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Regioselective preparation of (*R*)-2-(4-hydroxyphenoxy)propionic acid with a fungal peroxygenase

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ABSTRACT

The extracellular heme-thiolate peroxygenase of *Agrocybe aegerita* catalyzed the H_2O_2-dependent hydroxylation of 2-phenoxypropionic acid (POPA) to give the herbicide precursor 2-(4-hydroxyphenoxy)propionic acid (HPOPA). The reaction proceeded regioselectively with an isomeric purity near 98%, and yielded the desired *R*-isomer of HPOPA with an enantiomeric excess of 60%. ^{18}O-labeling experiments showed that the phenolic hydroxyl in HPOPA originated from H_2O_2, which establishes that the reaction is mechanistically a peroxygenation. Our results raise the possibility that fungal peroxygenases may be useful for a variety of organic oxidations.

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Selective hydroxylations of aromatic compounds are among the most challenging reactions in synthetic chemistry and have gained steadily increasing attention during recent years because hydroxylated aromatic precursors are used extensively in the chemical industry.[1] For example, (*R*)-2-(4-hydroxyphenoxy)propionic acid [(*R*)-HPOPA] is an intermediate in the synthesis of enantiomerically pure aryloxyphenoxypropionic acid-type herbicides, in which the crop protection activity normally derives from one enantiomer.[2] Although chemical syntheses of (*R*)-HPOPA from hydroquinone and an (*S*)-2-halopropionic acid are available, problems with the removal of byproducts prevent the cost-effective use of this approach.[3,4] Instead, (*R*)-HPOPA is currently prepared from (*R*)-2-phenoxypropionic [(*R*)-POPA] with whole cells of the ascomycete *Beauveria bassiana*, which produces regioselective oxidases that catalyze this hydroxylation.[5,6] The company BASF currently produces about 1000 tons per year of (*R*)-HPOPA in this way.[7–9] The required feedstock, (*R*)-POPA, is synthesized from (*S*)-2-chloropropionic acid isobutylester and phenol.[8]

A similar but simpler approach is to use purified microbial enzymes to hydroxylate POPA in one step. One possibility would be to use intracellular monooxygenases such as cytochrome P450 (cytP450),[10,11] but current applications of these enzymes are restricted to whole-cell biotransformations because cytP450s are not highly stable and their intracellular location makes them hard to produce in quantity.[12,13] Alternatively, modified hemoproteins such as microperoxidases might be used to catalyze aromatic hydroxylations by a cytP450-like oxygen transfer mechanism, but more research is needed to improve the performance of these catalysts.[14–19] We have taken a new approach by using a recently discovered heme-thiolate enzyme from the basidiomycete *Agrocybe aegerita*. This highly stable, secreted *A. aegerita* peroxidase/peroxygenase (AaP) has already been shown to oxidize a wide range of aromatic substrates and appears to be an unusually versatile oxidoreductase for biotechnological applications.[20,21] Here, we show that AaP hydroxylates POPA to HPOPA with complete regioselectivity and significant enantioselectivity.

The major isoform of AaP was produced in a 5-l stirred-tank bioreactor and purified by several steps of fast protein liquid chromatography (FPLC) as described previously.[20,21] The final enzyme preparations had a specific activity of around 60 U mg^{-1} with 3,4-dimethoxybenzyl alcohol as the substrate and an RZ ($A_{418\ nm}$/$A_{280\ nm}$) value near 1.7. Chemicals including racemic POPA and

Abbreviations: AaP, *Agrocybe aegerita* peroxygenase; cytP450, cytochrome P450; POPA, 2-phenoxypropionic acid; HPOPA, 2-(4-hydroxyphenoxy)propionic acid.

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HPOPA, ^{18}O-labeled H_2O, and ascorbic acid were purchased from Sigma–Aldrich. Separate enantiomers of POPA and HPOPA were purchased from Chemos. ^{18}O-labeled H_2O_2 (2% wt/vol) was obtained from Icon Isotopes.

The reaction mixtures (1 ml) contained various amounts of purified AaP, 50 mM potassium phosphate buffer (pH 3–10), and 0.5–2 mM of the substrate. The reactions were started by the addition of H_2O_2 (1 mM) and mixtures were stirred at room temperature. When indicated, ascorbic acid was added to a final concentration of 4 mM. The reactions were stopped with 0.1 ml of 50% wt/vol trichloroacetic acid. Products were identified and quantified against authentic standards.

Products were analyzed using high performance liquid chromatography (HPLC) on a Hewlett Packard HP Series 1100 instrument equipped with an Agilent 1100 Series DAD G1 diode array detector (DAD) and an Agilent LC/MSD VC electrospray ionization mass spectrometer (ESI-MS).

For routine nonenantioselective separations, the instrument was fitted with a Phenomenex Synergi 4u Fusion RP-80A reversed phase column (4.6 by 150 mm, 4 μm particle size). The column was eluted at 40 °C and 1 ml min^{-1} with aqueous phosphoric acid solution (15 mM, pH 3)/acetonitrile, 95:5, for 5 min, followed by a 10-min linear gradient to 100% acetonitrile.

Chiral separations were performed using an Agilent Zorbax SB-C18 Rapid Resolution Cartridge (2.1 by 30 mm, 3.5 μm particle size) connected in series with an Shodex CDBS-453 column (4.6 by 150 mm, 3 μm particle size). The isocratic mobile phase consisted of 10% acetonitrile and 90% aqueous 0.2 mM sodium chloride that contained 1% vol/vol acetic acid. The columns were operated at 10 °C and 0.5 ml min^{-1} for 35 min.

Liquid chromatography/mass spectroscopic (LC/MS) analyses were performed using a reversed phase Phenomenex Gemini 5u C6 Phenyl 110A column (4.6 by 150 mm, 5 μm particle size). The isocratic mobile phase consisted of 5% vol/vol acetonitrile and 95% aqueous 0.1% vol/vol ammonium formate that had been adjusted to pH 10 beforehand with NaOH. The column was operated at 40 °C and 1 ml min^{-1} for 5 min. Electrospray ionization was performed in the negative ionization mode.

To assess POPA hydroxylation, we treated a racemic mixture of POPA with purified AaP and two equivalents of H_2O_2 in the presence of ascorbic acid. This last ingredient was included to prevent HPOPA polymerization, an undesirable side reaction attributable to the general peroxidase activity of AaP.[22] The results showed that the reaction proceeded rapidly and regioselectively, giving HPOPA as the sole detectable product (Fig. 1, left; calculated isomeric purity was 98%), and that 27% conversion of the POPA occurred under these conditions (Fig. 1, right). Alternatively, the conversion could be increased to 43% by retaining the original stoichiometry, but by adding the H_2O_2 slowly via a syringe pump (Table 1). We found that the minimum molar ratio of ascorbic acid to HPOPA needed to prevent product polymerization was 3.25 to 1. Further work showed that HPOPA production occurred from pH 3 to 10, with a broad maximum between pH 5 and 8 (data not shown). Control reactions without AaP or with heat-inactivated AaP gave no conversion of POPA.

Chiral HPLC analyses after AaP-catalyzed oxidations of racemic POPA showed that both enantiomers were hydroxylated, but that (*R*)-POPA was clearly the preferred substrate. The resulting HPOPA contained a 60% enantiomeric excess (ee) of the *R*-enantiomer (Fig. 2). When the oxidations were performed on either of the pure POPA enantiomers, the corresponding HPOPA enantiomer was obtained as the sole detectable product in each case (data not shown).

The origin of the phenolic oxygen that results from AaP-catalyzed aromatic hydroxylation has until now remained unclear. To clarify this point, we compared natural abundance H_2O_2 with $H_2{}^{18}O_2$ as the oxidant (oxygen donor) in AaP-catalyzed hydroxylations of POPA to HPOPA. The mass spectra of the products (Fig. 3) show that the principal $[M-H]^-$ ion had an *m*/*z* of 181, as expected for the reaction with natural abundance H_2O_2, but shifted to *m*/*z* 183 with almost complete disappearance of the *m*/*z* 181 ion when $H_2{}^{18}O_2$ was used. A similar experiment using $H_2{}^{18}O$ gave no detectable ^{18}O incorporation (data not shown), as expected because phenolic oxygens are not readily exchangeable with water under our reaction conditions.[23] An additional experiment with natural abundance H_2O_2 in a N_2-purged reaction mixture showed that HPOPA production was not inhibited by depletion of O_2 (data not shown), and therefore molecular oxygen did not contribute significantly as an electron acceptor. These results show that the new phenolic oxygen in HPOPA originated from H_2O_2.

Our results show that AaP hydroxylates POPA regioselectively at the *para*-position to give HPOPA and that the reaction has a peroxygenative mechanism, that is, the transferred oxygen originates from peroxide and does not come from dioxygen (O_2) or water (H_2O) as usually occurs in oxygenation reactions. Some intracellular cytP450s perform similar oxidations when given H_2O_2 as an

Table 1
Conversion of racemic mixture of POPA (500 μM) to HPOPA by AaP (1 U ml^{-1}, 1.8 μM) in the presence of ascorbic acid and a final concentration of 1 mM H_2O_2

H_2O_2-addition	*C* (μM) rac. POPA	*C* (μM) rac. HPOPA	Σ (μM)
Direct	369	134	503
Syringe pump (0.5 mM h^{-1})	292	220	512

Reaction products were analyzed as described before.

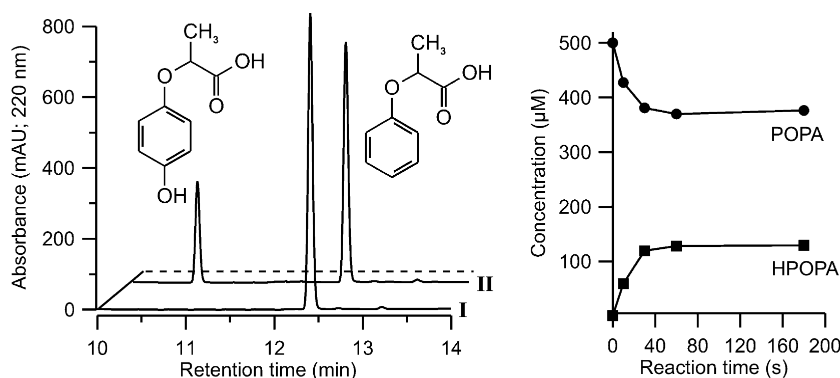

Figure 1. HPLC elution profile (left) of products formed by AaP (1 U ml^{-1}, 0.5 μM) during the conversion of POPA (500 μM) to HPOPA in the presence of ascorbic acid. Control without enzyme (**I**); complete reaction (**II**). The reaction was started by addition of H_2O_2 (1 mM) at pH 7.0. Time course of AaP-catalyzed hydroxylation of POPA (500 μM) to HPOPA (right).

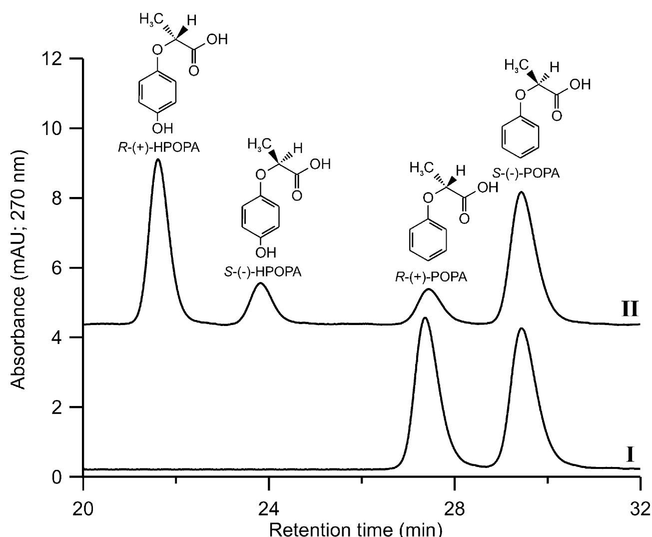

Figure 2. HPLC elution profile of products formed by AaP (1 U ml^{-1}, 0.5 μM) during the conversion of racemic POPA (1 mM) to HPOPA in the presence of ascorbic acid. Control without enzyme (**I**); complete reaction (**II**). The reaction was started by the addition of H_2O_2 (10 × 0.1 mM) at pH 7.0.

artificial oxidant.[24] This so-called 'shunt' pathway is a remarkable side reaction of a few cytP450s, in the course of which the substrate is directly oxidized by H_2O_2 to the hydroperoxo-ferryl state of the heme, requiring neither the stepwise activation of dioxygen nor an electron requirement from NAD(P)H.[1] Since cytP450 and AaP are both heme-thiolate enzymes, the catalytic cycle of AaP probably resembles the cytP450-catalyzed 'peroxidase shunt' pathway. However, AaP appears to be the better choice as a biocatalyst, because it not only performs diverse oxidations,[20] but is easier to produce and more stable than currently available cytP450s.

The data also show that AaP exhibits significant enantioselectivity toward POPA, with the industrially more important *R*-enantiomer reacting more rapidly. This property of AaP could be exploited to improve the yield of *R*-HPOPA from the POPA feedstock currently used, which although enriched in *R*-POPA is not enantiopure.[8,25] Further work is needed to ascertain how AaP recognizes the asymmetric center in POPA, but we surmise that a structural interaction between the enzyme's active site and the carboxylic acid moiety of the substrate may be important. We are currently investigating the possibility that the enantioselectivity of AaP may be useful with other aromatic substrates as well.

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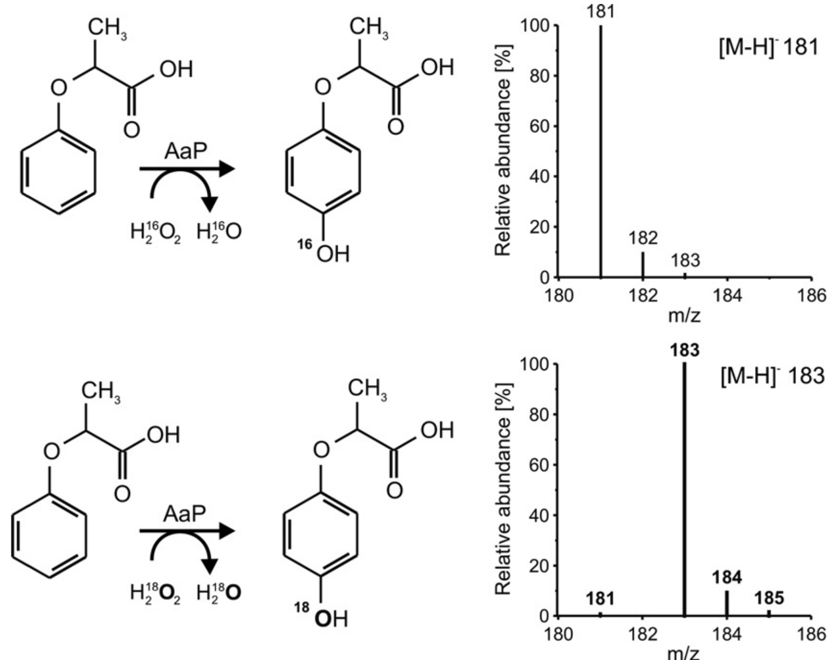

Figure 3. Mass spectra showing molecular ions of HPOPA obtained from the oxidation of POPA with AaP (2 U ml^{-1}, 0.9 μM) in the presence of natural abundance H_2O_2 (top) or $H_2{}^{18}O_2$ (bottom). Ascorbic acid was included in the reactions.

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