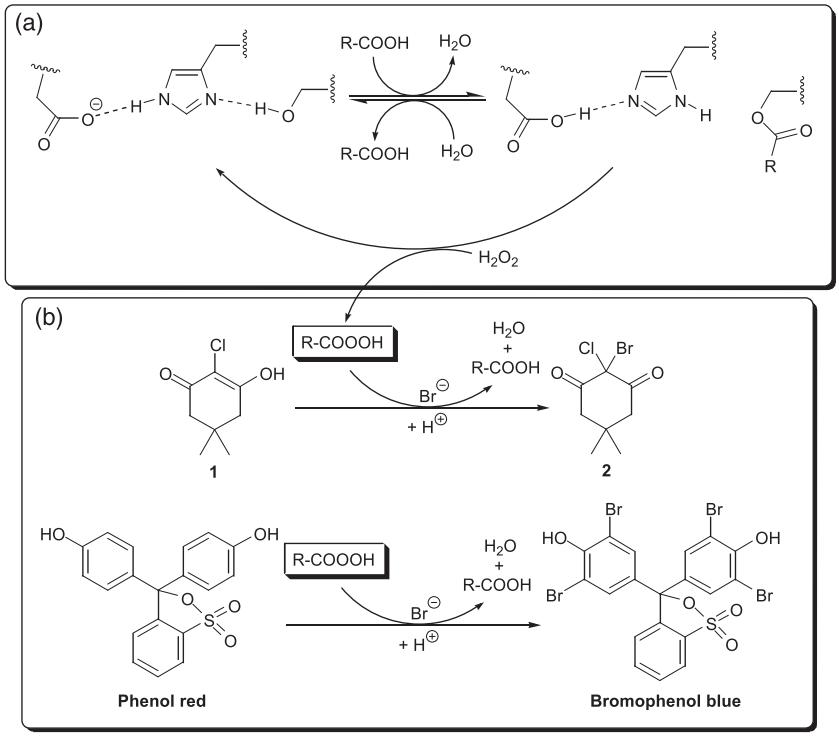

Figure 1. (a) Perhydrolase activity and (b) oxidative bromination

EXPERIMENTAL

Concentration determination of materials

The concentration of ca. 9%(w/v) AcOOH in an AcOH solution (Sigma-Aldrich Co., St. Louis, USA) was determined by iodometric titration using NaI, $Na_2S_2O_3$, and starch indicator and by cerimetric titration using 0.1 M $Ce(SO_4)_2$ solution (Kanto Chemical Co., Inc., Tokyo, Japan) and 1.5%(w/v) ferroin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) indicator.[19] The concentrations of ca. 9%(w/w) NaOBr aqueous solution (Kanto) and 1.01 g/mL Br_2 aqueous solution (Wako) were determined by the iodometric titration. The concentration of ca. 30%(w/w) H_2O_2 (Santoku Chemical Industries Co., Ltd., Tokyo, Japan) was determined by cerimetric titration.

Measurement on consumption of 1 and production of Br_3^-

Consumption of **1** ($\varepsilon = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$) and production of Br_3^- ($\varepsilon = 4.09 \times 10^4\ M^{-1}\ cm^{-1}$)[20] using three of the 45 μM oxidant reagents (AcOOH, NaOBr, and Br_2) in 1.0 M AcONa-H_2SO_4 buffer (pH 5.5) with or without additives (0.5 M NaBr and 10 mM H_2O_2) was assayed by measuring disappearance at 278 nm and appearance at 266 nm, respectively, using UV–Vis spectrophotometer at 25 °C. The three oxidants were used in dark room. Production of Br_3^- was performed in the absence of **1**. The initial consumption rate of **1** was assayed under 1.0 M AcONa-H_2SO_4 (pH 4.0–5.5), 0.1 M Na_2HPO_4-H_2SO_4 (pH 6.0–8.1), and 0.1 M H_3BO_3-NaOH (pH 8.3–10.0) buffers with 50 mM NaBr. The efficiencies and initial rates on the consumption and production were calculated using the three molar extinction coefficients of **1**, $\varepsilon_{(pH\ 4.0)} = 1.34 \times 10^4\ M^{-1}\ cm^{-1}$, $\varepsilon_{(pH\ 4.5)} = 1.35 \times 10^4\ M^{-1}\ cm^{-1}$, and $\varepsilon_{(pH\ 5.0–10.0)} = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$.

Structure determination analysis

The ^1H and ^{13}C NMR spectra were recorded by an ECS 400 NMR spectrometer (JEOL Ltd., Tokyo, Japan) at 400 and 100 MHz, respectively, using deuterated solvents such as $CDCl_3$ and D_2O. All of chemical shifts (δ) values are expressed in parts per million relative to trimethylsilane (TMS) as the internal standard, and all of the coupling constants (J) values are expressed in hertz. Signal multiplicities are represented as singlet (s), doublet (d), and double doublet (dd). The infrared (IR) spectra of the inorganic compounds were recorded by a Spectrum BX FT-IR system (Perkin-Elmer, Inc., MA, USA) using the KBr tablet method and were reported in wavenumbers (cm^{-1}). The IR spectra of the organic compounds were recorded by a FTIR-8400 spectrometer (Shimadzu Co., Kyoto, Japan) using a KBr fixed thickness liquid cell (0.1 mm) with dehydrated solvents.

Synthesis

2-Bromo-2-chloro-5,5-dimethyl-cyclohexan1,3-edione (**2**). (Method 1) To a solution of **1** (47.2 mg, 270 μmol) in 6 mL of 1.0 M AcONa-H_2SO_4 buffer (pH 5.5) containing 0.5 M NaBr was added AcOOH (771 μL, 594 μmol, 0.77 M solution containing AcOH). After the mixture was stirred at 25 °C for 10 min, the reaction was quenched with 9.4 mM H_2O_2. The product was filtered under reduced pressure and then the filtrate was washed using water. Drying the filtrate gave **2** (53.1 mg, 209 μmol) as a white powdery in 77% yield. (Method 2) To a solution of **3** (43.8 mg, 200 μmol) in 5 mL of CH_2Cl_2 was added *t*BuOCl (22.6 μL, 200 μmol) at room temperature. After the mixture was stirred at the temperature for 1 h, the mixture was diluted in 15 mL of CH_2Cl_2 and washed with 20 mL of water once. The extract was filtered with filter paper for dehydration and then removal of the solvent by evaporation gave **2** (48.1 mg, 190 μmol) in 95% yield. ^1H NMR ($CDCl_3$): δ 0.85 (3H, s, ax.CH_3), 1.20 (3H, s, eq.CH_3), 2.65 (2H, d, J = 14.6 Hz, eq.CH), 3.39 (2H, dd, J = 14.6, 0.9 Hz, ax.CH) ppm. ^{13}C NMR ($CDCl_3$): δ 25.9 (ax.CH_3), 29.7 (eq.CH_3), 30.6 (C5), 48.7 (C4 and C6), 74.0 (C2), 192.6 (C1 and C6) ppm. IR ($CHCl_3$): ν 577, 613, 1375, 1397, 1728, 1751, 2967 cm^{-1}. The ^1H and ^{13}C NMR values are consistent with those reported in the literature.[21,18b]

2,2-Dibromo-5,5-dimethyl-cyclohexane1,3-dione (**4**). To a solution of dimedone (37.8 mg, 270 μmol) in 6 mL of 1.0 M AcONa-H_2SO_4 buffer (pH 5.5) containing 0.5 M NaBr was added AcOOH (1.542 mL, 1.188 mmol, 0.77 M solution containing AcOH). After the mixture was stirred at 25 °C for 10 min, the reaction was quenched with 9.4 mM H_2O_2. The product was filtered under reduced pressure, and then the filtrate was washed using water. Drying the filtrate gave **4** (50.1 mg, 168 μmol) as a white powdery in 62% yield. ^1H NMR ($CDCl_3$): δ 1.02 (6H, s, $C(CH_3)_2$), 3.01 (4H, s, $CO\text{-}CH_2$) ppm. ^{13}C NMR ($CDCl_3$): δ 27.7, 30.6, 48.2, 66.4, 192.8 ppm. IR ($CHCl_3$): ν 546, 1200, 1457, 1722, 2322, 2359, 2965, 3030 cm^{-1}. The ^1H and ^{13}C NMR values are consistent with those reported in the literature.[22]

Titration of MCD for determination of pK_a

A stirred 45μM MCD aqueous solution (400 mL) was carefully neutralized by addition of a small amount of 1 M NaOH aqueous solution using an

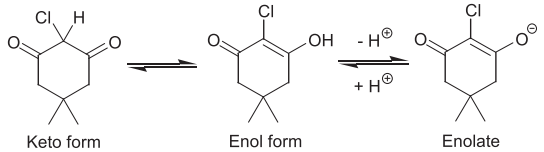

Figure 2. Tautomerism and ionization of **1**

appropriate micropipetor and then the afforded solution was titrated using 0.5 M HCl aqueous solution at 25 °C. After pH and the corresponding absorbance at 278 nm were measured for each dropping, the ε values were calculated by using Lambert–Beer equation with the resulting concentration of MCD. The minimum and maximam values assigned to enol ($\varepsilon_{enol} = 1.18 \times 10^4\ M^{-1}\ cm^{-1}$) and enolate ($\varepsilon_{enolate} = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$), respectively. Curve fitting of the plots was performed using SigmaPlot2001 software v7 (SPSS Inc., Chicago, IL, USA).

Detection of carbon dioxide produced from the reaction between AcOOH and Br^-

To a stirred 2.0 M NaBr solution (100 mL) in the absence and presence of 9.4 M H_2O_2 was added 0.2 M AcOOH-NaOH solution (pH 7.0) containing AcOH (100 mL) with dropping funnel under argon atmosphere at 25 °C. After stirring for 10 min, the reaction was quenched by the addition of 9.4 mM H_2O_2. Generated gas was passed through a trap with a saturated $Ca(OH)_2$ solution (50 mL) by pressing with balloon-filled argon. A given precipitate of $CaCO_3$ was filtered under reduced pressure and then washed using water and acetone. IR: ν 3468, 2967, 2878, 2513, 2363, 1799, 1422, 875, 712 cm^{-1}.

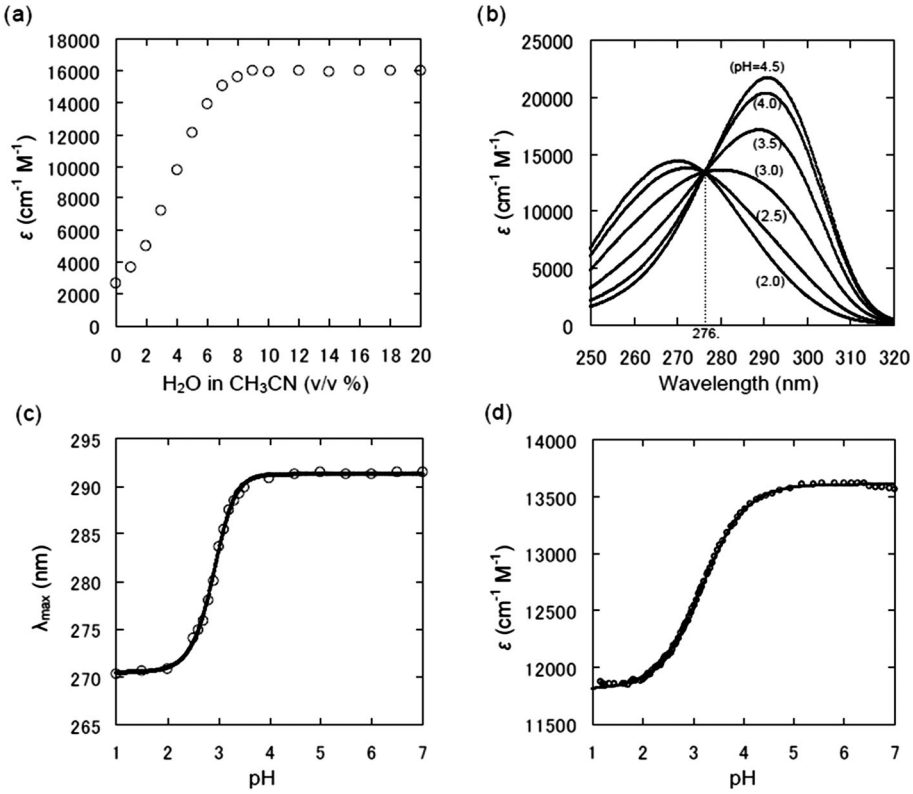

Figure 3. Relation between ionization and UV adsorption of **1**. The experiments about (a) effect of water on the ε value at 278 nm in CH_3CN, (b) determination of isosbestic point in water, (c) effect of pH on λ_{max} in water, and (d) determination of pK_a on **1** at 278 nm in water were performed in a 45 μM of **1** in solution at 25 °C

RESULTS AND DISCUSSION

MCD assay system

An MCD assay for perhydrolase is usually performed in 1.0 M AcONa-H_2SO_4 buffer (pH 5.5) with NaBr. It has been believed that the enol form of **1** equilibrates with the keto form in acidic aqueous solutions[13g,23] (Fig. 2). It may be that a conception about keto–enol tautomerism of **1** was associated with its tautomerism of dimedone.[24] However, the keto form of **1** was not observed by a detailed NMR analysis in several deuterated organic solvents. [22b] In fact, the keto form was not also observed by ^1H NMR analysis in 1.0 M CD_3COOD-NaOD buffer (pD 5.5).

The hyperchromic effect of **1** was caused by the deprotonation accompanied by an increase in water (Fig. 3(a)). The effect of pH on the UV spectra of **1** showed an isosbestic point (276.5 nm), which implied the existence of two compounds corresponding to the enol and enolate of **1** in the aqueous solution (Fig. 3(b)). The λ_{max} values of the enol and enolate were 270 and 291 nm, respectively (Fig. 3(c)). An acid dissociation constant of **1** (pK_a = 3.18) was determinated using Eqn 5 (Fig. 3(d)). Compound **2** produced by the bromination of **1** using AcOOH was confirmed by the chlorination of monobromodimedone (MBD, **3**)[25] using *t*BuOCl (Fig. 4). The bromination of the enolate of **1** without a consideration of the keto form in the assay was ensured by the results. Although we recently clarified that the MCD assay contains minor systematic error caused by decomposition of **2**,[18b] the relative discussion using apparent consumption efficiency of **1** enable to investigate the active species in the oxidative bromination.

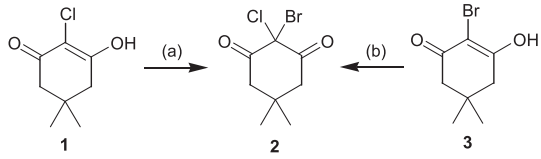

Figure 4. Synthesis of **2**. (a) AcOOH (2.2 eq), 1.0 M AcONa-H_2SO_4 buffer, 0.5 M NaBr, 25 °C, 10 min, 77% and (b) *t*BuOCl, CH_2Cl_2, r.t., 1 h, 95%

$$\varepsilon = \varepsilon_{enol} + \frac{\varepsilon_{enolate} + \varepsilon_{enol}}{1 + e^{\ln 10(pKa - pH)}} \quad (5)$$

Active species

The oxidative bromination using perhydrolase is usually performed at pH 5.5 in the presence of NaBr and H_2O_2. The chemical model system of the bromination using AcOOH instead of perhydrolase has simplified the study on the brominating activity in the presence of the enzyme. An active species produced from the reaction between AcOOH and NaBr at pH 5.5 was investigated by a UV–Vis analysis. The investigation was mainly performed in the absence of H_2O_2 because Br_2 equilibrating HOBr and Br_3^- in the presence of Br^- (Eqns 6 and 7)[20a,26] are rapidly

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decomposed by H_2O_2 (Eqns 8 and 9).[27] The λ_{max} values of the product in CCl_4 and the acetate buffer were 415 and 267 nm, respectively. These values corresponded to Br_2[28] and Br_3^-,[26b,29] respectively. This result was the same to the bromination under a strong acidic pH.[30]

$$Br_2 + H_2O \rightleftharpoons HOBr + H^+ + Br^- \quad (6)$$

$$Br_2 + Br^- \rightleftharpoons Br_3^- \quad (7)$$

$$HOBr + H_2O_2 \rightarrow O_2 + H^+ + Br^- + H_2O \quad (8)$$

$$Br_2 + H_2O_2 \rightarrow O_2 + 2H^+ + 2Br^- \quad (9)$$

The consumption efficiency of **1** and production efficiency of Br_3^- using oxidant such as AcOOH, NaOBr, and Br_2 are shown in Table 1. It was reported that **1** is consumed by oxygen of a strong oxidant.[31] AcOOH is also a strong oxidant; however, **1** was not consumed by AcOOH (Entries 3a and 4a). AcOOH and Br_2 strongly contributed to the bromination of **1** in the presence of NaBr and absence of H_2O_2 (Entries 1a and 9a), whereas the contribution of NaOBr was weak (Entry 5a). Although the bromination of **1** using NaOBr remained low activity, the bromination using AcOOH was proportional to the equivalent of the oxidants in stoichiometry as well as the bromination using Br_2 (Fig. 5(a)). A reason for the weak brominating power of NaOBr was supposedly due to the decomposition of HOBr as shown by Eqns 10 and 11.[20b,32] The results indicated that HOBr was not an active species on the oxidative bromination following perhydrolase activity.

$$2HOBr \rightleftharpoons HBrO_2 + H^+ + Br^- \quad (10)$$

$$HOBr + HBrO_2 \rightleftharpoons BrO_3^- + 2H^+ + Br^- \quad (11)$$

It is believed that the production of Br_2 from the reaction between AcOOH and Br^- undergoes the production of HOBr (Eqns 1 and 12).[30] In the case that appearance of HOBr is assumed in production of Br_2, the production efficiency of Br_3^- using NaOBr must be higher than that using AcOOH. However, the production efficiency using NaOBr (Entry 5b) was significantly lower than that using AcOOH (Entry 1b) and Br_2 (Entry 9b). Comparison between AcOOH and Br_2 on the brominating activity against phenol was also performed in a 1.0 M AcONa-H_2SO_4 buffer (pH 5.5) containing 0.5 M NaBr and 30 %(v/v) MeOH. The consumption

Table 1. Chemical bromination in the model system

Entry	Oxidants	NaBr	H_2O_2	a (%)	b (%)
1	AcOOH	+	−	89	74
2	AcOOH	+	+	59	1>
3	AcOOH	−	−	1>	0
4	AcOOH	−	+	1>	0
5	NaOBr	+	−	8	5
6	NaOBr	+	+	1>	1>
7	NaOBr	−	−	5	1>
8	NaOBr	−	+	1>	1>
9	Br_2	+	−	78	67
10	Br_2	+	+	5	1>
11	Br_2	−	−	77	1>
12	Br_2	−	+	1	1>

The brominations using oxidants (AcOOH, NaOBr, and Br_2) were performed in the buffer solution with or without additives (NaBr and H_2O_2) for 1 min. Final concentrations of oxidants, NaBr, and H_2O_2, are 45 μM, 0.5 M, and 10 mM, respectively. (a) Consumption efficiency of **1** and (b) production efficiency of Br_3^- were calculated using the corresponding ε value.

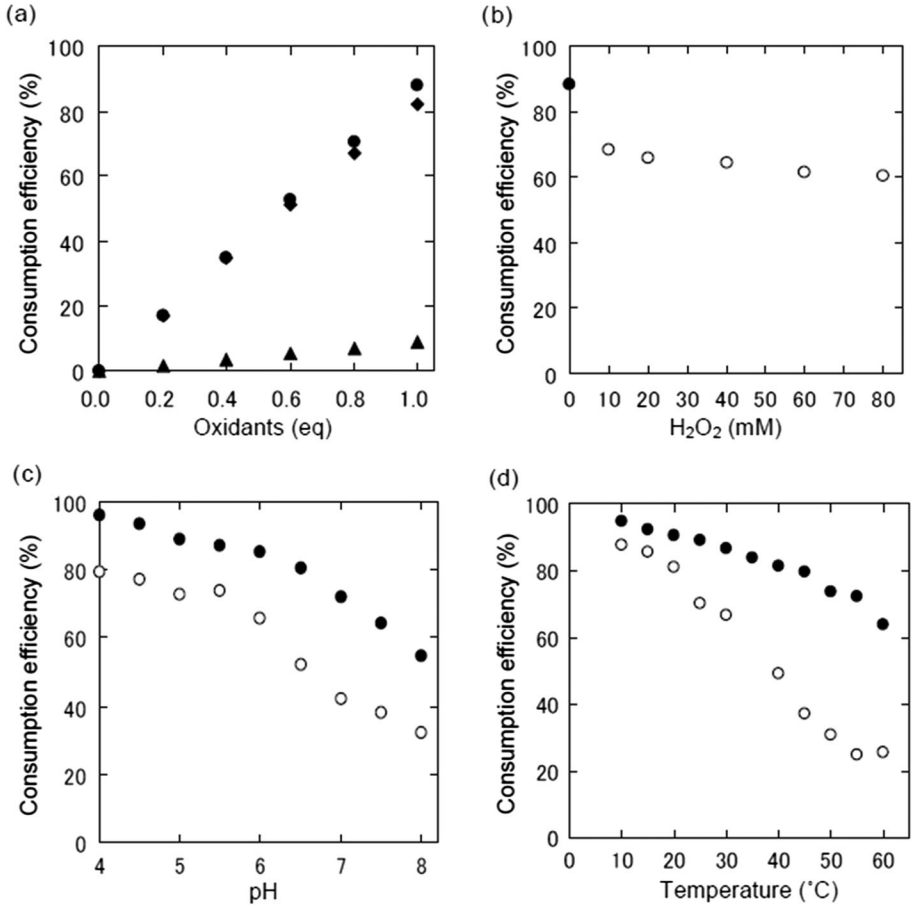

Figure 5. Relation between consumption efficiency of **1** and various factors such as (a) amount of oxidants, (b) concentration of H_2O_2, (c) pH, and (d) temperature. The brominations using AcOOH (circle symbols), Br_2 (diamond symbols), and NaOBr (triangle symbols) in the presence (open symbols) and absence (closed symbols) of 10 mM H_2O_2 were observed without 5 min.

efficiency of phenol using AcOOH, 66%, was higher than that using Br_2, 34%. These facts let us expect that new active species is involved with the oxidative bromination.

$$HOBr + H^+ + Br^- \rightarrow Br_2 + H_2O \quad (12)$$

For the bromination using AcOOH in a solution with NaBr and without H_2O_2, the initial consumption rate of **1**, 0.248 mMs^{-1}, was faster than the net initial production rate of Br_3^-, 0.110 mMs^{-1}. The reaction between Br_2 and Br^- (Eqn 7) will not become a rate-determining step because the reaction is too fast. Although the bromination of **1** using Br_2 was strongly interfered with H_2O_2 (Entry 10a), the bromination using AcOOH maintained the high consumption efficiency (Entry 2a, Fig. 5(b)–(d)). The results indicated that a brominating species produced by the reaction between AcOOH and Br^- possesses some tolerance against H_2O_2. On the other hand, CO_2 produced in its reaction was detected as $CaCO_3$ using $Ca(OH)_2$ solution. The reaction between AcOOH and Br^- in the absence and presence of H_2O_2 gave $CaCO_3$ in 1.27 ± 0.02 and 0.20 ± 0.04 % yields, respectively. It can be interpreted that the cause of the suppression on the production of $CaCO_3$ by H_2O_2 is responsible for the decrease in abundance of active species accompanied with decarboxylation. Acetyl hypobromite (AcOBr), which is well known as an intermediate of the Hunsdiecker reaction (Eqn 13), is decomposed into an alkyl bromide (RBr) and CO_2 via halodecarboxylation by heating.[33] A decrease in the consumption efficiency of **1** with the increasing temperature (Fig. 5(d)) reflected in the instability of the active species. Therefore, AcOBr was suggested to be a significant active species for the oxidative bromination.

$$AcOBr \rightarrow CH_3Br + CO_2 \quad (13)$$

Reaction species

It was suggested that the bromination of **1** using haloperoxidase undergoes the radical chain reaction depending on the MCD free radical (MCD), which was produced by the one-electron oxidation with H_2O_2 (Eqns 14–16).[31] This is the reason that the α-position of **1** is activated by the chlorine substituent. However, the initial rate for the bromination of **1** in the presence of 450 μM hydroxyl-TEMPO as a radical scavenger (0.243 mMs^{-1}) hardly decreased. The bromination of dimedone without a substituent using AcOOH readily proceeded affording 2-dibromodimedone (DBD, **4**) in 62% yield.

$$2MCD + H_2O_2 \rightarrow 2MCD\cdot + H_2O \quad (14)$$

$$MCD\cdot + Br_2 \rightarrow MBMCD + Br\cdot \quad (15)$$

$$Br\cdot + MCD \rightarrow MCD\cdot + H^+ + Br^- \quad (16)$$

On the other hand, the increase in the consumption efficiency of **1** using AcOOH in the presence of NaBr accompanied with the decreasing pH (Fig. 5(c)) implied that the production of active species was driven by the protonation of AcOOH (Eqn 17). The reaction in the presence of 0–2.5 M NaBr showed that the production efficiency of Br_3^- increased with the increasing concentration of Br^- despite a decreasing consumption efficiency of **1** (Fig. 6(a)). The brominating power of Br_3^- is significantly lower

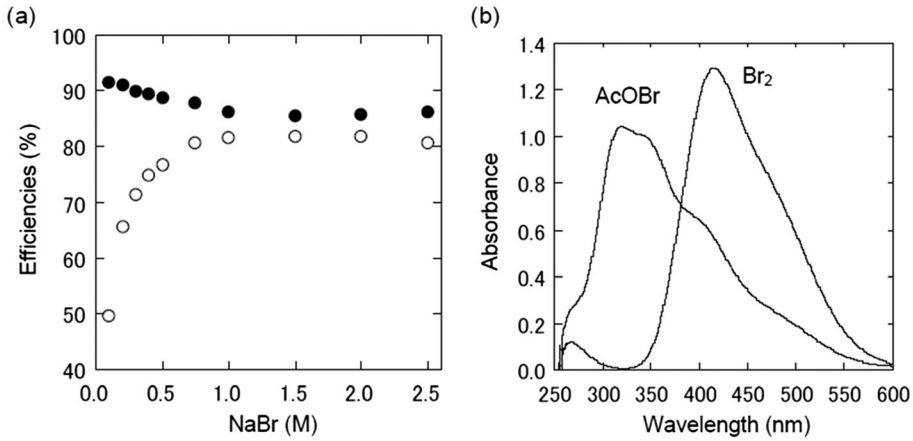

Figure 6. Behavior of active species for bromination via an ionic reaction. (a) Relation between concentration of NaBr and the efficiencies on consumption of **1** (closed circle symbols) and production of Br_3^- (open circle symbols). The experiments were performed in a 1.0 M AcONa-H_2SO_4 buffer (pH 5.5) with 50 mM NaBr at 25 °C for 1 min. (b) UV–Vis spectra of AcOBr and Br_2. Change from AcOBr to Br_2 was observed by the addition of excess of NaBr to 5 M AcOBr ($\varepsilon = 203 \pm 10$ M^{-1} cm^{-1},[28b] $\lambda = 320$ nm) in CCl_4 prepared by mixing AcOAg and Br_2 ($\varepsilon = 198.8$ M^{-1} cm^{-1},[35] $\lambda = 415$ nm)

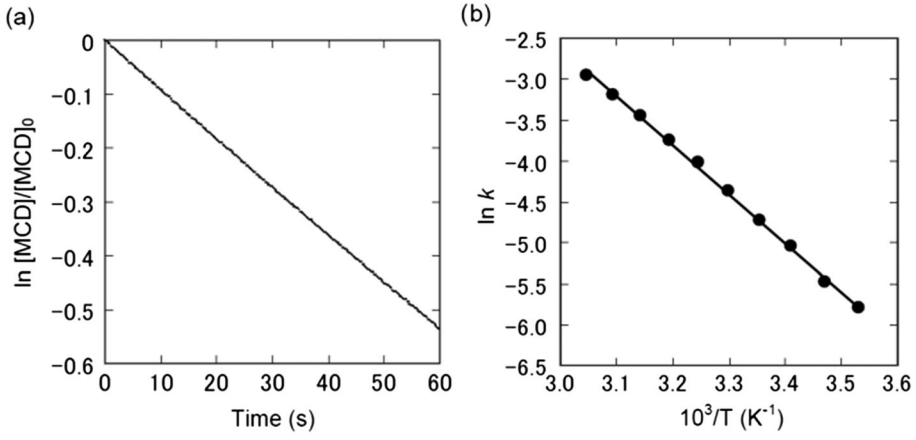

Figure 7. Determination about (a) $k_{25\,°C}$ and (b) E_a on the production of the active species from the reaction between AcOOH and Br^-. The reaction was performed in 1.0 M AcONa-H_2SO_4 buffer (pH 5.5) with 50 mM NaBr for 1 min

than that of Br_2.[34] The conversion from AcOBr to Br_2 was confirmed by mixing AcOBr and NaBr in CCl_4 (Fig. 6(b)). These facts indicated that the active species produced from the reaction between AcOOH and Br^- was the brominium cation (Br^+) donor and implied that the non-enzymatic step for the bromination using perhydrolase is an ionic reaction.

$$AcOOH + H^+ + Br^- \rightarrow AcOBr + H_2O \quad (17)$$

Rate-determining step

It was presumed that the oxidative bromination step is divided into the production of AcOBr (Eqn 17) and consumption of AcOBr and **1** (Eqn 18). A linear relationship between ln $[MCD]/[MCD]_0$ and time showed a pseudo-first-order reaction with a kinetic constant at 25 °C ($k_{25°C} = 8.707 \times 10^{-3} s^{-1}$) for the consumption of **1** (Fig. 7(a)). The initial rate of the bromination depended on pH (Fig. 8(a)), temperature (Fig. 8(b)), and concentrations of AcOOH (Fig. 8(c)) and NaBr (Fig. 8(d)), however, was not dependent on the concentration of **1** (Fig. 8(e)). These results indicating that the consumption step is faster than the production step reflected in high activity of the active species on the bromination. Indeed, the direct bromination of **1** using AcOBr in CCl_4 is the rapid reaction beyond our measurement limit. Thus, the production step is the rate-determining step represented as Eqn 19, which is agreed with the steady-state approximation for the bromination, whereas the bromination was inactivated by H_2O_2 (Fig. 8(f)).

$$MCD + AcOBr \rightarrow MBMCD + AcOH \quad (18)$$

$$v = -d[MCD]/dt = d[MBMCD]/dt = d[AcOBr]/dt \quad (19)$$

Production mechanism of AcOBr

The production mechanism of AcOBr was discussed by referring to the three-reaction mechanisms of production, hydrolysis, and spontaneous decomposition for AcOOH. It is known that the three reactions undergo the protonation of a carbonyl group for the formation of an active intermediate in the first step (Fig. 9(A)).[36] The protonated AcOOH ($AcOOH_2^+$) with low energy structure is affirmed by a proton affinity value of AcOOH (−775.9 kJmol^{-1}).[37] These facts implied that $AcOOH_2^+$ intermediate is readily produced in an aqueous weak acidic solution; that is to say, the protonation for the first step in the production mechanism is not the decisive rate-determining step. On the other hand, it was proved that the second step on the three-reaction mechanisms are the rate-determining steps under strong acidic condition (Fig. 9B(b)–(d)).[36] There is a possibility that the three reactions occur under the mild conditions of the MCD assay for perhydrolase. The activation energy ($E_a = 49.5$ kJmol^{-1}) and pre-exponential factor ($A = 4.14 \times 10^6$ M^{-2}s^{-1}) for the production of the active species from the reaction between AcOOH and Br^- were obtained by Arrhenius plots (Fig. 7(b)). It is reasonable that the E_a values of the three reactions were higher than the E_a value of the production and that the $k_{25°C}$ values of the three reactions were lower than the $k_{25°C}$ value of the production (Table 2). The initial rate of the bromination was directly

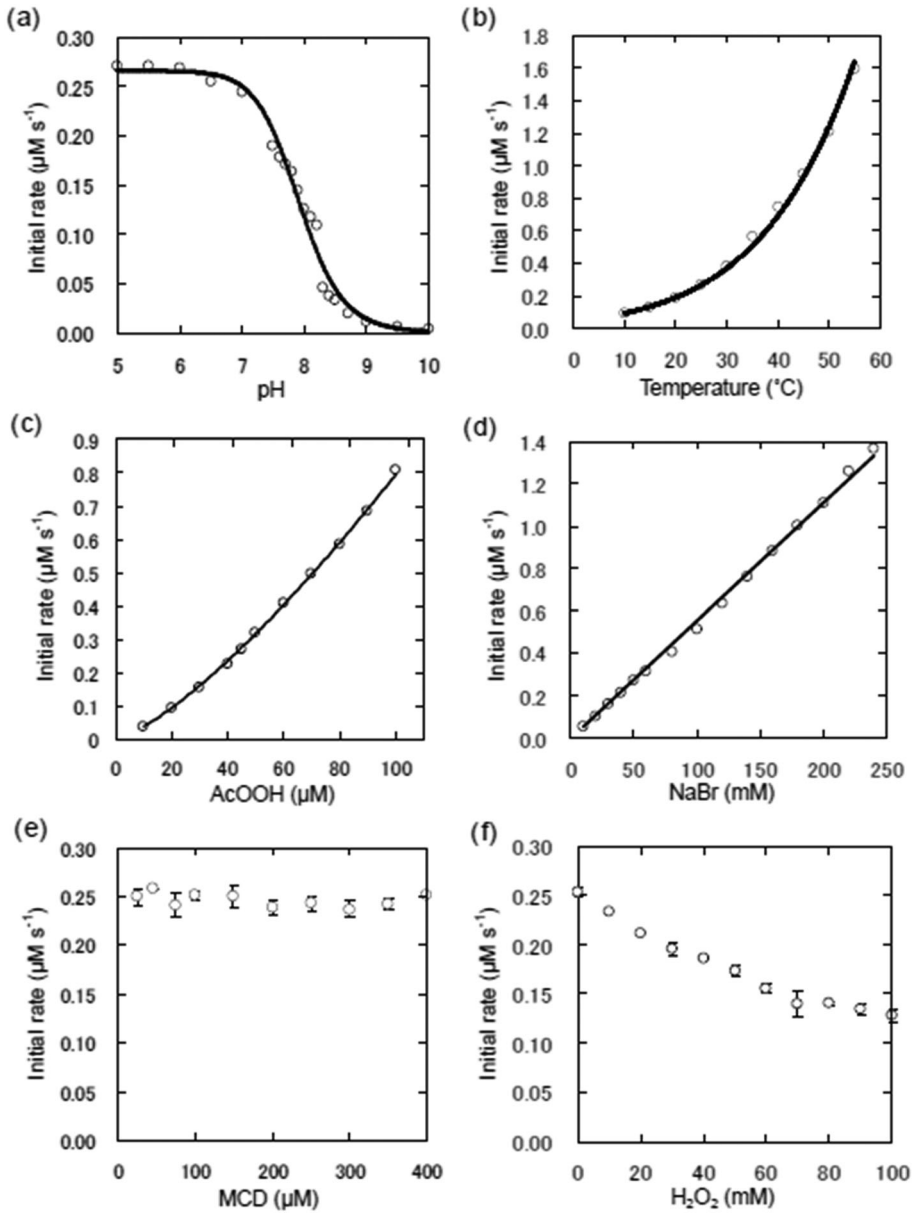

Figure 8. Initial rate for the oxidative bromination of **1** using AcOOH in the presence of Br^-. Dependences on (a) pH, (b) temperature, and concentrations of (c) AcOOH, (d) NaBr, (e) **1**, and (f) H_2O_2 were observed within 10 min. Standard condition for the experiments is 45 μM of **1**, 45 μM AcOOH, and 50 mM NaBr in the absence of H_2O_2 at pH 5.5 and 25 °C

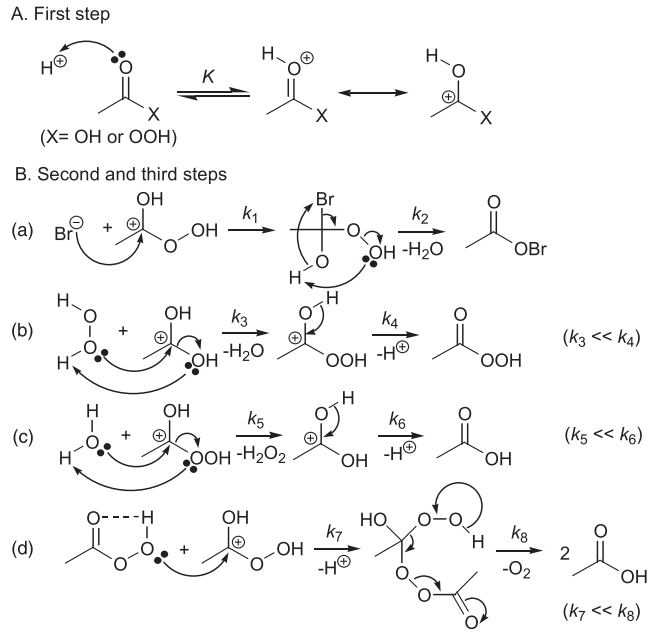

Figure 9. The mechanism on (A) formation of carbocation intermediate by protonation at first step, (B) intermolecular reaction between carbocation intermediate and counterparts at second step, and intramolecular reaction at third step. The counterparts about (a) production of AcOBr, (b) production of AcOOH, (c) hydrolysis of AcOOH, and (d) decomposition of AcOOH are Br^-, H_2O, H_2O_2, and AcOOH, respectively

Table 2. The values of E_a and $k_{25°C}$ for the four reactions

Entry	Reaction	E_a (kJ mol^{-1})	$k_{25°C}$ (s^{-1})
1	Production of AcOBr	49.5	8.707×10^{-3}
2[36c]	Production of AcOOH	57.8	1.393×10^{-6}
3[36c]	Hydrolysis of AcOOH	60.4	4.878×10^{-6}
4[36d]	Decomposition of AcOOH	88.4	9.797×10^{-6}

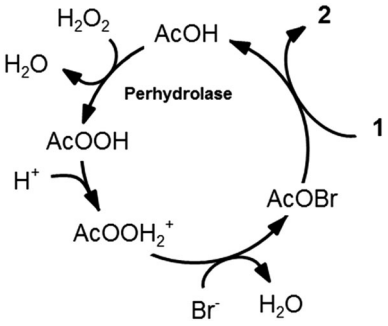

Figure 10. The catalytic cycle of an oxidative bromination using perhydrolase

proportional to the concentration of NaBr (Fig. 8(d)). Therefore, it can be interpreted that the reaction between $AcOOH_2^+$ and Br^- during the production of AcOBr is rate-determining step (Fig. 9B(a)).

CONCLUSIONS

The investigation about the active species on the oxidative bromination following perhydrolase activity is applied the chemical model system using oxidant, because its oxidative bromination undergoes non-enzymatic mechanism. In the chemical bromination in the presence of Br^-, the activity of HOBr and Br_2 was lower than that of AcOOH instead of the enzyme. HOBr and Br_2 have been described as the active species in the oxidative bromination using the enzyme was excluded by the comparison of the oxidants on the activity, then AcOBr was suggested as a new active species on the bromination by detection of the decarboxylation in the reaction between AcOOH and Br^- and the strong brominating power with some tolerance against H_2O_2. Kinetic analysis of the bromination with a pseudo-first-order reaction in MCD assay clarified the ionic mechanism and rate-determining step during the production of the active species. The production of the active species, possessing a strong dependent on the concentration of NaBr, was superior to the three reactions as the production, hydrolysis, and spontaneous decomposition of AcOOH in terms of $k_{25°C}$ and E_a. The rate-determining step about the production mechanism of AcOBr was interpreted as the reaction between $AcOOH_2^+$ and Br^-. Therefore, the oxidative bromination following perhydrolase activity was summarized with catalytic cycle (Fig. 10).

MCD assay that depends on the halogenation is necessary to characterization of perhydrolase. However, the production mechanism of the active species indicated that the evaluation of perhydrolase activity is influenced on pH dependence of the non-enzymatic bromination. In principle, net perhydrolase activity at basic condition is not detected by this method. Indeed, perhydrolase with the activity at basic condition has not been observed. Thus, the assay for perhydrolase should be improved in the future. On the other hand, production of AcOBr possessing strong brominating power in aqueous solution under mild condition is very interesting. AcOBr, which is vulnerable to the proton source, is generally synthesized in a toxic aprotic solvent such as CCl_4. The oxidative bromination using AcOOH in aqueous solution with halide salt is quick and safe. Employment of perhydrolase with low concentration of H_2O_2 in the bromination enhances its safety. The production mechanism of the active species under mild condition will provide a significant key for the application of perhydrolase.

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