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A useful propionate cofactor enhancing activity for organic solvent-tolerant recombinant metal-free bromoperoxidase (perhydrolase) from *Streptomyces aureofaciens*

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ABSTRACT

The oxidative brominating activity of an organic solvent-tolerant recombinant metal-free bromoperoxidase BPO-A1 with C-terminal His-tag (rBPO-A1), from *Streptomyces aureofaciens* found to depend on various additives. These included carboxylic acids, used as cofactors and alcohols, used as water-miscible organic solvents. Enzyme activity was significantly enhanced by using propanoic acid (PA) as a cofactor, which had a high Log *D* at pH 5.0 and ethylene glycol with a low Log *P*. The positional specificity of oxidative hydroxybromination for olefins, using rBPO-A1 and PA in the presence of methanol, was higher compared to a non-enzymatic reaction using peracetic acid. The oxidative bromination step, occurring after enzymatic peroxidation step, is suggested to be pseudoenzymatic.

1. Introduction

Bromination is an important chemical reaction for forming carbon-carbon bonds and substitution of the functional groups. Reagents such as molecular bromine (Br_2), *N*-bromosuccinimide (NBS), dibromoisocyanuric acid (DBI), and 2,4,4,6-tetrabromo-2,5-cyclohexadione (TABCO), are widely used for bromination. These sacrificial reagents are required for the reactions in stoichiometric quantities. Although bromination reactions using these reagents contribute to the synthesis of various compounds, the development of the oxidative bromination reactions, using bromide salt and green oxidant such as H_2O_2 and O_2, reduce wasteful byproducts and improve safety [1]. Using haloperoxidase (HPO) including bromoperoxidase (BPO) and chloroperoxidase (CPO) to catalyze the oxidative bromination has attracted attention for developing functional enzyme mimics [2].

Metal-free HPO or perhydrolase is an enzyme used for oxidative bromination. Various bromocompounds [3,4] can be produced by oxidative bromination using metal-free HPO [5–7] in the presence of H_2O_2 and Br^-. Metal-free HPO contains a Ser-His-Asp catalytic triad at the active site, which catalyzes the reaction that converts carboxylic acid to percarboxylic acid in the presence of H_2O_2. During catalytic peroxidation, covalent bonding of the carboxylic acid to the nucleophilic Ser residue in the active site produces an ester; percarboxylic acid is then produced by perhydrolyzation of the ester with H_2O_2 [6–11]. Acetic acid is usually used as a cofactor for this enzymatic catalysis. Other cofactors, such as propanoic acid (PA) and hexanoic acid, are also useful for bromoperoxidase-esterase (BPO-EST) from *Pseudomonas putida* [4], CPO-P from *P. pyrrocinia* [3], CPO-T from *Streptomyces aureofaciens* Tü24 [3], CPO from *Serratia marcescens* [12], and lipases from *Fusarium oxysporum*, *Humicola langinosa*, and *Candida antarctica* [13]. On the other hand, oxidative bromination step has been explained by a non-enzymatic reaction between percarboxylic acid and Br^-. A highly brominating acylhypobromite, generated by the non-enzymatic reaction, may contribute to oxidative bromination [14].

BPO-A1 from *S. aureofaciens* ATCC 10762 possesses thermal stability and highly brominating activity [15]. The *bpo-A1* gene was cloned in the pIJ699 vector and expressed in *S. lividans* TK64 [16]. Recently, a His-tagged recombinant BPO-A1 (rBPO-A1) was over-expressed in *Escherichia coli* Rosetta™2 (DE3) with pET42 encoding the *bpo-A1* gene [17,18]. Its thermal and organic solvent stabilities were improved by random mutagenesis [17,18]. This enzyme has high practical application because of its high activity in the presence of organic solvents. In this paper, we describe the influence of

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external factors, including various carboxylic acids as cofactors and several alcohols as water-miscible organic solvents, on the activity of the organic solvent-tolerant rBPO-A1.

2. Materials and methods

2.1. Preparation of purified rBPO-A1

E. coli Rosetta™2 (DE3) cell transformed with pET42a(+)_BPO-A1-His [14], were cultured in 200 mL of liquid Luria-Bertani (LB) medium (Miller) and supplemented with chloramphenicol (17 mg/L) and kanamycin (30 mg/L) at 37 °C. When culture OD_{660} reached 0.6, *bpo-A1-his* gene expression was induced by addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After incubation at 37 °C for 6 h, the transformed cells were collected by centrifugation and re-suspended in 10 mL of 0.2 M Tris-H_2SO_4 buffer (pH 8.3). The suspended cells were disrupted by ultrasonic disintegration using an ultrasonic disruptor UD-200 (Tomy Seiko Co., Ltd., Tokyo, Japan) at 80 W for 10 min, intermittently, in an ice bath. Cell debris was removed by centrifugation at 27,700 × *g* at 4 °C for 10 min, and the supernatant was obtained.

Crystalline ammonium sulfate was slowly dissolved in the supernatant by stirring at 4 °C for 30 min to be 30% (w/v) final saturation. After the precipitate was removed by centrifugation at 17,700 × *g* at 4 °C for 10 min, additional crystalline ammonium sulfate was also slowly dissolved in the resulting supernatant by stirring at 4 °C for 30 min to be 50% (w/v) final saturation. The resulting precipitate was collected by centrifugation at 17,700 × *g* at 4 °C for 10 min and re-dissolved in 1 mL of 0.2 M Tris-H_2SO_4 buffer (pH 8.3).

The rBPO-A1 solution obtained by ammonium sulfate fractionation, was diluted to 5% (v/v) with a binding buffer (pH 7.1) composing 20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole. It was then applied to a HisTrap column (1 mL, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The bound proteins were eluted from the column using a stepwise imidazole gradient (5 mL fractions using 20, 40, 60, 100, and 300 mM imidazole). Active fractions, which had been eluted using 300 mM imidazole, were collected. After immobilized metal ion adsorption chromatography using a HisTrap column, the collected eluent was desalted with a PD-10 column (GE Healthcare Bio-Sciences AB), pre-equilibrated with 0.02 M Tris-H_2SO_4 buffer (pH 8.3).

2.2. Activity assay

The oxidative brominating activity was assayed by measuring monochlorodimedone (MCD; Fluka Biochemmka, St. Gallen, Switzerland) disappearance at 278 nm at 25 °C [19] using a UV-2500PC UV-VIS recording spectrophotometer equipped with a thermostat (Shimadzu Co., Kyoto, Japan). One unit (U) of oxidative brominating activity was defined as the amount of enzyme that catalyzed the consumption of 1 μmol of MCD in 1 min. The standard reaction mixture for this assay was composed of 0.06 mg/L rBPO-A1, 44 μM MCD, 10.4 mM H_2O_2, 300 mM propanoic acid-NaOH buffer (pH 5.0), 300 mM NaBr, and 10 mM NaN_3. The molar extinction coefficient (ε) of the enolic MCD anion ($\varepsilon = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$) [14] was used for the assay because the stable enol form exists as an enolic anion without the ketoic isomer at reaction pH [20,21]. The net specific activity of the enzyme in this assay including a systematic error was obtained by multiplying 1.12 to the obtained value of the apparent specific activity [20].

The molar concentration of the purchased 30% H_2O_2 solution (Wako Pure Chemical, Industries, Ltd., Osaka, Japan) was determined by cerimetric titration using 0.1 M cerium (IV) sulfate solution (Kanto Chemical Co., Inc., Tokyo, Japan) with 1.5% (w/v) ferroin (Wako Pure Chemical, Industries, Ltd.) as an indicator [22]. The protein concentration was determined by the Bradfold method using bovine serum albumin as a standard [23].

2.3. Non-enzymatic and enzymatic brominating activities

Non-enzymatic oxidative bromination using peracetic acid (AcOOH), was performed to identify the products. The reaction mixture was composed of 1.0 M AcOH-NaOH buffer (pH 5.5), 10 mM H_2O_2, 0.5 M NaBr, 30% (v/v) MeOH, 20 mM substrate (cyclohexene, indene, or nerol), and 20 mM AcOOH. After the mixture was stirred at 1 °C for 0.5 h, organic compounds were extracted thrice with 50 mL of dichloromethane. The organic phase was washed twice with 100 mL of water, and the extract was then filtered through a filter paper for dehydration. After removing of the solvent by evaporation below 10 °C, the residue was purified by silica gel chromatography with AcOEt/hexane (2/8 and 3/7). The isolated products were identified by ^1H and ^{13}C NMR analyses.

Enzymatic oxidative bromination using rBPO-A1 was performed to detect of the products. The reaction mixture was composed of 0.3 M propanoic acid-NaOH buffer (pH 5.5), 500 mM NaBr, 21 mM H_2O_2, 10 mM NaN_3, 25% (v/v) MeOH, 1% (v/v) substrates (cyclohexene, indene, or nerol), and 8 U rBPO-A1. After the reaction mixture was incubated at 30 °C for 24 h, organic compounds were extracted thrice with dichloromethane. The organic phase was washed with water, and the extract was filtered through a filter paper for dehydration. After removal of the solvent by evaporation below 10 °C, the crude products were identified by ^1H NMR analysis.

3. Results and discussion

3.1. Effect of cofactors on peroxidating activity

The enzyme activity of metal-free HPO is usually measured as the oxidative brominating activity by using an MCD assay. Oxidative bromination reaction proceeds after enzymatic peroxidation reaction. The resulting value may involve enzymatic and/or non-enzymatic oxidative bromination. However, non-enzymatic oxidative bromination's influence can be disregarded under acidic condition of pH 6.0 or lower because generation of a strongly brominating active species is not the rate-limiting step under acidic conditions [14]. Thus, all investigations of carboxylic acids' influences as cofactors on peroxidating activity of rBPO-A1 were performed at pH 5.0.

The following carboxylic acids could be shown to function as active cofactors during peroxidating activity: acetic acid (AA), propanoic acid (PA), 1-butanoic acid (BA), 1-pentanoic acid (PeA), 1-hexanoic acid (HexA), 1-heptanoic acid (HepA), 1-octanoic acid (OA), 2-methylpropanoic acid (MPA), 2,2-dimethylpropanoic acid (DMA), methoxyacetic acid (MAA), 2-chloropropanoic acid (2CPA), and 3-chloropropanoic acid (3CPA). The following carboxylic acids, including hydroxyacetic acid (HAA), cyanoacetic acid (CyAA), bromoacetic acid (BAA), chloroacetic acid (CAA), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), succinic acid (SA), and malic acid (MA), and amino acids, such as glycine, aspartic acid, glutamic acid, histidine, lysine, and arginine, were inactive as cofactors. In conditions of 100 mM AA at pH 5.0 at 25 °C, the specific activity of rBPO-A1 (18.1 U/mg) was higher than that of BPO-EST from *Pseudomonas putida* (11.7 U/mg) [4]. The specific activities in the presence of 100 mM PA, MPA, and DMA were 248.6, 145.8, and 2.6 U/mg, respectively. Consequently, PA, MPA, and BA enhanced rBPO-A1's activity by 13.7-, 8.0-, and 4.6-fold, respectively, compared to that obtained with AA (Fig. 1A). The relative activity of BPO-EST was increased 1.18-fold upon substitution of AA to PA as the cofactor, whereas activity was decreased by 0.60- and 0.40-fold after

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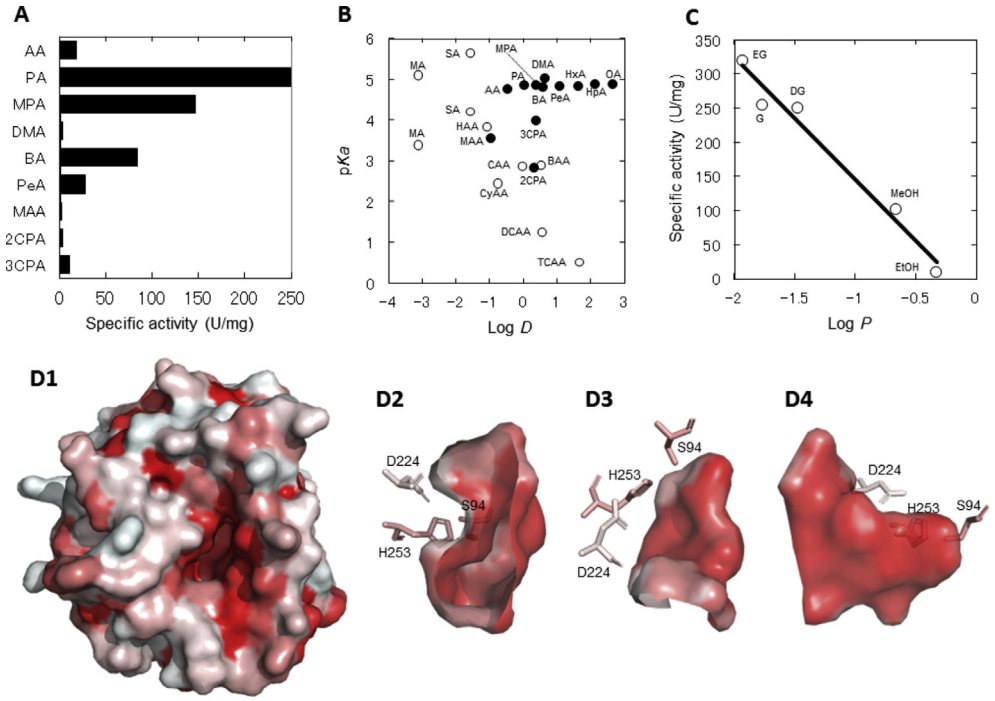

Fig. 1. (A) Reactivity of carboxylic acids as cofactors on peroxidating activity of rBPO-A1 (Supplementary Table 1). Activity was measured using 100 mM carboxylic acid-NaOH buffers (pH 5.0). (B) The relation between Log *D* of the carboxylic acids at pH 5.0 and p*Ka* of that on the activity of rBPO-A1 in the presence of the carboxylic acids as cofactors (Supplementary Table 1). Carboxylic acids, either functioning as cofactors or not, are shown using closed and open circles, respectively. (C) The relation between Log *P* of alcohols and specific activity of rBPO-A1 in the presence of a cofactor (Supplementary Table 2). The Log *P* values were those specified by International Chemical Safety Cards (CDC, Atlanta, GA, USA). The experiments were performed in 30% (v/v) alcohol solutions. (D) Three-dimensional structure of native BPO-A1 from *S. aureofaciens*. The diagrams of BPO-A1 (PDB code 1A8Q) were created using PyMOL Visualizer version 2.0.6 (Schrödinger Inc., NY, USA). Surface and stick representations of overall structure (D1) and cavity with Ser-His-Asp catalytic triad in the active site (D2-4) are shown. D3 and D4 in this figure are turned 90 clockwise around the horizontal axis and 90 counterclockwise around the vertical axis, respectively, relative to D2. Hydrophobicity of the residues, 1.38−0.64, 0.62~ −0.78, and −0.85~ −2.53, are colored red, pink, and white, respectively. The amino acid residues' hydrophobicity values are described in D. Eisenberg et al. [24]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

substitution from AA to MPA and BA, respectively [4]. Comparing rBPO-A1 and BPO-EST, regarding on the cofactor's influence, indicated rBPO-A1 was superior. The decrease in the activity associated with changes from primary to tertiary fatty chains can be attributed to increased steric hindrance.

We further investigated the relation between peroxidating activity and hydrophobicity. It was showed that carboxylic acids' high p*Ka* and Log *D* values were required for peroxidating activity (Fig. 1B). A weak acidity of cofactors is reasonable because a nucleophilic attack of Ser residue on the carboxylic acid is required to form ester intermediates. High hydrophobicity of cofactor is also justified for the cofactor's easy access to the active center. The rBPO-A1 has activity in the presence of organic solvents such as ethylene glycol (EG), glycerol (G), diethylene glycol (DG), methanol (MeOH), and ethanol (EtOH). The specific activity of the enzyme was weakened by increasing Log *P* of the organic solvent (Fig. 1C). In other words, the cofactor's access to the active center was hindered by interaction with the organic solvent suggesting involvement of a hydrophobic interaction in this process. Indeed, the hydrophobic active center tunnel and cavity are shown in the three-dimensional structure (Fig. 1D). Thus, peroxidating activity is related to hydrophobicity of the cofactor and organic solvent.

3.2. Effect of pH and temperature on peroxidating activity

The optimal pH of rBPO-A1, with PA as the cofactor, was observed to be 5.0 (Fig. 2A). Carboxylic acids such as MPA, BA, PeA, and AA, showed no significant changes to the optimal pH (data not shown). In addition, the non-enzymatic brominating activity with AcOOH in the presence of NaBr, showed no significant changes in pH dependence around pH 5.0−7.0 [14]. Therefore, the optimal pH for enzyme activity is not depended on the cofactor. It was reported that the optimal pH for metal-free HPOs' activities were in the weak acid range of 4.0−5.5 (Supplementary Table 3). It has been proposed that the hydroxyl group of serine in the Asp-His-Ser catalytic triad at the active site is protonated under weak acidic conditions [25,26]. The reason for the sharp decline in rBPO-A1's peroxidating activity at pH 4.0 was probably the inactivation of the Asp-His-Ser triad at the active site by protonation of the serine hydroxyl group. Conversely, the reason for a gradual decline in enzymatic activity around pH 5.0−7.0 was related to p*Ka* of carboxylic acids by the fact that high p*Ka* values of carboxylic acids were required for activity (Fig. 1B). Formation of ester intermediate during peroxidation is hindered by ionization of the cofactor: in other words, the cofactor's reduced access to the active site can also be caused by ionization decreasing hydrophobicity of the carboxylic acid.

Reaction activity in the presence of rBPO-A1 peaked at 60 °C whereas peak in the non-enzymatic activity of H_2O_2 was not observed in temperature range of 10−70 °C (Fig. 2B). The increase in the activity of the enzyme with PA around 10−50 °C (Fig. 2B) is due to the peroxidation step because high activity in the non-enzymatic oxidative bromination step was maintained at low temperature, which suppress the decomposition of the active species generated by the reaction between peracid and Br^- [14]. This indicated that the active species was heat-labile. The significant decrease in activity around 65−70 °C (Fig. 2B) was attributed to decomposition of the active species. Indeed, non-enzymatic oxidative bromination, using AcOOH and NaBr in the absence of

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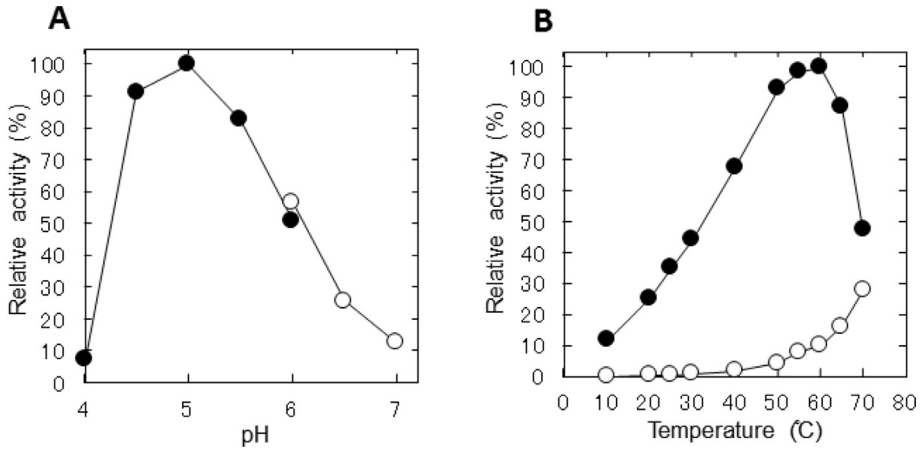

Fig. 2. Effects of pH (A) and temperature (B) on the relative activity of rBPO-A1 in the presence of PA as cofactor. (A) Activities were measured in 0.1 M citric acid-NaOH buffer at pH 4.0–6.0 (closed symbols) and 0.1 M sodium phosphate buffer at pH 6.0–7.0 (open symbols) in the presence of 10 mM PA at 25 °C. (B) The activities in the presence (closed symbols) and absence (open symbols) of the enzyme were measured in 0.1 M citric acid-NaOH buffer (pH 5.0) in the presence of 100 mM propanoic acid-NaOH buffer (pH 5.0) at 25 °C.

enzyme, was not observed around 65–70 °C (data not shown). The native BPO-A1 possesses high stability up to 80 °C [15]. These facts indicated that rBPO-A1 possesses high peroxidating activity at high temperatures.

3.3. Effect of additives on peroxidating and brominating activities

We further investigated the effects of varying concentrations of organic solvents, PA, H_2O_2, NaBr, and MCD on rBPO-A1 activity. There was organic solvent concentration-dependent activity; the activity increased with increased concentration of EG whereas the activity decreased with increased concentration of MeOH (Fig. 3A). The enhanced enzyme activity upon the addition of EG may have been caused by decrease in hydrophilic interactions between PA and water without an accompanying increase in hydrophobic interaction between PA and EG. The non-enzymatic oxidative bromination of MCD with AcOOH was not affected by MeOH and EG (data not shown). These results indicated that organic solvent

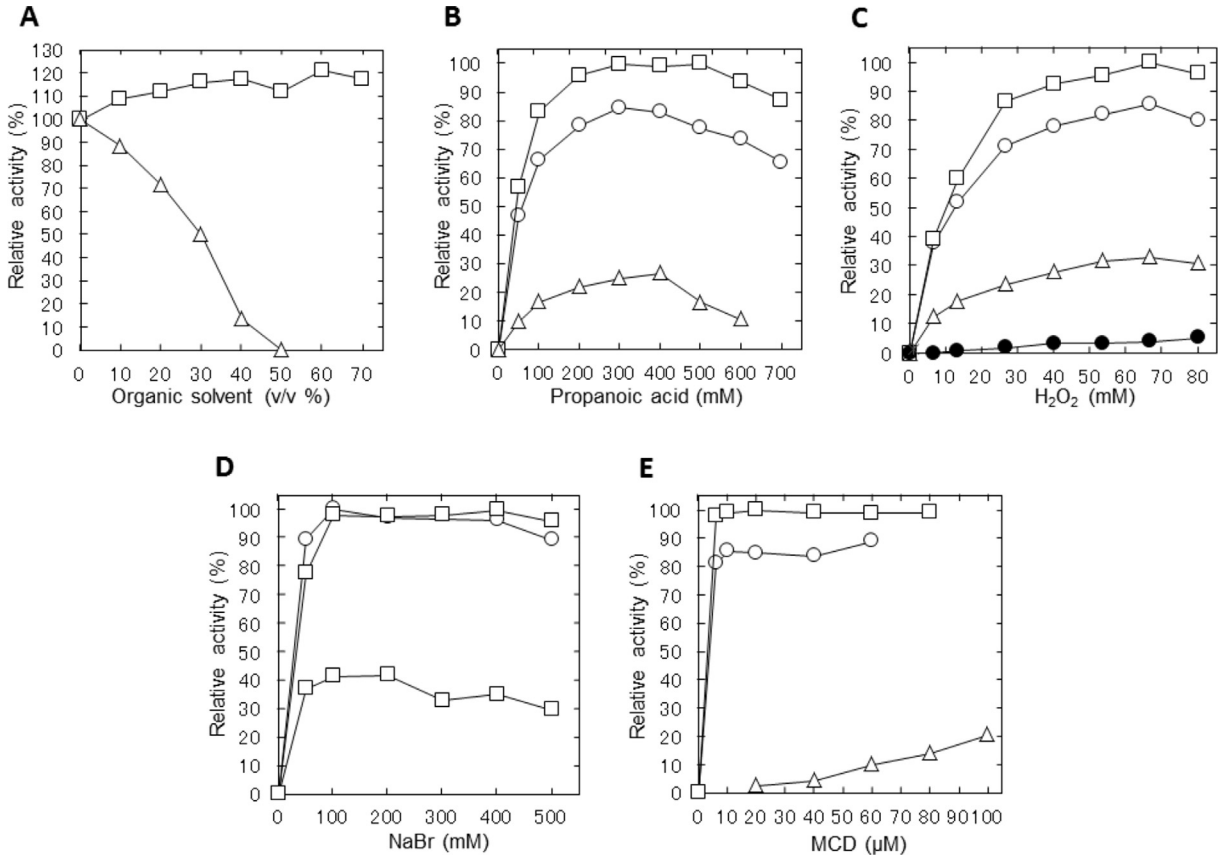

Fig. 3. Effects of concentration of organic solvent (A), PA (B), H_2O_2 (C), NaBr (D), and MCD (E) on the oxidative brominating activity of rBPO-A1 using PA as cofactor. The activities in the presence (open symbols) and absence (closed symbols) of enzyme were measured at pH 5.0 at 25 °C. Measurements were performed in the absence of organic solvent (circle symbols) and presence of EG (square symbols) and MeOH (triangle symbols). (A–E) For investigations in the presence of organic solvent, 30% (v/v) MeOH and 40% (v/v) EG were used.

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indirectly contributed to the peroxidation step.

The concentration dependence of PA and H_2O_2 is reflected in the peroxidation step in principle. Maximum activity peaks were obtained at varying concentrations of PA solutions (Fig. 3B). The optimal PA concentration for activity, in the absence of organic solvents, was 300 mM. Enzyme activities in the absence and presence of MeOH or EG also were decreased by excess addition of PA because it led to denaturation of the enzyme and inhibition of the cofactor is caused by increasing solution hydrophobicity. Thus, excessive use of PA is not recommended in this enzyme reaction system. The enzyme was also denatured by excess amounts of H_2O_2. The optimal concentration of H_2O_2, both with and without organic solvent, was 70 mM (Fig. 3C). The net enzymatic activities were calculated by removing the non-enzymatic activity of H_2O_2 from the apparent activity.

On the other hand, the concentration dependence of NaBr and MCD is reflected in the oxidative bromination step in principle. The NaBr concentration dependence in the absence and presence of MeOH or EG did not give a limiting factor at 100 mM and over (Fig. 3D). Although the MCD concentration dependence in the absence and presence of EG also did not give a limiting factor at 10 mM and over, MeOH significantly affected the MCD concentration dependence as a limiting factor (Fig. 3E) due to the hydrophobic interaction between MeOH and MCD. These implied that the critical factors for activity in the reaction system were the cofactor and organic solvents, rather than H_2O_2, NaBr, and substrate. The organic solvent influences the cofactor's access to the active center by hydrophobic interaction with the cofactor. Therefore, PA binding to the nucleophilic Ser residue in the active site during the peroxidation step, made stronger contributions to activity than H_2O_2 undergoing perhydrolyzation at the peroxidation step and NaBr and MCD at the oxidative bromination step.

3.4. Substrate- and positional-specificities of rBPO-A1using PA in the presence of MeOH

The oxidative bromination step is thought to proceed non-enzymatically. This is mainly because substrate structures such as MCD, phenol red, phenols, and olefins, which are HPO substrate, are considerably different from each other. Evidence for the binding of metal-free HPOs and these substrates at the oxidative bromination step are unclear, whereas the substrate binding site of organic acids at the peroxidation step have been revealed by X-ray crystal structure analysis of the CPO-P/propionate complex [1A8S] and CPO-T/benzoate complex [1A8U] [11]. Metal-free CPO can catalyze chlorination and bromination whereas metal-free BPO can only catalyze bromination, despite both HPOs produce same peracid as an intermediate for the corresponding oxidative halogenation. This fact includes the possibility that the bromination step is enzymatic. Here, we investigated substrate- and positional-specificities of rBPO-A1 for hydrophobic substrates. The enzyme using PA was active for 2-nitrophenol and inactive for 3-nitrophenol and 4-nitrophenol despite non-enzymatic bromination of the three nitrophenols using AcOOH instead of rBPO-A1 proceeded. The enzyme using PA was active for aliphatic olefins of cyclohexene and nerol as well as olefins with aliphatic moiety such as indene. It was inactive for aromatic olefins such as styrene and cinnamic acid. The positional-specificity for cyclohexene, styrene, and nerol was examined by comparing the non-enzymatic method, using AcOOH, and the enzymatic method, using rBPO-A1. Both methods resulted in corresponding bromohydrins (**1**, **5**, **9**) (Fig. 4). However, the proportion of byproducts to the main product in the non-enzymatic method was higher compared to that from the enzymatic method. The byproducts namely, 1,2-dibromocompounds (**2**, **6**, **10**), 1-bromo-2-methoxycompounds (**3**, **7**, **11**), and 1-bromo-2-acethoxycompounds (**4**, **8**, **12**) undergo a nucleophilic addition reaction for bromonium ion intermediate by bromide ion, MeOH, and AA, respectively. Byproduct formation was suppressed in the enzymatic method. The enzyme with PA was inactive for oxidative chlorination, which implied that enzymatic chlorination was not achieved by enhancing the peroxidating activity. Indeed, no correlation was observed between the specific activity of metal-free HPOs and applicability of the halogen ion species (Supplementary Table 3). These facts indicated that the oxidative bromination step proceeds via an enzymatic factor.

On the other hand, rBPO-A1 was deactivated under substrate

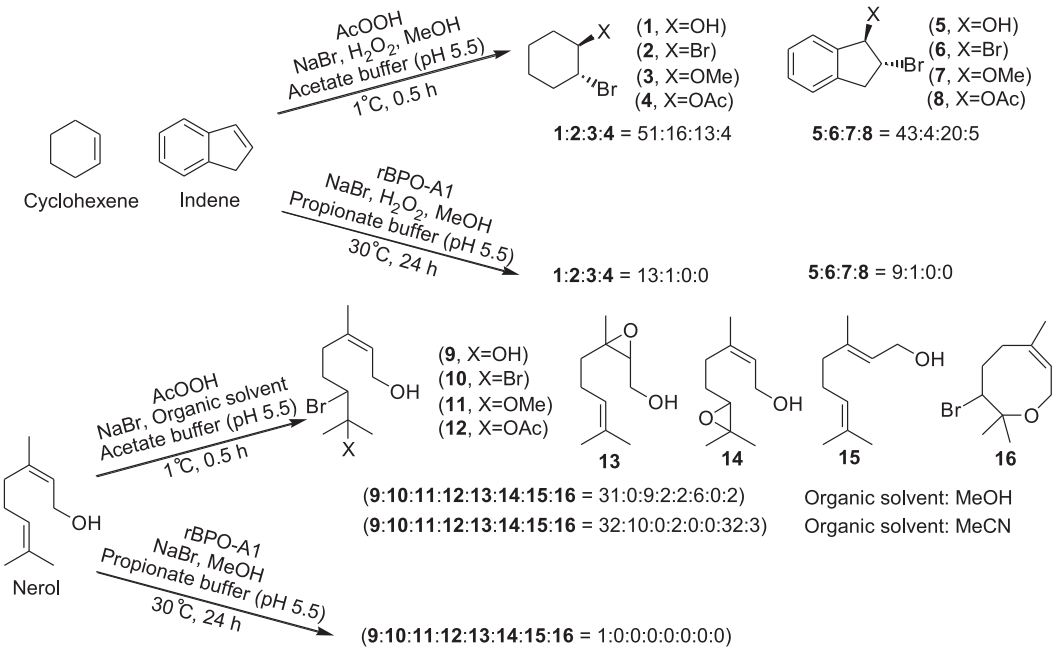

Fig. 4. Non-enzymatic oxidative bromination using AcOOH and enzymatic oxidative bromination using rBPO-A1 for cyclohexene, indene, and nerol.

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deficient conditions. Bromination of the added substrate did not occur after incubating the enzyme in the reaction mixture without substrate. Therefore, generated brominating active species had a harmful effect on the enzyme. This deactivation was probably caused by random self-bromination, which might have led forming NBr bonds from the NH bond at several peptide bonds in the enzyme. The brominating reagent generating Br^+ readily converts the NH bond to an NBr bond. A potential halide ion-binding site at R58 and W205 in the M99T mutant of BPO-A2 [1BRT] was accidentally detected as an oxyanion hole by the three-dimensional structural analysis [11]. The Arg residue in BPO-A1 at the corresponding position (R54) is highly conserved in other metal-free HPOs (Supplementary Fig. 1). It seems reasonable to suppose that guanidinium group in R54 is a temporary stabilizer of the Br^+ and a regioselective activator by formation of the NBr bond.

Although the existence of a substrate binding site in rBPO-A1 remains unknown, our study revealed that rBPO-A1 possesses enzymatic factor for the substrate- and positional-specificities in the oxidative hydroxybromination following peroxidation. Furthermore, rBPO-A1 includes a hydrophobic tunnel, a cavity, and a potential halide ion-binding site around the active center of the enzyme. These attributes can explain that oxidative bromination occurs near the active center. In conclusion, oxidative bromination can be considered as a pseudoenzymatic reaction.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.06.036.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.06.036.

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