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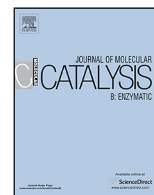

Towards preparative peroxygenase-catalyzed oxyfunctionalization reactions in organic media

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ABSTRACT

The peroxygenase from *Agrocybe aegerita* (*Aae*UPO) has been evaluated for stereoselective oxyfunctionalization chemistry under non-aqueous reaction conditions.

The stereoselective hydroxylation of ethylbenzene to *(R)*-1-phenylethanol was performed in neat substrate as reaction medium together with the immobilized biocatalyst and ^{tert}BuOOH as oxidant.

Stability and activity issues still have to be addressed. Nevertheless, gram-scale production of enantiopure (*R*)-1-phenylethanol was achieved with respectable 90,000 turnovers of the biocatalyst.

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1. Introduction

Peroxygenases catalyze a broad range of synthetically interesting oxyfunctionalization reactions. [1,2] Amongst them, stereospecific hydroxylation of alkyl benzenes is worth mentioning as chemical catalysts with comparable selectivity and activity are still missing [3]. Furthermore, peroxygenases excel over the well-known P450 monooxygenases by their simplicity needing simple hydrogen peroxide or organic hydroperoxides as cosubstrates instead of the nicotinamide cofactor and complicated electron transport chains [4,5].

The choloroperoxidase from *Caldariomyces fumago* (*Cf*UPO) represents the first example of an 'unspecific' peroxygenase (E.C. 1.11.2.1) exhibiting significant P450-like activity (e.g. C—H-bond activation) [6,7]; and major research efforts had been devoted to the exploration of its properties, product spectrum and possible applications. Unfortunately, however, *Cf*UPO's catalytic activity towards non-activated or poorly activated C—H bonds is comparably low impairing its preparative usefulness. In 2004 the group around Hofrichter reported another peroxygenase from the fungus *Agrocybe aegerita* (*Aae*UPO) exhibiting significantly higher activity [8]. Today, more than 300 substrates have been reported for *Aae*UPO that often are converted highly chemo- and enantioselectively [1,2]. Furthermore, recombinant expression systems for *Aae*UPO are available [9] enabling protein engineering [10,11]. Also a crystal structure of *Aae*UPO is known facilitating (semi-)rational protein engineering [12].

Overall, *Aae*UPO is an extremely promising candidate biocatalyst for preparative-scale, selective oxyfunctionalization chemistry.

One major limitation of *Aae*UPO (and of peroxygenase-catalysis in general) however still is its limitation to aqueous reaction conditions, which poses a major challenge to the conversion of poorly water soluble, hydrophobic starting materials such as alkyl benzenes. Both, from an economical and an environmental point of view, higher substrate loadings than traditionally used are highly desirable [13–15]. The use of cosolvents to increase the water solubility of the hydrophobic starting materials or two-liquid-phase approaches using a hydrophobic cosolvent as substrate reservoir and product sink have been proposed [16–19]. Avoiding additional solvents at all and performing the transformations in neat conditions (*i.e.* without any cosolvent whatsoever) would be the most elegant methodology.

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Therefore, in the present study, we set out to evaluate the feasibility of peroxygenase-catalyzed oxyfunctionalization reactions under non-aqueous conditions. As the model enzyme we chose *Aae*UPO, recombinantly expressed in *Pichia pastoris* (r*Aae*UPO) [9], the model reaction was the stereoselective hydroxylation of ethylbenzene to *(R)*-1-phenylethanol.

2. Materials

2.1. Chemicals and enzymes

All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) in the highest purity available and used without further purification except ethylbenzene that was freshly distilled prior use to remove the traces of phenylethanol and acetophenone. The enzyme carrier ReliZyme^{TM} HA403/M, a macroporous PMMA resin with amino-functionalizations, was obtained from Resindion S.r.l., Italy.

2.2. Enzyme production

The recombinant peroxygenase from the basidiomycetous fungus *Agrocybe aegerita* (r*Aae*UPO) was produced *via* heterologous fermentation in *Pichia pastoris* following a previously described procedure [9].

2.3. Concentration of rAaeUPO

The concentration of r*Aae*UPO was determined using the molar extinction coefficient of 115 mM^{-1} cm^{-1} at 420 nm. Absorption spectra in the UV/vis range was recorded in a Biomate5 (Thermo) spectrophotometer (Fig. 1). The Reinheitszahl (Rz value) is the ratio of absorbance due to hemin (A420, Soret region) to absorbance due to protein (A280) and therewith a measure for the protein purity. The Rz-value of the current r*Aae*UPO preparation was 1.6 corresponding well to values reported in the literature [9–11].

2.4. Immobilization of rAaeUPO on Relizyme^{TM} HA403/M resin

To immobilize the peroxygenase the following procedure was used: Relizyme^{TM} HA 403/M resin (1 g) was treated with 50 mL of 0.125% glutaraldehyde solution in water for 2.5 h in a shaking device at 16 °C. The glutaraldehyde solution was then removed by centrifugation, and the resin was washed three times with 0.1 M phosphate buffer at pH 7. The buffer was then removed, and 3 mL of pure r*Aae*UPO (1.35 mg) and 1 mL of 0.1 M phosphate buffer at pH 7 were added to the activated support. The mixture was incubated in a shaker for 24 h at 16 °C. The residual enzymatic activity in the solution was monitored by using the ABTS oxidation assay (*vide infra* for details). The resin was then washed with 50 mM phosphate buffer at pH 7, dried and stored at 4 °C.

2.5. rAaeUPO concentration in the beads

The amount of the r*Aae*UPO bound to the resin was determined by subtracting the amount of enzyme present in the supernatant after immobilization from the total amount of enzyme present originally (prior addition of the resin). The measurement was done spectrophotometrically (Fig. 1) using the molar extinction coefficient of 115 mM^{-1} cm^{-1} at 420 nm. Quite reproducibly, 1.35 mg r*Aae*UPO per gram of resin was bound (i.e. quantitative immobilization).

2.6. Activity determination of rAaeUPO

In order to quantify the specific activity of r*Aae*UPO, we used ABTS as a substrate in aqueous media. Absorbance changes during ABTS oxidation in 0.1 M citrate buffer pH 5 were recorded at 25 °C in a Biomate5 (Thermo) spectrophotometer. The reactions were initiated by the addition of 5 mM H_2O_2. Oxidation of ABTS was followed by the formation of the cation radical (ε_{405} 36.8 mM^{-1} cm^{-1}). Different concentrations of r*Aae*UPO were used to create a calibration line based on the activity towards ABTS (0.5 mM ABTS in 0,1 M citrate buffer and 5 mM H_2O_2).

The activity of the immobilized enzyme was estimated in a reaction mixture of 5 mL containing 7 mg beads, 0.5 mM ABTS and 5 mM H_2O_2 in citrate buffer (pH 5.0) magnetically stirred at room temperature. Aliquots were withdrawn every 30 s and measured at 405 nm. Reactions were performed in duplicates.

2.7. Hydroxylation of ethylbenzene

Reactions were performed at 30 °C and ambient atmosphere in 1 mL ethylbenzene containing different amount of immobilized r*Aae*UPO. Every 30 min ^{tert}BuOOH was added into the reaction mixture and samples were collected. Samples were mixed with ethyl acetate (containing 5 mM 1-octanol as internal standard) and analysed by GC. Reactions were performed in duplicates.

2.8. Stability of immobilized rAaeUPO in ethylbenzene

The immobilized r*Aae*UPO was incubated in ethylbenzene at 30 °C for 24 h and the residual activity was measured following ethylbenzene hydroxylation after the addition of 10 mM ^{tert}BuOOH and 1 h of incubation.

2.9. Stability of immobilized rAaeUPO against peroxide

The immobilized r*Aae*UPO (7 mg) was incubated in phosphate buffer (pH 5.0) containing 10 mM ^{tert}BuOOH and the residual activity towards ABTS was measured as described above.

2.10. Immobilization of PpAOx on Relizyme^{TM} HA403/M resin

The Relizyme^{TM} HA 403/M resin (1 g) was treated with 50 mL of 0.125% glutaraldehyde solution in water for 2.5 h in a shaking device at 18 °C. The glutaraldehyde solution was then removed by centrifugation, and the resin was washed three times with 0.1 M phosphate buffer at pH 7. The buffer was then removed, and 9 mL of *Pp*AOx (15 mg) and 1 mL of 0.1 M phosphate buffer at pH 7 were added to the activated support. The mixture was incubated in a shaker for 24 h at 18 °C: the residual enzymatic activity in the solution was assayed by ABTS oxidation assay (0.1 M phosphate pH 7, 0.033% methanol, 2 mM ABTS and 2.5U HRP) described by Sigma Aldrich. The resin was then washed with 50 mM phosphate buffer at pH 7, dried and stored at 4 °C.

2.11. Concentration of PpAOx on the beads

The amount of the enzyme added for the immobilization and the enzyme retained in the supernatant was quantify with the BSA assay. Based on this assay, 15 mg of protein was bound per mg of beads (*i.e.* quantitative immobilization).

2.12. Activity of PpAOx

In order to quantify the specific activity of the immobilized *Pp*AOx we used the assay recommended by the supplier (Sigma Aldrich): the reaction conditions were: 0.1 m phosphate

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