Monatshefte für Chemie 131, 687–695 (2000)

Monatshefte für Chemie
Chemical Monthly

Enantioselective Sulfoxidations Catalyzed by Horseradish Peroxidase, Manganese Peroxidase, and Myeloperoxidase

Antonin Tuynman[1], **Hans E. Schoemaker**[2], and **Ron Wever**[1,*]

[1] E.C. Slater Institute, BioCentrum, University of Amsterdam, NL-1018 TV Amsterdam, The Netherlands
[2] DSM Research, Bio-Organic Chemistry, NL-6160 MD Geleen, The Netherlands

Summary. Horseradish peroxidase (HRP), myeloperoxidase (MPO), and manganese peroxidase (MnP) have been shown to catalyze the asymmetric sulfoxidation of thioanisole. When H_2O_2 was added stepwise to MPO, a maximal yield of 78% was obtained at *pH* 5 (*ee* 23%), whereas an optimum in the enantiomeric excess (32%, (*R*)-sulfoxide) was found at *pH* 6 (60% yield). For MnP a yield of 18% and a high enantiomeric excess of 91% of the (*S*)-sulfoxide were obtained at *pH* 5 and a yield of 36% and an *ee* of 87% at *pH* 7.0. Optimization of the conversion catalyzed by horseradish peroxidase at *pH* 7.0 by controlled continuous addition of hydrogen peroxide during turnover and monitoring the presence of native enzyme as well as of intermediates I, II, and III led to the formation of the sulfoxide in high yield (100%) and moderate enantioselectivity (60%, (*S*)-sulfoxide).

Keywords. Enantioselective sulfoxidation; Horseradish peroxidase; Myeloperoxidase; Manganese peroxidase.

Introduction

General

The use of enzymes in oxidative conversions is a potentially attractive method for the synthesis of optically active compounds. In this way oxidative processes employing stoichiometric amounts of heavy metal salts can be avoided, whereas the high regio- and/or enantioselectivity of enzymatic processes is a promising feature which may be exploited in various ways. Other oxidative bioconversions involve the use of whole cells, making the whole process rather cumbersome and being therefore often restricted to conversions leading to compounds with a high added value like pharmaceuticals [1]. Peroxidases, however, might be potentially attractive biocatalysts for fine chemicals production since cosubstrate recycling procedures are not necessary and hydrogen peroxide is used as a clean and cheap oxidant.

Various peroxidases have been shown to catalyze the formation of a number of alkyl arylsulfoxides or dialkylsulfoxides in an enantioselective manner [2–5]. The

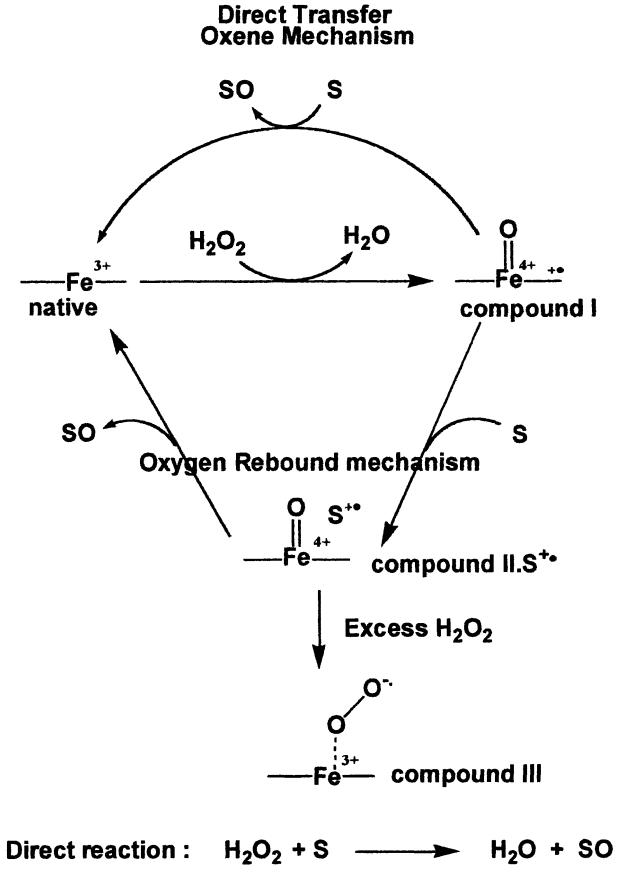
* Corresponding author

compounds are useful intermediates in organic synthesis, both as chiral auxiliaries in catalytic processes and as stoichiometric intermediates. Also epoxidations [6, 7], benzylic [8] and propargylic [9] hydroxylation, and indole oxidations [10] are catalyzed by peroxidases. The potential application in organic synthesis is still hampered by the moderate stability of the peroxidases under turnover conditions, the limited solubility of organic reactants in water, and the oxidative inactivation of the heme. The catalytic performance of peroxidases may be improved by continuous controlled addition of hydrogen peroxide [11, 12], by variation of *pH* [4], and by the addition of some organic solvents [11]. We recently have shown that under carefully choosen conditions also lactoperoxidase (a commercially available enzyme) is capable of enantioselective sulfoxidation of thioanisole, affording the (*R*) isomer in a yield of 85% with an *ee* of 80% [5]. We have also shown that the opposite antipode, the (*S*)-sulfoxide, could be obtained in a yield of 84% with an *ee* of 73% using the commercially available fungal peroxidase from *Coprinus cinereus*. In addition, we have described a spectroscopic method to monitore the active enzyme intermediates during turnover. In this way we were able to optimize the conditions for maximum yield and optimum *e.e.* [5].

The aim of the present research was twofold. First we investigated if we also could use our spectroscopic method to further optimize the sulfoxidation of thioanisole to form the (*S*)-sulfoxide in high yield and high *ee* using horseradish peroxidase. Second, we investigated two other peroxidases (myeloperoxidase and manganese peroxidase) to see if these enzymes could also be used in enantioselective sulfoxidation reactions. Myeloperoxidase was expected to give the (*R*)-sulfoxide due to its structural ressemblance with lactoperoxidase [13]. Moreover, in analogy to the heme CPO it is able to oxidize chloride to hypochlorous acid [14]. The manganese peroxidase is also a fungal enzyme like CiP, and it was expected that with this enzyme – the mnp-gene has recently been cloned and expressed [15] – we would obtain the (*S*)-sulfoxide.

Mechanistic considerations

The general mechanism of peroxidases starts by the addition of H_2O_2 to the enzyme in its resting state, the native Fe^{3+} state [16]. Upon release of a molecule of water, an oxoferryl species ($Fe(IV)=O\ P^{+\cdot}$) called compound I is created containing two oxidative equivalents, one of which is located in the porphyrin or on a protein residue as a radical-cation. The substrate is oxidized to a substrate radical in this step. Addition of an equivalent of a traditional peroxidase substrate, a one-electron donor, will reduce compound I to a $Fe(IV)=O$ species that is one oxidative equivalent above the resting state, the so-called compound II, which does not contain an electron hole an either porphyrin or protein anymore. A second equivalent of substrate will reduce this species back to the native enzyme upon the release of a water molecule and a second substrate radical. The enzyme can also return directly from compound I back to the native enzyme upon reaction with a second molecule of H_2O_2, releasing a molecule of oxygen and a molecule of water. This is called the catalase activity of the enzyme. Another way for the direct reconversion of compound I to the native enzyme proceeds *via* oxygen-transfer to a suitable substrate such as a sulfide or an alkene (Scheme 1). This is called the oxo-



Scheme 1. Oxene and oxygen-rebound mechanisms for the oxygen transfer to sulfides; the formation of compound III under a large excess of H_2O_2 is indicated as well as the direct racemic reaction between H_2O_2 and the sulfide

ferryl or oxene mechanism [17]. Alternatively, oxygen-transfer may take place *via* a two-step mechanism: in this so-called oxygen-rebound mechanism [17–22] a molecule of substrate is first oxidized by compound I to a substrate radical-cation that forms a complex with compound II (compound II · $S^{+\cdot}$ Scheme 1). Subsequently the oxygen of compound II is transferred to this substrate radical-cation, a molecule of oxygenated product is released, and the enzyme returns to its native state. In the case of an oxygen-rebound mechanism the rate of transition from compound I to compound II should depend linearly on the sulfide concentration, whereas in an oxene mechanism the formation of compound II should not be observed. As we have shown, lactoperoxidase and myeloperoxidase operate *via* an oxygen-rebound mechanism [5].

At high H_2O_2 concentrations a third intermediate of the enzyme, compound III, can be formed (Scheme 1). In most reactions this intermediate is believed to be a catalytic inactive, mostly a dead-end species and leading to inactivated enzyme for most peroxidases [23]. However, compound III is not necessarily irreversible formed and may return to the native state [23]. Under operational conditions,

involving discontinuous addition of hydrogen peroxide [3], compound III may well be transiently formed. It is of importance to avoid the presence of compound III, since it is indicative of a high H_2O_2 concentration [5]. This will lead to an enhanced racemic direct reaction between the sulfide and H_2O_2 and therefore lower the *ee* of the sulfoxides formed [5].

Results and Discussion

Several studies have been published on the catalysis of asymmetric sulfoxidation by horseradish peroxidase [3, 4, 24–28]. Yet there is no agreement on the best method to perform these incubations, and therefore the yield and the enantiomeric excess of the sulfoxide formed differ from report to report. This may also be due to the experimental conditions used in these studies. Differences include amount of enzyme, concentration of the sulfide, open reaction vessels *vs*. closed ones, size headspace to prevent evaporation of the substrate, *pH* values [3, 4, 24–28], and the way H_2O_2 is added (in one step [24] or stepwise [3, 4, 25–28]).

For horseradish peroxidase, we have attempted to optimize the sulfoxidation with respect to the yield whilst maintaining a high enantioselectivity. Therefore, the method previously described in detail for CiP and LPO [5] of continuously adding H_2O_2 and simultaneously monitoring the enzyme intermediates, in particular compound III, was applied. This method guarantees that no excessive accumulation of H_2O_2 will occur. The rate of addition of H_2O_2 is to be lowered when a small amount of compound III is formed, thus minimizing the direct racemic reaction between H_2O_2 and the sulfide. The reactions were carried out at $pH = 7.0$, since at that *pH* value the highest *ee* for this conversion had been reported. Initially it was found that by incubating 19.2 μ*M* HRP with 1.7 m*M* thioanisole at *pH* 7.0 at a H_2O_2 influx of 1 μmol/h, after 5 hours a small amount of compound III could be detected in the mixture of compound I and II. Therefore, the rate of H_2O_2 addition was lowered to 0.4 μmol/h, and consequently only compound I and II were present. The presence of a mixture of compounds I and II is indicative of an oxygen-rebound mechanism which could be confirmed by stopped-flow studies [21, 22]. Nonetheless, during the incubations the absorbance of the *Soret* band decreased. About 20 μ*M* enzyme were necessary to fully convert the substrate before the enzyme was inactivated.

After 48 hours all of the thioanisole was converted into the sulfoxide with an enantioselectivity of 60% of the (*S*)-enantiomer. Variation of the substrate or the enzyme concentration did not lead to an altered *ee* provided that the reaction was quenched as soon as compound III started to be formed.

It is intriguing that the enantioselectivity does not change when the substrate concentration is changed. *Kobayashi et al*. have shown that 10% of the oxygen incorporated in the sulfide derives from water [17]. It is believed that the incorporation of an oxygen atom from water occurs when a sulfide radical-cation recombines with a second molecule of radical-cation to give a molecule of sulfide and a sulfide dication; the latter will react with water. The fact that the *ee* is independent on the sulfide concentration implies that these processes take place in the active site. Indeed, the stopped-flow experiments of *Dunford et al*. are indicative of the presence of two molecules of substrate in or near the active site [22].

From the decrease in the absorption at 280 nm we determined an initial turnover frequency of 0.3 min^{-1}. This is significantly lower than the turnover frequency of 3 min^{-1} determined by *Savenkova et al.* [29, 30]. The turnover number obtained with our optimization technique is 52, which is significantly higher than those previously reported [3, 4]. It must be mentioned that we did not use sulfide concentrations exceeding the solubility limits and that the reaction vessels were completely filled and sealed to prevent evaporation of the sulfide.

Using less favourable conditions resulted in lower yields and/or lower *ee*s. When the reactions were carried out by stepwise addition of H_2O_2 and were quenched after one hour [25–28], the yields did not exceed 11% (results not shown). After quenching, the enzyme is still active, and higher yields could have been obtained if the procedure would have been carried out for a longer time. In Refs. [25–28] higher enantioselectivities were claimed, but the amont of sulfoxides formed in a parallel reaction without enzyme was subtracted. This is not correct since the H_2O_2 concentration in the enzyme catalyzed reaction is much lower than in the absence of enzyme due to its catalase activity. In a later publications of the same group the subtraction of the racemic parallel reaction has been omitted, leading to enantioselectivities of 58% [29, 30].

When the reactions were carried out as described by *Morishima et al.* [24], *i.e.* by a single addition of 1.0 m*M* H_2O_2 at the start of the reaction, a yield of only 4% of the (*S*)-sulphoxide was obtained in one hour with an *ee* of only 52%. The low *ee* is probably due to a significant contribution of the direct reaction. Figure 1 shows that using this method the conversion of thioanisole only takes place during the first

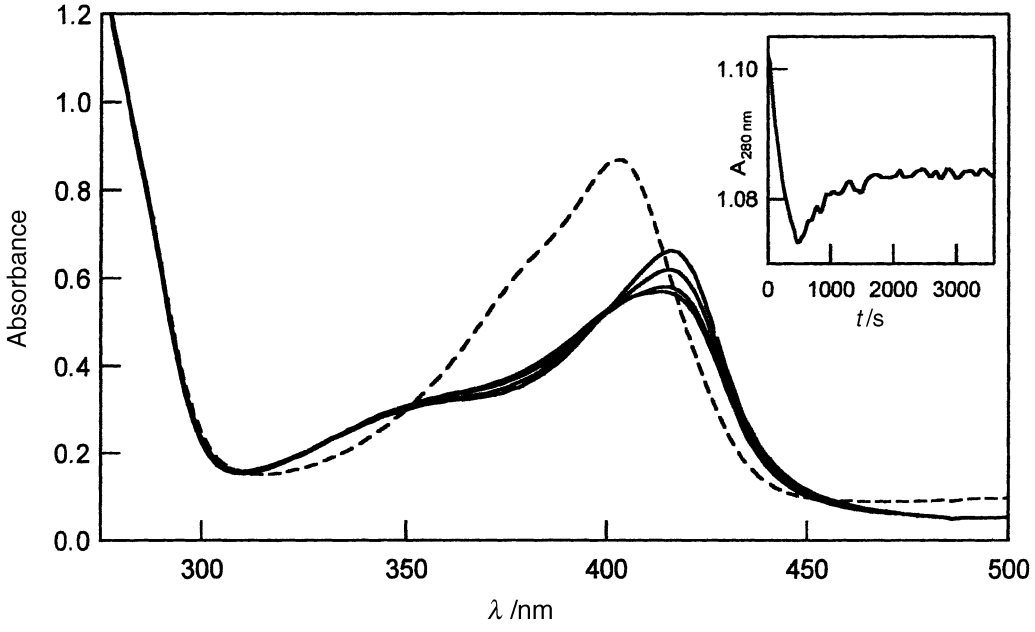


Fig. 1. Spectra of the enzyme intermediate of HRP in the sulfoxidation of thioanisole after 10, 130, 250, and 490 s (——); hereafter, the enzyme starts to return to its native state, the spectrum of which is represented after one hour (- - - -); in the insert the conversion of thioanisole is shown by the decrease in absorption at 280 nm; see experimental procedures for details

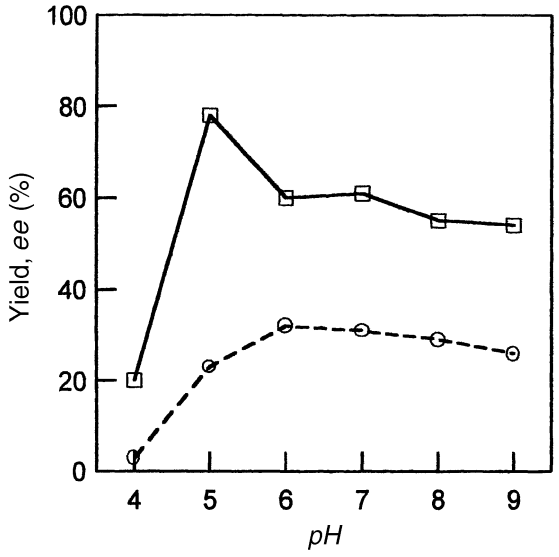


Fig. 2. *pH* dependence of the formation of (*R*)-methyl phenyl sulfoxide and its *ee* by MPO (O: *ee*, □: yield); see experimental procedures for details

500 seconds as judged from the decrease in absorption at 280 nm (insert). The following slower increase in absorption is probably due to conversion of compound I or II into native enzyme: the enzyme is mostly present as compound II and starts to return to its native state after the first 500 seconds.

In conclusion, *ee* and yield of the sulfoxidation of thioanisole by HRP depend strongly on the conditions and the design of the experiment. It was possible to convert the thioanisole completely into the sulfoxide using a slow continuous influx of H_2O_2 (on average 0.46 μmol/h) for 48 h. This influx is considerably slower than that for the same reaction catalyzed by LPO and CiP [5]. Continuous addition of H_2O_2 only is not sufficient to obtain a high *ee* it is also imperative to follow the enzyme intermediates spectroscopically to prevent the accumulation of H_2O_2 and the formation of catalytically inactive species as *e.g.* compound III.

The sulfoxidation of thioanisole by MPO was carried out *via* the traditional method of adding H_2O_2 stepwise. The *pH* dependence of the enantioselectivity and the yield are presented in Fig. 2. Although the yield is maximal at *pH* 5 (78%), the best enantioselectivity is obtained at *pH* 6.0 (32%). At higher *pH* values the enantiomeric excess remains fairly constant. The yield decreases showly at increasing *pH* values. These enantioselectivities are much higher than previously reported (4–8%) for the sulfoxidation of *p*-methylthioanisole by MPO [31]. In these experiments, much lower enzyme and substrate concentrations have been used. In agreement with Ref. [31] the rate for this reaction is maximal at *pH* 5.0. For MPO we have not optimized the yield *via* our method of controlled addition of H_2O_2 and monitoring at the same time the enzyme intermediates present. The high yields obtained employing the traditional stepwise addition of H_2O_2 are in contrast to our findings with other heme peroxidases.

Here we also report for the first time the enantioselective sulfoxidation by MnP. Due to a limited amount of purified enzyme available we were only able to carry out the

experiment at *pH* 5.0 and 7.0. Best results were obtained at *pH* 5.0. Although the yield was very low (18%), the enantioselectivity was very high (91% *ee* of (*S*)-sulfoxide). This is the highest value reported for a heme peroxidase producing the (*S*)-sulfoxide and significantly higher than reported for structurally related enzymes [32] (CiP: 73% *ee* HRP: 60% *ee*). At *pH* 7.0 the yield was considerably higher (36%), but the *ee* dropped to 87%. CPO also produces the sulfoxide with a high *ee* of even 99% [11, 12], but in this case the (*R*)-enantiomer is formed.

Unfortunately, the very limited amount of MnP available did not permit us to optimize this reaction with respect to *pH* value and controlled continuous addition of H_2O_2. Small genetic modifications for MnP (such as the F41L mutation for HRP [25]) might enhance the *ee* and make this enzyme one of its mutants a promising catalyst for the production of (*S*)-sulfoxides.

Experimental

MPO was purified from human leukocytes as described ($A_{428nm/280nm} = 0.8$) [33]. Enzyme concentrations were determined using a molar extinction coefficient of $102\,mM^{-1} \cdot cm^{-1}$ at 403 nm for HRP [34], $89\,mM^{-1} \cdot cm^{-1}$ at 428 nm for MPO [33], and $127\,mM^{-1} \cdot cm^{-1}$ at 406 nm for MnP [35]. Absorption spectra in the UV/Vis range were recorded on a Hewlett Packard 8452 A spectrophotometer. Figure 3 shows the spectra of native MPO, MPO-I, MPO-II, and MPO-III. The spectra of native HRP, HRP-1, HRP-II, and HRP-III are shown in Fig. 4.

To determine the *pH* optimum for the sulfoxidation of thioanisole by MPO, reactions were carried out in 100 m*M* buffer at 25°C. Buffers used were sodium acetate ($pH = 4.0$ and 5.0), potassium phosphate ($pH = 6.0$–8.0), and sodium carbonate ($pH = 9.0$).

The reaction mixture contained 10 μ*M* MPO and 1 m*M* thioanisole. During one hour, $20 \times 10\,mm^3$ of 10 m*M* H_2O_2 were added. The same procedure was applied to the reaction with MnP as a catalyst, however only at $pH = 5.0$ and 7.0 due to the very limited amount of enzyme available. Kinetic experiments following the sulfoxidation of thioanisole by HRP were conducted using a HP 8452 A spectrophotometer at 280 nm. The difference of the molar extinction coefficients of methyl phenyl sulfide and the methyl phenyl sulfoxide was determined to be $0.84\,mM^{-1} \cdot cm^{-1}$ at 280 nm. The amounts of compound I, II, or III and of native enzyme were followed simultaneously. Hydrogen

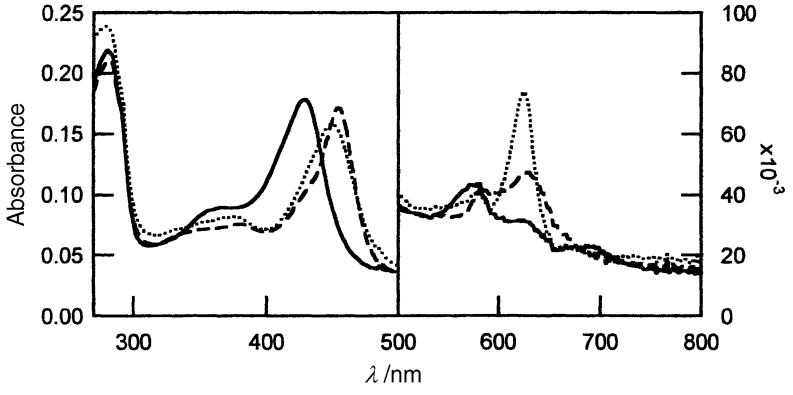


Fig. 3. Optical absorption spectra of MPO, native enzyme, compound II, and compound III; ——: 2 μ*M* MPO in 100 m*M* potassium phosphate (*pH* 7.0), - - - -: MPO-II generated by addition of 40 μ*M* hydrogen peroxide to 2 μ*M* MPO, · · · · · ·: MPO-III generated by addition of 4 m*M* hydrogen peroxide to 2 μ*M* MPO

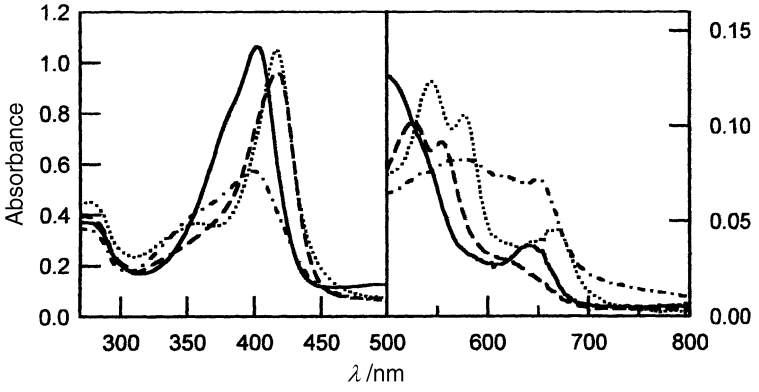


Fig. 4. Optical absorption spectra of native HRP, HRP-I, HRP-II, and HRP-III; ——: 10 μ*M* HRP in 100 m*M* potassium phosphate (*pH* 6.5), - - - - - - - - - -: HRP-I generated by addition of 10 μ*M* hydrogen peroxide, - - - - - -: HRP-II generated by addition of 10 μ*M* ferrocyanide to HRP-I, · · · · ·: HRP-III generated by addition of a large excess hydrogen peroxide (2.5 m*M*)

peroxide was added continuously *via* a syringe pump (Cole Parmer 74900-10) with a 250 mm^3 Hamilton syringe with a teflon luer lock connected to the reaction cuvet *via* a PEEK tubing 1/16″OD/0.20″ID that went through a capillary in the teflon cap that sealed the cuvet.

For HRP, the average rate of hydrogen peroxide addition was 0.46 μmol/h. Typically, reactions were carried out in a 1.67 cm^3 quartz cuvet sealed with a teflon cap with two capillaries: one to add the hydrogen peroxide solution and one to dispose of the overflow. The cuvets were completely filled with the reaction mixture in order to prevent partitioning of the methyl phenyl sulfide into a gas-phase headspace. The contents of the cuvet were stirred continuously. General conditions were 10 μ*M* enzyme, 100 m*M* potassium phosphate buffer, and 0.15–1.7 m*M* methyl phenyl sulfide.

The reactions were quenched with sodium sulfite [3] to consume the excess of H_2O_2 after 1 h for MPO and MnP and for HRP when the sulfoxidation process had ended as judged from a constant absorbance at 280 nm.

1 μmol of acetophenone was added as internal standard, and the reaction mixture was extracted twice with 3.4 cm^3 CH_2Cl_2. The organic layer was concentrated under a stream of nitrogen to a volume of about 20 mm^3 and diluted with 1 cm^3 of 80% hexane/20% isopropyl alcohol. A 20 mm^3 sample was loaded onto a chiracel OD HPLC column (Daicel Chemical Industries, 0.46 cm × 25 cm) equipped with a Pharmacia LKB-HPLC pump 2248 and a LKB Bromma 2140 rapid spectral detector connected to a PC. The Borwin™ program was used to evaluate peak areas. The (*R*)- and (*S*)-methyl phenyl sulfoxides were eluted isocratically with the same solvent mixture at a flow rate of 0.5 ml/min and detected at 254 nm. Retention times of methyl phenyl sulfide, acetophenone, and the (*R*)- and (*S*)-methyl phenyl sulfoxides were 8.4, 9.4, 15.0, and 17.8 min, respectively [5].

H_2O_2 solutions were freshly prepared by dilution of a 30% stock solution (Merck). The concentration was determined spectrophotometrically using an absorption coefficient of 43.6 $M^{-1} \cdot cm^{-1}$ at 240 nm [36]. All other chemicals were of the highest purity. Methyl phenyl sulfide was purchased from Fluka, methyl phenyl sulfoxide and acetophenone from Aldrich.

Acknowledgements

We wish to thank Prof. *M. H. Gold* from the Oregon Graduate Institute of Science and Technology for a gift of manganese peroxidase. This work was supported by the BioCentrum and the Innovation Oriented Research Programs Catalysis (IOP catalysis). We also received financial support from the Council for Chemical Sciences of the Netherlands Organisation for Scientific Research (NWO) and the Netherlands Technology Foundation (STW).

References

[1] Holland HL (1992) In: Holland HL (ed) Organic synthesis with oxidative enzymes. VCH, New York
[2] Colonna S, Gaggero N, Manfredi A, Casella L, Gulotti M, Carrea G, Pasta P (1990) Biochemistry **29**: 10465
[3] Colonna S, Gaggero N, Carrea G, Pasta P (1992) J Chem Soc Chem Commun 357
[4] Colonna S, Gaggero N, Richelmi C, Carrea G, Pasta P (1995) Gaz Chim Ital **125**: 479
[5] Tuynman A, Vink MKS, Dekker HL, Schoemaker HE, Wever R (1998) Eur J Biochem **258**: 906
[6] Allain AJ, Hager LP, Deng L, Jacobsen EN (1996) J Am Chem Soc **117**: 4415
[7] Tuynman A, Lutje Spelberg J, Kooter IM, Schoemaker HE, Wever R (2000) J Biol Chem **275**: 3025
[8] Miller VP, Tschirret-Guth, RA, Ortiz de Montellano PR (1995) Arch Biochem Biophys **319**: 333
[9] Hu S, Hager LP (1999) J Am Chem Soc **121**: 872
[10] Corbett MD, Chipko BR (1979) Biochem J **183**: 269
[11] Van Deurzen MPJ, Remkes IJ, van Rantwijk F, Sheldon RA (1997) J Mol Cat A: Chemical **117**: 329
[12] Van Deurzen MPJ, Seelbach K, van Rantwijik F, Kragl U, Sheldon RA (1997) Biocat Biotrans **15**: 1
[13] De Gioia L, Ghibaudi EM, Laurenti E, Salmona M, Ferrari RP (1996) J Bioinorg Chem **1**: 476
[14] Klebanoff SJ (1968) J Bacteriol **95**: 2131
[15] Mayfield MB, Kishi K, Alic M, Gold MH (1994) Appl Environ Microbiol **60**: 4303
[16] Ortiz de Montellano PR (1992) Annu Rev Pharmacol Toxicol **32**: 89
[17] Kobayashi S, Nakano M, Goto T, Kimura T, Schaap AP (1986) Biochem Biophys Res Commun **135**: 166
[18] Kobayashi S, Nakano M, Kimura T, Schaap PA (1987) Biochemistry **26**: 5019
[19] Casella L, Gullotti M, Ghezzi R, Poli S, Beringhelli T, Colonna S, Carrea G (1992) Biochemistry **31**: 9451
[20] Baciocchi E, Lanzalunga O, Malandrucco S (1996) J Am Chem Soc **118**: 8973
[21] Perez U, Dunford HB (1990) Biochem Biophys Acta **1038**: 98
[22] Perez U, Dunford, HB (1990) Biochemistry **29**: 2757
[23] Huwiler M, Jenzer H, Kohler H (1986) Eur J Biochem **158**: 609
[24] Tanaka M, Ishimori K, Mukai, M, Kitagawa T, Morishima I (1997) Biochemistry **36**: 9889
[25] Ozaki S, Ortiz de Montellano PRO (1994) J Am Chem Soc **116**: 4487
[26] Harris RZ, Newmyer SL, Ortiz de Montellano PR (1993) J Biol Chem **268**: 1637
[27] Ozaki S, Ortiz de Montellano PR (1995) J Am Chem Soc **117**: 7056
[28] Newmyer SL, Ortiz de Montellano PR (1995) J Biol Chem **270**: 19430
[29] Savenkova MI, Ortiz de Montellano PR (1998) Arch Biochem Biophys **351**: 286
[30] Savenkova MI, Ortiz de Montellano PR (1998) Biochemistry **37**: 10828
[31] Capeillère-Blandin C, Martin C, Gaggero N, Pasta P, Carrea G, Colonna S (1998) Biochem J **335**: 27
[32] Baunsgaard L, Dalbøge H, Houen G, Rasmussen EM, Welinder KG (1993) Eur J Biochem **213**: 605
[33] Bakkenist ARJ, Wever R, Vulsma T, Plat H, van Gelder BF (1978) Biochim Biophys Acta **524**: 45
[34] Schonbaum GR, Lo S (1972) J Biol Chem **247**: 3353
[35] Millis CD, Cai D, Stankovich MT, Tien M (1994) Biochemistry **28**: 8484
[36] Beers RF Jr and Sizer IW (1952) J Biol Chem **195**: 133

Received November 18, 1999. Accepted January 21, 2000