CHEMISTRY
A EUROPEAN JOURNAL

DOI: 10.1002/chem.201002265

# Ostensible Enzyme Promiscuity: Alkene Cleavage by Peroxidases

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**Abstract:** Enzyme promiscuity is generally accepted as the ability of an enzyme to catalyse alternate chemical reactions besides the 'natural' one. In this paper peroxidases were shown to catalyse the cleavage of a C=C double bond adjacent to an aromatic moiety for selected substrates at the expense of molecular oxygen at an acidic pH. It was clearly shown that the reaction occurs due to the presence of the enzyme; furthermore, the reactivity was clearly linked to the hemin moiety of the peroxidase. Comparison of the transformations catalysed by peroxidase and by hemin chloride revealed that these two reactions proceed equally fast; additional experiments confirmed that the peptide backbone was not obligatory for the reaction and only a single functional group of the enzyme was required, namely in this case the prosthetic group (hemin). Consequently, we propose to define such a promiscuous activity as 'ostensible enzyme promiscuity'. Thus, we call an activity that is catalysed by an enzyme 'ostensible enzyme promiscuity' if the reactivity can be tracked back to a single catalytic site, which on its own can already perform the reaction equally well in the absence of the peptide backbone.

**Keywords:** alkenes · enzyme catalysis · oxygen · oxidation · promiscuity

## Introduction

Enzyme promiscuity, also called cross-reactivity or secondary activity, is the ability of an enzyme to catalyse alternate chemical reactions besides the 'natural one'.[1–4] Several attempts have been made to classify enzyme promiscuity, such as the promiscuity of the wild-type enzymes (accidental promiscuity) or the promiscuity induced by mutations.[2,5] Furthermore, promiscuous activity was subdivided into three groups, namely 1) condition-, 2) substrate- and 3) catalytic-promiscuity.[6,7]

In a recent article entitled 'How enzymes work',[8] it was stated that the tasks of an enzyme are 1) to bring the reacting species together in a geometry that favours the reaction, 2) to distort the substrate and hence to stabilise one substrate conformation better than the others and to permit it to follow a specific reaction mechanism and 3) to create an inner microenvironments in the protein core leading to altered p$K_a$'s of the involved amino acid residues. All these apects contribute to the enzymatic catalysis. Thus, several functional groups of an enzyme cooperate to enable catalysis. Consequently, also a promiscuous enzymatic activity should rely on the presence of several functional groups of the enzyme.

Peroxidases are known to possess various promiscuous activities. Most of the peroxidases[9] have an iron(III) protoporphyrin IX as a prosthetic group (hemin), although flavin[10] or other metals, such as selenium,[11] vanadium[12] and manganese,[13] can constitute the active centre. Peroxidases have three catalytic activities: peroxidase, peroxigenase and oxidase. A peroxidase reaction occurs with electron-deficient substrates, such as phenols or aromatic amines,[14] which are oxidised by one-electron transfer to form radical coupling products. Furthermore, catalase and other related peroxidases carry out the disproportion of $H_2O_2$ and hydroperoxides, respectively.[15,16] Similarly, chloroperoxidase and myeloperoxidase generate halogenating species through the interaction of compound I ($Fe^V$=O porphyrin) with halide ions.[16,17] A peroxigenase reaction involves a direct oxygen transfer from compound I to the substrates, such as thioethers, alkenes and aromatic alkylamines.[16,17] In contrast, in the absence of $H_2O_2$ or peroxides, an oxidase reaction takes place. For instance, horseradish peroxidase performs the hydroxylation of phenols (e.g. L-ty-

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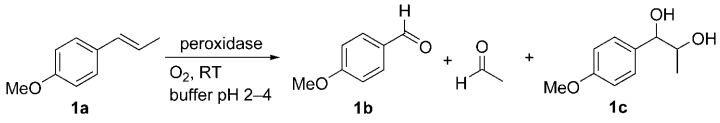
rosine, adrenaline) in the presence of dihydroxyfumaric acid as a hydrogen donor.[18] Aerobic α-oxidation of aldehydes produces carbonylic products, which are postulated to derive from a dioxetane intermediate.[19]

## Results and Discussion

Continuing our previous studies on enzymatic alkene cleavage by using the fungus *Trametes hirsuta*,[20,21] we noticed by serendipity that peroxidases[22] cleave the C=C double bond of *t*-anethole in the presence of molecular oxygen as the main reaction (Scheme 1). It has to be noted, that the previously observed alkene cleavage by *Trametes hirsuta* did not result from a peroxidase.




Scheme 1. Biotransformation of *t*-anethole (**1a**) to *p*-anisaldehyde (**1b**) by employing peroxidases at the expense of molecular oxygen. 1-(4′-Methoxyphenyl)propane-1,2-diol (**1c**) was formed as a byproduct.

Testing 13 commercial peroxidases from various sources employing *t*-anethole (**1a**) as a test substrate, three peroxidases catalysed this cleavage leading to *p*-anisaldehyde (**1b**) at ambient temperature and acidic pH: horseradish peroxidase (HRP), lignin peroxidase (LiP) and a peroxidase from *Coprinus cinereus* (CiP). In contrast to our previous studies on alkene cleavage with *Trametes*,[20] a side product, namely the diol 1-(4′-methoxyphenyl)propane-1,2-diol (**1c**) was formed.

**Optimisation**: For the optimisation of the pH, the three active peroxidases were tested at varied pH values (pH 1 to 8) at a substrate concentration of 6 g $L^{-1}$ (Figure 1). The best conversion was achieved at pH 2 with HRP; at lower pH the activity rapidly vanished. The conversion gradually decreased with increasing pH. CiP and LiP also showed the best conversion at an acidic pH, although the pH optimum was at pH 3 and 4, respectively. All three peroxidases showed negligible activity at neutral pH.

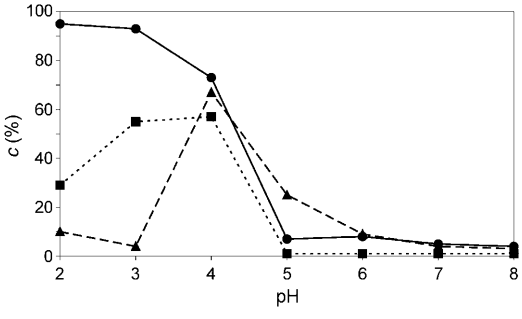



Figure 1. Influence of pH on the alkene cleavage of *t*-anethole (**1a**) with peroxidises (●: HRP; ■: CiP; ▲: LiP). Lines were only drawn to simplify reading of the graphs and do not give the course of data in-between the measured points. Substrate concentration: 6 g $L^{-1}$, peroxidases: 3 mg or 20 μL (depending on the preparation), 20 mM buffer (pH 2, 3) or 50 mM buffer (pH 4, 5, 6, 7, 8), 22 °C, 170 rpm, 24 h.

To ensure an optimal oxygen supply in the aqueous buffer, the reaction was tested at a varied oxygen pressure between one and six bar. For all three peroxidises, the highest conversion was achieved at 2 bar molecular oxygen (Figure 2). Nevertheless, HRP and CiP also tolerated a higher pressure. Both enzymes still showed good conversion at 6 bar. In contrast, LiP already lost its activity at 4 bar oxygen pressure. As a consequence, all the following experiments were performed at 2 bar of dioxygen pressure. These experiments demonstrated clearly that without an 'active' enzyme the background reaction is negligible (see Figure 2, LiP at 4–6 bar $O_2$).

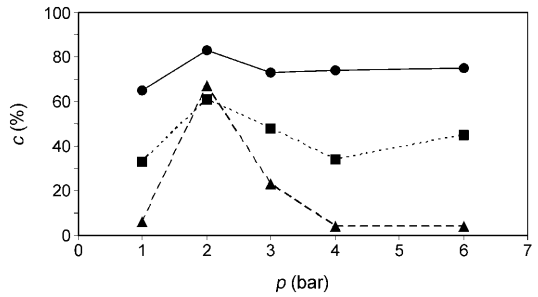



Figure 2. Influence of pressure on the alkene cleavage of *t*-anethole with peroxidises (●: HRP; ■: CiP; ▲: LiP). Experiments were performed at pH 2 (HRP) and pH 4 (CiP and LiP). *t*-Anethole: 6 g $L^{-1}$, peroxidases: 3 mg or 20 μL (depending on the preparation), $NMe_3$/HCOOH buffer (20 mM, pH 2) or AcONa/AcOH buffer (50 mM, pH 4), 22 °C, 170 rpm, 24 h.

Since the lipophilic substrate **1a** shows only limited solubility in aqueous buffer, various cosolvents were tested to improve the availability/solubility of the substrate in aqueous solution. Various cosolvents (e.g. DMSO, DMF, THF, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 1,4-dioxane, acetone and acetonitrile) were tested at 1.8 % v $v^{-1}$. At this low cosolvent concentration all solvents were tolerated; nevertheless, only DMSO led to a slightly enhanced conversion. Consequently, the formation of *p*-anisaldehyde (**1b**) was investigated at a varied concentration of DMSO by employing HRP at pH 2 (HCOOH/$NMe_3$, pH 2, 20 mM) at a total volume of 1 mL. Best conversions were obtained between 5 to 18 % v $v^{-1}$ DMSO (Figure 3). The addition of DMSO improved the conversion from 50 % in the absence of cosolvent to 70 % at 5–18 % DMSO. Further addition of DMSO provoked a slight decrease of conversion up to 30 % v $v^{-1}$ DMSO and then a significant drop, which led to complete enzyme deactivation at 60 % v $v^{-1}$.

Monitoring the course of the reaction at a substrate concentration of 6 g $L^{-1}$ employing HRP (0.2 mol %) showed that 90 % conversion was reached after 24 h (see Figure 4).

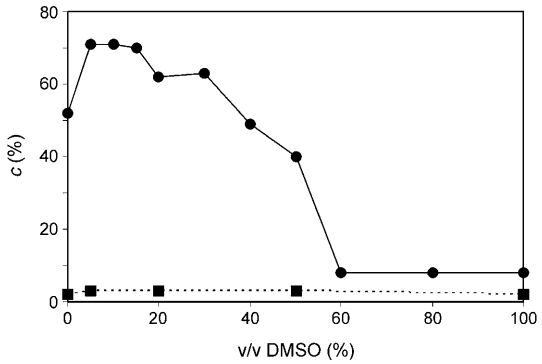



Figure 3. Conversion of *t*-anethol (**1a**) at varied cosolvent concentration (DMSO; ●: HRP; ■: blank). *t*-Anethole: 6 g$L^{-1}$, peroxidases: 3 mg or 20 μL (depending on the preparation), $NMe_3$/HCOOH buffer, 20 mM, pH 2, 22 °C, 170 rpm, 2 bar $O_2$, 24 h.

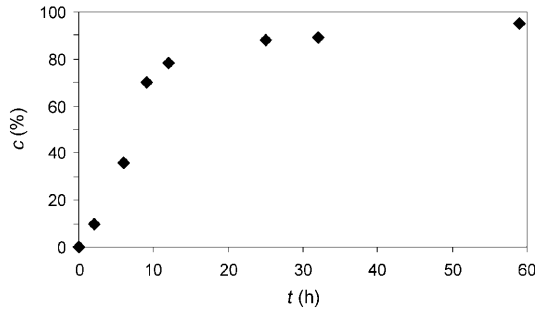
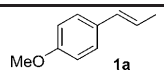
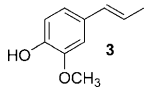



Figure 4. Time course of alkene cleavage of *t*-anethole (**1a**) with HRP. *t*-Anethole: 6 g$L^{-1}$, peroxidases: 3 mg or 20 μL (depending on the preparation), $NMe_3$/HCOOH buffer, 20 mM, pH 2, 22 °C, 170 rpm, 24 h.

**Substrate spectrum**: To understand the structural requirements for alkenes to be accepted as a substrate, a broad range of compounds[23] were tested with different substituents on the phenyl ring for styrene-type substrates or different substitution patterns at the double bond (i.e. mono-, 1,1-di-, 1,2-di- and trisubstituted alkenes). Additionally, also substrates with isolated double bonds and cyclic alkenes were tested. However, it became clear that the peroxidases are highly specific. The substrate spectrum encompassed *t*-anethol (**1a**) (Table 2, entry 1), indene (**2**) and isoeugenol (**3**). HRP turned out to be the most active enzyme and **1a** the substrate transformed fastest (Table 1).

**Structural changes of the enzyme**: Peroxidases are employed in general at pH 4–6.5; however, since here the reactions had to be performed at a rather acidic pH, it can be expected that the observed activity is due to a change in the enzyme structure. To clarify that, CD spectra of the HRP were recorded at pH 2 and 6.5 for comparison, since the crystal structure of HRP[24] has been determined at pH 6.5 (see Table 2). The striking difference in the percentage of α helix passing from the physiological pH to pH 2 indicated that the protein underwent an unfolding process. Therefore,

Table 1. Substrates transformed by peroxidises.[a]

| Entry | Substrate | HRP | | CiP | | LiP | |
|---|---|---|---|---|---|---|---|
| | | *c* [%][b] | sel. [%][c] | *c* [%][b] | sel. [%][c] | *c* [%][b] | sel. [%][c] |
| 1 |  **1a** | 90 | 92 | 68 | 90 | 71 | 94 |
| 2 |  **2** | 12 | >99 | 3 | >99 | 0 | 0 |
| 3 |  **3** | 72 | 77 | 51 | 85 | n.d. | n.d. |

[a] Reaction conditions: 20 mM buffer pH 2, and 2 bar $O_2$. Substrate concentration: 6 g$L^{-1}$, peroxidases: 3 mg or 20 μL (depending on the preparation), $NMe_3$/HCOOH buffer, 20 mM, pH 2, 22 °C, 170 rpm, 24 h. [b] Conversion of substrate determined by GC analysis. [c] Chemoselectivity ratio of formed aldehyde to all formed compounds (aldehyde and side products).

Table 2. CD data for HRP (pH 2 and 6).[a]

| pH | α Helix | β Strand | Loop | Unordered | Total |
|---|---|---|---|---|---|
| 6.5 | 62 | 12 | 2 | 24 | 100 |
| 2.0 | 33 | 32 | 10 | 25 | 100 |

[a] Recorded data analysed by the software DICHROWEB.

it can be concluded that the biocatalytic alkene cleavage activity stems from a rearrangement of the overall secondary and tertiary structure of HRP.

**Proving 'enzymatic' activity**: Although all experiments described above indicate enzymatic activity, such as the fact that the increase of DMSO concentration led to a loss of activity or the defined pH profile, additional experiments should unambiguously show the enzyme activity. If the alkene cleavage occurs in the enzyme core, in which is buried the hemin cofactor, denaturing the enzyme at a high temperature will lead to a collapse of the tertiary structure and, therefore, a loss of the activity. By using denatured CiP and HRP, negligible product formation was detected; therefore, it can be concluded that functionalities located on the outside of the protein are not responsible for the reaction and the transformation occurs in the enzyme core.

In a next step, it should be tested whether unspecific oxygen activation occurs. Enzymes possessing transition metals with two stable and consecutive oxidation states (e.g. $Fe^{II}/Fe^{III}$ as for peroxidases) can transfer one electron to dioxygen and hence generate a superoxide radical anion $O_2^{\bullet-}$,[25] which could attack the alkene outside the enzyme in an unspecific side reaction. The superoxide dismutase (SOD) is known to catalyse efficiently the disproportion of free $O_2^{\bullet-}$. Therefore, alkene cleavage experiments were carried out in the absence and presence of SOD. In all the experiments performed, *t*-anethol was converted at a comparable rate; therefore, $O_2^{\bullet-}$ is not the species responsible for the alkene cleavage and alkene cleavage is subsequently an activity that is directly connected to the peroxidases.

Finally to prove that the reaction occurs indeed at the hemin in the enzyme, the hemin moiety was blocked.[26,27] Blockage of the hemin was performed according to the Ortiz de Montellano protocol with slight modifications,[26] by using 1-aminobenzotriazole as a blocking agent and hydrogen peroxide to generate the iron–oxo species (compounds I and II), which promote the formation of the active inhibitor moiety. The experiments were also performed in the absence of hydrogen peroxide, to see whether the reagent on its own could already lead to deactivation. Nevertheless, the blocking experiment in the absence of $H_2O_2$ provoked only a drop of the conversion to 14%, whereas the hemin moiety was completely inhibited when $H_2O_2$ was added to stimulate the blockage of the prosthetic group.

In summary, the alkene cleavage by peroxidase is unambiguously an enzymatic activity catalysed in the active site at the hemin. The hemin is tightly bound to the peptide backbone.

**Contribution of the peptide backbone**: Since the alkene cleavage was clearly taking place at the hemin in the core of the enzyme, the question was raised as to what extent the peptide backbone improves or contributes to the transformation. For this purpose the reaction was first performed just by using $Fe^{II}$ and $Fe^{III}$ salts in the absence of peroxidase to see whether these salts are able to activate dioxygen under our standard reaction conditions. Hence, solutions of $FeSO_4$ and $FeCl_3$ in aqueous buffer ($HCOOH/NMe_3$, pH 2, 20 mM) were tested at 10 mol% concentration with respect to the substrate. *t*-Anethole (**1a**) was not cleaved at all in presence of $Fe^{III}$, whereas $Fe^{II}$ led to an expected stoichiometric amount of *p*-anisaldehyde (**1b**) (see formation of superoxide radical anion above). In contrast, HRP employed at a concentration between 0.2–0.3 mol% led always to full conversion within 24 h. Obviously the iron salts tested cannot catalyse the described transformation on their own. In a next step, hemin chloride was tested at a low concentration (0.1, 0.01 and 0.001 mM, which corresponds to 0.28, 0.028 and 0.0028 mol%) for its catalytic activity. To solubilise hemin chloride in aqueous solution, DMSO was used as a cosolvent (5% v $v^{-1}$). For comparison, HRP was tested at the same concentrations as the hemin chloride considering that HRP is a single polypeptide chain containing one hemin prosthetic group (44 kDa).[24] Reactions were run simultaneously in the same apparatus by using **1a** as a substrate. The conversion was monitored over 24 h. To our surprise, the peroxidase (HRP) as well as the hemin chloride showed exactly the same time course when applied at comparable concentration (Figure 5). For instance, in both cases 50% of **1a** was converted within 24 h by employing 0.028 mol% of the catalyst. Additionally, in the experiments performed with HRP and with hemin chloride, **1b** was formed with identical chemoselectivity (85%) and in all cases the same byproduct was formed, namely **1c**.

Additional support for the hypothesis that the hemin chloride and the peroxidise-catalysed reaction behave the same was obtained by measuring the conversion versus pressure for hemin chloride. The conversion obtained by employing hemin chloride was identical within error to the one obtained for HRP above 2 bar oxygen (Figure 2), if comparable catalyst concentrations were applied. Measuring the dependence of conversion on DMSO concentration for the hemin reaction revealed a maximum at 20% v/v DMSO and a decrease of conversion at increasing DMSO concentration similar to that observed for HRP (Figure 3).

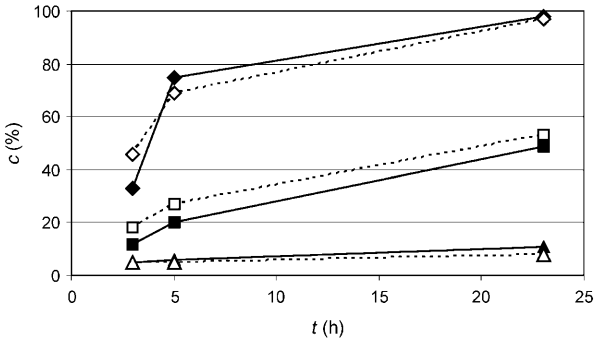



Figure 5. Comparison of the catalytic activity between HRP and hemin chloride (HRP: ----- and open symbols; hemin chloride: —— and full symbols; ◆/◇: 0.1 mM; ■/□: 0.01 mM; ▲/△: 0.001 mM). Reactions were run at pH 2, 22 °C, 2 bars $O_2$. DMSO (5% v $v^{-1}$) was added to the samples with hemin chloride.

Since the hemin chloride might be less hindered than the heme moiety bound into the enzyme, we speculated that the substrate spectrum of hemin chloride could be broader. However, testing all substrates, which were first tested for the peroxidase,[23] we noticed that the substrate spectrum is exactly the same. Thus, only substrates transformed previously by the peroxidase were also transformed by hemin chloride. The transformations led to comparable conversion and identical distribution between cleavage products and side products.

These data clearly showed that the peptide backbone did not contribute to the 'enzymatic' transformation, except for solubilising the hemin. Thus, the peptide chain ensured that the hemin remains in aqueous solution; the same effect could be achieved by addition of DMSO (5% v $v^{-1}$) in case only hemin chloride was used as a catalyst.

CD spectra suggested that the activity at pH 2 is due to a structural change of the protein probably making the hemin site accessible for the transformation. Another option is that the alkene cleavage can only occur at an acidic pH anyway. To test this, the alkene cleavage was performed by employing the hemin chloride as the sole catalyst at a varied pH (Figure 6). The experiment showed that hemin chloride could cleave **1a** over a broad pH range with a continuous decrease of conversion from pH 2 (conv. 92%) to 7 (conv. 64%). The conversion decreased significantly at pH 8 (conv. 35%). Therefore, the observed activity employing HRP at pH 2 is only due to a change of the structure of the peptide backbone.

As a consequence, the peptide backbone was not obligatory for the reaction, thus the enzymatic reaction did actually not take advantage of any feature of an enzyme.[8] Actual-

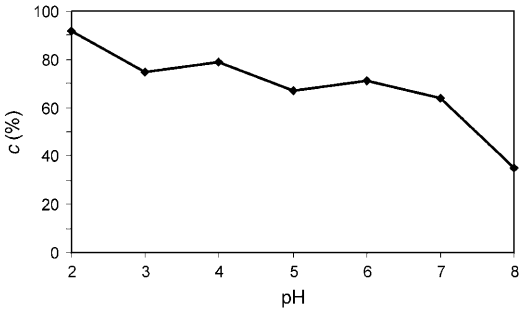



Figure 6. pH profile for the alkene cleavage of *t*-anethole (**1a**) with hemine. *t*-Anethole: 6 g L−1, hemin chloride: 0.28 mmol %, 22 °C, 170 rpm, 5 h.

ly only a single functional group of the enzyme is required, namely in this case the prosthetic group—the hemin. Although we have clearly shown for HRP that it catalyses the alkene cleavage with promiscuous activity, the observed promiscuous activity does not benefit from the peptide backbone. Therefore, we propose to define such promiscuous activity as 'ostensible enzyme promiscuity'. Thus, we call an activity that is catalysed by an enzyme 'ostensible enzyme promiscuity' if the reactivity can be tracked back to a single catalytic site, which on its own can already perform the reaction equally well as in the absence of the peptide backbone.

## Conclusion

It was shown that peroxidases are able to cleave selected C=C double bonds adjacent to activated phenyl moieties at the expense of molecular oxygen at an acidic pH with promiscuous activity. We could unambiguously prove that exclusively the hemin moiety present in the enzyme is responsible for this activity; thus, the peptide backbones of the peroxidases did not have any additional benefit for the reaction. We propose that such an activity should be called 'ostensible enzyme promiscuity'. Such a classification allows one to distinguish between a promiscuous activity due to enzyme features, such as the collaboration of various functional groups[4,8] and promiscuous activity just due to the presence of a single active moiety in the enzyme. Since enzymes consist of an amino acid backbone, various activities described in organocatalysis can be expected to be 'catalysed' by such a peptide backbone too. Therefore, we think that a classification such as 'ostensible enzyme promiscuity' is required, to indicate that a single catalytic moiety on its own can already perform the reaction equally well as in the absence of the peptide backbone.

## Experimental Section

**General**: GC analyses were carried out on a Varian 3800 gas chromatograph equipped with FID and a DB 1701 capillary column (30 m, 0.25 mm, 0.25 μm film, $N_2$). GCMS analyses were carried out on a Hewlett Packard 6890 equipped with FID and a HP Mass Selective Detector 5973 attached with a HP 5 MS capillary column (30 m, 0.25 mm, 0.25 μm film) and on an Agilent 7890A equipped with FID and a Mass Selective Detector 5975C attached with an Agilent 19091S-433 capillary column (30 m, 0.25 mm, 0.25 μm film). Helium was used as a carrier gas. CD spectra were recorded on a Jasco J-715 spectropolarimeter. Reactions under $O_2$ pressure were performed as previously described.[20]

**Substrates and enzymes**: Substrates and reference materials were purchased from Aldrich, Lancaster and Acros with the highest purity available. The peroxidases tested were: 1) Novozyme 51004, 2) Peroxidase from *Coprinus cinereus* (produced by *Aspergillus oryzae*), 3) Novozymes OON00008, 4) Baylase assist, peroxidase from *Coprinus cinereus*, Biesterfeld Chemiehandel GmbH & Co, Germany, 5) P8250 Peroxidase Typ II from horseradish, Sigma–Aldrich, 031K74711, 6) P8125–5KU Peroxidase Typ I from horseradish, Sigma–Aldrich, 031K7465, 7) HRP: POD10814407001, Roche, lot.:93396221, 8) HRP: POD10108090001, Roche, lot.:93350720, 9) HRP: peroxidase SP 502 Batch PPX 3829, Novonordisk A/S, 10) Lactoperoxidase from bovine milk, L-2005, Sigma–Aldrich, lot. 16H38311, 11) Chloroperoxidase from *Caldaromyces fumago*, 25810, Biochemika, 12) Bromoperoxidase from *Corallina officinalis*, B2170, Sigma–Aldrich, 123 K3783 and 13) Lignin peroxidase, Fluka, lot. & fillingcode: 1239384.32506 171.42603. Experiments were performed in general in triplet.

**General procedure for the catalytic alkene cleavage with peroxidases**: The enzyme (3 mg solid preparation or 20 μL liquid preparation) was transferred into the corresponding reaction vessel (riplate LV). Buffer (900 μL) and substrate (6 μL, 0.04 mmol) were added. The samples were placed into an $O_2$-pressure reactor in an upright position. The reactor was flushed with pure $O_2$ and then the pressure was adjusted to 2 bar. After 24 h at 170 rpm and 22 °C, the content of the reaction vessels was transferred to Eppendorf tubes (2 mL) and the aqueous solutions were extracted with AcOEt (2×500 μL). The combined organic phases were dried with $Na_2SO_4$ anhydrous and analysed by GC analysis.

**General procedure for the catalytic alkene cleavage with hemin chloride**: A stock solution of hemin chloride (3.5 mg, 0.0054 mmol) was prepared in DMSO (2.5 mL) to give a homogeneous solution (2 mM). The hemin chloride solution (50 μL) was transferred into the corresponding vessel (riplate LV). Buffer (950 μL) and substrate (6 μL, 0.04 mmol) were added. Reactions were run, worked-up, and analysed according to the procedure with enzymes.

**pH optimization**: Conversion of *t*-anethol (**1a**) versus pH was determined for HRPs, CiP and LiP by using the general procedure for the alkene cleavage by enzymes. The different buffers employed were Bis-Tris (pH 7, 50 mM), Bis-Tris (pH 6, 50 mM), NaOAc/HOAc (pH 5, 50 mM), NaOAc/HOAc (pH 4, 50 mM), $NMe_3$/$HCO_2H$ (pH 3, 20 mM), $NMe_3$/$HCO_2H$ (pH 2, 20 mM) and $HCO_2H$ (pH 1, 20 mM).

**$O_2$ pressure**: Dependence of conversion versus dioxygen pressure was determined for HRP, CiP and LiP by following the general procedure for the alkene cleavage by enzymes. The reactions were run at different pressure (1, 2, 3, 4 and 6 bars).

**Reaction in organic solvents**: Experiments were performed with HRP and CiP, by using the general procedure for the catalytic alkene cleavage by enzymes. Different organic solvents (17 μL, 1.8 % v v$^{-1}$) were added before starting the reaction.

Particularly, stability of HRP was tested with DMSO. Solutions (1 mL) of buffer $NMe_3$/HCOOH (pH 2, 20 mM) with increasing amounts of DMSO were prepared (0–5–10–15–20–30–40–50–60–80–100 % v v$^{-1}$), and then reactions were run by following the general procedure.

**Time study**: Experiments were performed with HRP, by using the general procedure for the biocatalytic alkene cleavage and taking samples following this time schedule (0–1–2–3–4–5–6–8–11–14–22.5–25.5–28.5–31.5–34.5–46.5–50.5 h).

**CD spectra**: The CD spectra of HRP was recorded in buffer $NMe_3$/HCOOH (pH 2, 20 mM) and in buffer Bis-Tris (pH 6.5, 50 mM). Blank CD spectra at pH 2 and 6.5 were also acquired. Spectra were compared and analysed with the software DICHROWEB.

**Denatured enzyme**: Solutions of CiP (20 μL) and HRP (3.5 mg) were prepared in buffer $NMe_3$/HCOOH (1 mL, pH 2, 20 mM) and heated at 99 °C for 10 min in an Eppendorf Thermomixer. Then, the preparations were cooled down to room temperature and *t*-anethole **1a** (6 μL) was added. The biotransformation was carried out by using the general procedure for the enzymatic alkene cleavage.

**Reactions in the presence of superoxide dismutase (SOD)**: Experiments were performed by using CiP and by following the general procedure for the alkene cleavage by enzymes. Two samples were prepared: one containing SOD (1–2 mg) from horseradish and the other from *E. Coli*. The conversion in the presence of SOD was compared with the one obtained in the usual reaction conditions.

**Hemin inhibition**

*Method a*: In the absence of $H_2O_2$, HRP (1 mg) was dissolved in HCOOH/$NMe_3$ buffer (900 μL, pH 2, 20 mM) and 1-aminobenzotriazole (2 mg, 0.015 mmol) was added to give an inhibitor/catalyst molar ratio of 650:1 and the system was shaken for 30 min (170 rpm). *t*-Anethole (6 μL, 0.04 mmol) was poured into the solution and the reaction was run by using the general procedure for the enzymatic alkene cleavage.

*Method b*: In the presence of $H_2O_2$, HRP was dissolved in HCOOH/$NMe_3$ buffer (900 μL, pH 2, 20 mM) and 1-aminobenzotriazole (2 mg, 0.015 mmol) was added to give an inhibitor/catalyst molar ratio of 650:1. Then $H_2O_2$ (30% v v$^{-1}$; 150 μL) was added and the system was shaken for 30 min (170 rpm) leading to complete iron–hemin inhibition. Then, *t*-anethole (6 μL) was added and reaction was run by using the general procedure for the enzymatic alkene cleavage.

**Comparison of the activity between HRP and hemin chloride**: A 0.1 mM stock solution of HRP (5 mg mL$^{-1}$) was prepared in HCOOH/$NMe_3$ buffer (pH 2, 20 mM). Then, 0.01 and 0.001 mM solutions were obtained by subsequent dilutions (1:10, v/v). Hemin chloride (7 mg, 0.01 mmol), which is not soluble in pure aqueous buffer, was dissolved in DMSO (5 mL) and then HCOOH/$NMe_3$ buffer (95 mL, pH 2, 20 mM) was added to give a homogeneous solution (0.1 mM). Then, 0.01 and 0.001 mM solutions were obtained by subsequent dilutions (1:10, v:v).

*t*-Anethole (6 μL, 0.04 mmol) was used as a substrate and the reaction was performed by using the general procedure for the alkene cleavage. The conversion of the substrate was monitored during over time, by following the schedule (3, 5, 23 h) for both HRP and hemin chloride at all catalyst concentrations (0.001, 0.01 and 0.1 mM).

Conversion of *t*-anethol versus pH was determined by using hemin chloride (0.1 mM) as the catalyst in aqueous buffer and DMSO as the cosolvent (5% v v$^{-1}$). The following buffers were used: Tris-HCl (pH 8, 50 mM), Bis-Tris (pH 7, 50 mM), Bis-Tris (pH 6, 50 mM), NaOAc/HOAc (pH 5, 50 mM), NaOAc/HOAc (pH 4, 50 mM), $NMe_3$/HCOOH (pH 3, 20 mM), and $NMe_3$/HCOOH (pH 2, 20 mM). Reactions were run by using the general procedure for the alkene cleavage with hemin chloride.

## Acknowledgements

This study was financed by the Austrian Science Fund (FWF Project P18381) and the European community (RTN Network (R)Evolutionary Catalysis MRTN-CT-2006-035866). Financial support by the European community (THEME KBBE-2009-3-3-02, Project: AmBioCas, grant agreement no.: 245144 and MC-ITN Biotrains, grant agreement no.: 238531) is acknowledged.

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Received: August 6, 2010
Published online: October 27, 2010

  