COMMUNICATION TO THE EDITOR

Chloroperoxidase-Catalyzed Enantioselective Oxidation of Methyl Phenyl Sulfide with Dihydroxyfumaric Acid/Oxygen or Ascorbic Acid/Oxygen as Oxidants

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Abstract: The chloroperoxidase catalyzed oxidation of methyl phenyl sulfide to (*R*)-methyl phenyl sulfoxide was investigated, both in batch and membrane reactors, using as oxidant H_2O_2, or O_2 in the presence of either dihydroxyfumaric acid or ascorbic acid. The effects of pH and nature and concentration of the oxidants on the selectivity, stability, and productivity of the enzyme were evaluated. The highest selectivity was displayed by ascorbic acid/O_2, even though the activity of chloroperoxidase with this system was lower than that obtained with the others. When the reaction was carried out in a membrane reactor, it was possible to reuse the enzyme for several conversion cycles. The results obtained with ascorbic acid/O_2 and dihydroxyfumaric acid/O_2 as oxidants do not seem to be compatible with either a mechanism involving hydroxyl radicals as the active species or with the hypothesis that oxidation occurs through the initial formation of H_2O_2. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **62**: 489–493, 1999.
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INTRODUCTION

Peroxidases are heme proteins that catalyze the oxidation of a large number of organic and inorganic substrates (Dunford and Stillman, 1976; Frew and Jones, 1984; van Deurzen et al., 1997). In particular, chloroperoxidase (CPO; EC 1.11.1.10) not only catalyzes reactions characteristic of peroxidases, namely hydrogen peroxide or other peroxide-supported oxidations of a variety of organic compounds, but can also use chloride, bromide, or iodide ions as donors for halogenation reactions (Hewson and Hager, 1978; Libby et al., 1982) and catalyze the dismutation of hydrogen peroxide (Frew and Jones, 1984). In addition, CPO catalyzes some P-450-type reactions, such as N-dealkylation of alkylamines (Kedderis et al., 1986), enantioselective epoxidation of styrene and styrene derivatives (Colonna et al., 1993; Ortiz de Montellano et al., 1987), N-oxidation of arylamines (Corbett et al., 1980), and oxidation of organic sulfides. The enantioselective oxidation of sulfides has been investigated by several research groups and seems to take place through a direct transfer of oxygen from compound I to the substrate (Casella et al., 1992; Colonna et al., 1990, 1992; Doerge, 1986; Kobayashi et al., 1987; Pasta et al., 1994).

However, in spite of the remarkable synthetic potentialities of CPO (Colonna et al., 1993; van Deurzen et al., 1997), commercial processes based on this enzyme have not yet been developed. One of the reasons for this is that H_2O_2 (or other peroxides), which is generally used as the oxidant, rapidly inactivates the enzyme by oxidation of the porphirin ring (van Deurzen et al., 1997). Enzyme stability can be improved by keeping H_2O_2 concentration low, through stepwise or continuous addition of the oxidant (Colonna et al., 1992; van Deurzen et al., 1994). Furthermore, the enantioselective oxidation of the various substrates catalyzed by CPO is in competition with their spontaneous oxidation by H_2O_2, which reduces the enantiomeric purity of the products.

Previously, several research groups have reported that horseradish peroxidase, in the presence of reducing equivalents (dihydroxyfumaric acid, DHFA) and O_2, can catalyze the hydroxylation of a number of aromatic compounds (Buhler and Mason, 1961; Courteix and Bergel, 1995;

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Dordick et al., 1986). According to Dordick et al. (1986), hydroxylation occurred through superoxide anion and compound III; however, in Courteix and Bergel (1995), the responsible species was DHFA radicals. In both cases, reaction would eventually take place by means of hydroxyl radicals that oxidize phenolic substrates via noncatalyzed reactions. Very recently, van de Velde et al. (1998) found that CPO can selectively oxidize indole to 2-oxindole and methyl phenyl sulfide to (*R*)-methyl phenyl sulfoxide in the presence of DHFA and O_2. The regio- and enantioselectivity displayed by CPO in these reactions cannot be explained by a mechanism in which the active species would be hydroxyl radicals, as suggested for HRP. Therefore, van de Velde et al. (1998) hypothesized that oxidation occurred through a mechanism involving the initial formation of H_2O_2 via autoxidation of DHFA.

In the present work, we investigate the CPO-catalyzed oxidation of methyl phenyl sulfide using H_2O_2 as oxidant, or O_2 in the presence of either DHFA or ascorbic acid (AA). The reaction was carried out both in batch and in membrane reactors and the effects of pH and nature and concentration of the oxidants on the selectivity, stability, and productivity of the enzyme are evaluated.

MATERIALS AND METHODS

Materials

Dihydroxyfumaric acid, ascorbic acid, hydrogen peroxide, methyl phenyl sulfide, and chloroperoxidase from *Caldariomyces fumago* (RZ 0.8, 2000 U/mg protein) were obtained from Sigma. All other reagents and compounds were of analytical grade.

Batch Reactor Experiments

Methyl phenyl sulfide (8 to 16 m*M*) CPO (400 to 500 U), and H_2O_2 (16m*M*) or DHFA (60 m*M*) or AA (60 m*M*) were gently stirred, in an uncapped vessel, in 5 mL of 0.05 *M* sodium citrate buffer, pH 5, 25°C. At scheduled times, the reactions were quenched with sodium sulfite (Colonna et al., 1992) and extracted with four portions (5 mL each) of ethyl acetate. The organic phase was dried over sodium sulfate and analyzed by HPLC. The influence of pH on sulfide oxidation was studied using 0.05 *M* citrate buffer, pH 3 to 6. The effect of AA concentration was investigated at pH 5 using 50 to 180 m*M* AA.

Membrane Reactor Experiments

The reactions were carried out, under gentle stirring, at 25°C, in a 10-mL uncapped ultrafiltration cell (Amicon) furnished with a membrane with a MW cutoff of 3000 (Diaflo YM3). The reactor contained 10 mL of 0.05 *M* sodium citrate buffer (pH 5) 2000 U of CPO, and 16 to 64 m*M* H_2O_2 or 60 m*M* DHFA or AA. In the case of AA, the reactor was continuously bubbled with air. At scheduled times, the reaction mixture was ultrafiltered and the reactor furnished with a fresh solution containing the aforementioned reagents with the exception of CPO. The ultrafiltered solution was extracted with ethyl acetate and analyzed by HPLC.

HPLC Analyses

HPLC analyses were performed on a Chiralcel OB column (Daicel) employing a Jasco instrument (Model 980-PU pump, Model 975-UV detector) and *n*-hexane/2-propanol 85/15 as the mobile phase. The flow rate was 1 mL/min and readings were made at 254 nm. The data were computed by a HP-3395A integrator. The retention times for methyl phenyl sulfide, (*S*)-methyl phenyl sulfoxide and (*R*)-methyl phenyl sulfoxide were 6, 14, and 30 min, respectively.

RESULTS AND DISCUSSION

Investigated Reactions

The CPO-catalyzed oxidation of methyl phenyl sulfide was carried out using H_2O_2 or DHFA and O_2 as oxidant (Fig. 1). The formation of diketosuccinate from DHFA has been suggested by Klibanov et al. (1981) for the horseradish peroxidase-catalyzed oxidation of phenolic compounds in the presence of DHFA and O_2. For the enzymatic oxidation of sulfide, the ascorbic acid/O_2 system was also employed and, in this case, the oxidation product formed from AA is likely to be dehydroascorbic acid (Seib and Tolbert, 1982).

Batch Reactor Experiments

Preliminary experiments aimed at the optimization of reaction conditions were carried out in batch reactors. Table I shows that all three oxidizing systems, namely H_2O_2, DHFA/O_2, and AA/O_2, were able to bring about the oxidation of the substrate. With DHFA and AA, the oxidant (O_2) was provided, unless stated otherwise, by simply stirring the reaction mixture in an uncapped vessel in an atmosphere of air. The reaction rates were, at least under the conditions employed, in the order H_2O_2 > HDFA/O_2 > AA/O_2. The optical purity of the product, (*R*)-methyl phenyl sulfoxide,

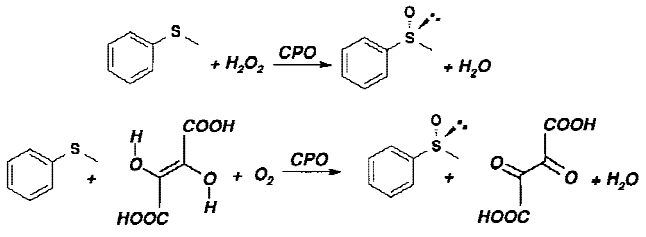

Figure 1. Scheme of the CPO-catalyzed oxidation of methyl phenyl sulfide to (*R*)-methyl phenyl sulfoxide using H_2O_2 as oxidant, or DHFA and O_2.

Table I. CPO-catalyzed conversion of methyl phenyl sulfide into (*R*)-methyl phenyl sulfoxide using different oxidation systems.[a]

	Conversion (%)		
Time (h)	H_2O_2	DHFA/O_2	AA/O_2
3	70	34	22
6	100	52	35
24	100	100	92

[a]The reactions were carried out, under gentle stirring, at 25°C, in 5 mL of sodium citrate buffer (pH 5) containing 500 U of CPO, 8 m*M* methyl phenyl sulfide, and 16 m*M* H_2O_2 or 60 m*M* of either DHFA or AA.

was very high in all cases, with enantiomeric excess values ranging between 95% and 98%.

With H_2O_2 as oxidant it has been shown previously that the optimum pH for enantioselective sulfide oxidation is 5 (Colonna et al., 1992; Fu et al., 1992; Kobayashi et al., 1987); however, for the DHFA/O_2 and AA/O_2 systems the optimum pH is not known. Therefore, the influence of pH on sulfide conversion and enantiomeric excess of the product was investigated for both systems. With DHFA/O_2 the highest degree of conversion was obtained at pH 3, whereas, with AA/O_2, the optimum was at pH 5 (Table II). The enantiomeric excess of the sulfoxide produced was equal to or higher than 98% in all cases except for DHFA/O_2 at pH 3 and 4, where it was 93%.

The effect of AA concentration on the CPO-catalyzed oxidation of methyl phenyl sulfide was also investigated. Figure 2 shows that the degree of conversion increased as a function of AA concentration, and that, especially at 6 h reaction time (where the conditions are closer to those of initial reaction), saturation was not reached, even at 180 m*M* AA. Because of the lower solubility of DHFA, it was not possible to employ it at concentrations higher than 60 m*M*.

The precise determination of the apparent K_m of DHFA and AA by spectrophotometric methods was prevented by the high absorbance of both substances at 272 nm (ε = 2560 M^{-1} cm^{-1} for DHFA and 7540 M^{-1} cm^{-1} for AA), where the transformation of methyl phenyl sulfide into the corresponding sulfoxide was monitored (Colonna et al., 1992).

The enantiomeric excesses of the product were ⩾98% at

Table II. Effect of pH on the CPO-catalyzed conversion of methyl phenyl sulfide using DHFA/O_2 or AA/O_2 as oxidant.[a]

	Conversion (%)	
pH	DHFA/O_2	AA/O_2
3	80	13
4	72	20
5	58	36
6	52	34

[a]The reactions were carried out overnight under gentle stirring, at 25°C, in 5 mL of 0.05 *M* sodium citrate buffer (pH 3 to 6) containing 400 U of CPO, 16 m*M* methyl phenyl sulfide, and 60 m*M* of DHFA or AA.

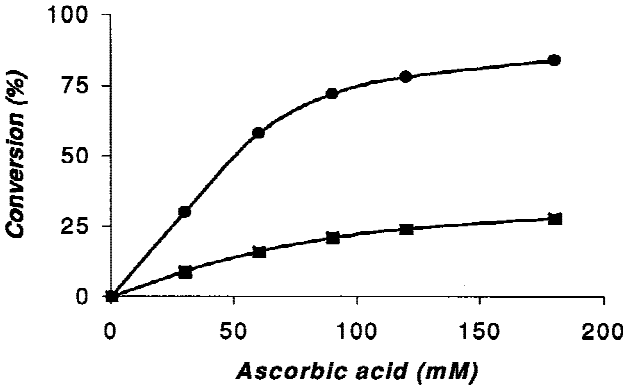

Figure 2. Effect of AA concentration on the CPO-catalyzed conversion of methyl phenyl sulfide. The reactions were carried out under gentle stirring, at 25°C, in 5 mL of 0.05 *M* sodium citrate buffer (pH 5), containing 500 U of CPO, with 8 m*M* sulfide and 30 to 180 m*M* AA. Reaction time: 6 h (■) and 16 h (●).

all the AA concentrations tested. It should be emphasized that control experiments carried out in the presence of 60 m*M* DHFA or AA up to 1 *M* showed that no oxidation of the sulfide occurred in the absence of CPO, even after 24-h incubation at pH 5. In the same conditions, no substantial decrease of CPO activity was observed, which indicates that the two oxidizing systems were not harmful for the enzyme.

Membrane Reactor Experiments

The oxidation of methyl phenyl sulfide to the corresponding sulfoxide by CPO and H_2O_2, DHFA/O_2, or AA/O_2 was also carried out in a membrane reactor, which made it possible to reuse the enzyme for several conversion cycles. The reactor was operated in a discontinuous mode; that is, it was emptied by filtration at scheduled times and then replenished with a fresh solution containing the substrate and the oxidizing system.

The results obtained using H_2O_2 as oxidant are reported in Table III. It can be seen that both degree of conversion and optical purity of the product were markedly dependent

Table III. CPO-catalyzed oxidation of methyl phenyl sulfide in membrane reactor with H_2O_2 as oxidant.[a]

	Conversion (%)		
	H_2O_2 concentration		
Cycle no.	16 m*M*	32 m*M*	64 m*M*
1	66 (⩾98)[b]	91 (⩾98)[b]	88 (97)[b]
2	50 (⩾98)	79 (96)	90 (88)
3	42 (⩾98)	70 (94)	96 (32)
4	32 (⩾98)	57 (90)	98 (21)

[a]The reactions were carried out for 20 h, at 25°C, in a membrane reactor using 10 mL of 0.05 *M* sodium citrate buffer (pH 5) containing 2000 U of CPO, 16 m*M* sulfide, and 16 to 64 m*M* H_2O_2. For details see Methods.

[b]In parentheses are the percent enantiomeric excesses of the (*R*)-methyl phenyl sulfoxide obtained.

on the concentration of H_2O_2 and cycle number. At 16 to 32 m*M* H_2O_2, the conversion degree decreased as a function of the cycle number, whereas the opposite was true at 64 m*M* H_2O_2. This result, which is rather puzzling, could be ascribed to the fact that, at 16 and 32 m*M* H_2O_2, there was CPO inactivation, but the oxidation was still predominantly catalyzed by the enzyme. This hypothesis is confirmed by the high optical purity of the product, which points to a low incidence of aspecific oxidation. Instead, at 64 m*M*, enzyme inactivation occurred more rapidly, and thus in the third and fourth conversion cycle aspecific oxidation prevailed, yielding a product with low optical purity. The increase of conversion as a function of cycle number at 64 m*M* H_2O_2 was probably due to the fact that, because of enzyme inactivation, there was no depletion of H_2O_2 caused by the catalase activity of CPO (Frew and Jones, 1984). As a consequence, substrate oxidation, although aspecific, was quite fast.

Tables IV and V show the results obtained in the membrane reactor employing DHFA/O_2 or AA/O_2 as oxidizing systems. In both cases, almost complete conversion was obtained up to the seventh or eighth cycle, with an optical purity that, especially for the AA/O_2 system, was practically complete for all the reaction cycles. This was a consequence of the high stability of CPO with the two systems and of the absence of aspecific oxidation of the substrate.

General Considerations

The present study has demonstrated that CPO can be employed in a membrane reactor for several conversion cycles to carry out in a selective and effective way the oxidation of methyl phenyl sulfide to (*R*)-methyl phenyl sulfoxide. Of the three oxidizing systems investigated, namely H_2O_2, DHFA/O_2, and AA/O_2, the highest selectively was displayed by AA/O_2, even though the activity of CPO with this system was lower than that obtained with the others.

Concerning the reaction mechanism by which CPO acts in the presence of DHFA/O_2 or AA/O_2, no conclusive explanation can be given. In fact, from one side, the involvement of hydroxyl radicals as active species, suggested for HRP-catalyzed oxidation of phenolic substrates (Courteix and Bergel, 1995; Dordick et al., 1986), should be ruled out by the high enantioselectivity shown by the oxidation process. On the other hand, oxidation through a mechanism implying the formation of H_2O_2 via autoxidation of DHFA (or AA) (van de Velde et al., 1998) is not completely convincing for the following reasons: first, as reported by van de Velde et al. (1998), catalase had no effect on CPO-catalyzed oxidations carried out with the DHFA/O_2 system; and, second, the high stability and selectivity found when using CPO with the DHFA/O_2 or AA/O_2 does not seem to be compatible with the presence of consistent concentrations of H_2O_2. Concerning the first point, however, it should be mentioned that catalase is a relatively poor scavenger of H_2O_2 due to its very high K_m. Thus, small concentrations of H_2O_2 generated in situ through DHFA oxidation cannot be explicitly ruled out.

We found that, upon reacting CPO with excess ascorbic acid in an anaerobic optical cell, the enzyme is reduced to the Fe(II) state. This reduced form reacts rapidly with dioxygen to fully regenerate the native Fe(III) state. The capacity of the enzyme to undergo reactions with O_2 in the presence of reducing agents raises the possibility that it may perform oxygen transfer reactions to exogenous substrates according to a true monooxygenase pathway. To shed light on the mechanism of this enzymatic reaction, kinetic and spectroscopic studies are currently being carried out in our laboratories.

Table IV. CPO-catalyzed oxidation of methyl phenyl sulfide in a membrane reactor with the DHFA/O_2 system as oxidant.[a]

Cycle no.	Conversion (%)	Enantiomeric excess (%)
1	99	⩾98
2	96	⩾98
3	93	97
4	92	97
5	93	⩾98
6	94	96
7	92	93

[a]The conditions are the same as those described in the footnote to Table III, but with 60 m*M* DHFA instead of H_2O_2.

Table V. CPO-catalyzed oxidation of methyl phenyl sulfide in membrane reactor with the AA/O_2 system as oxidant.[a]

Cycle no.	Conversion (%)	Enantiomeric excess (%)
1	92	≥98
2	91	≥98
3	90	≥98
4	87	≥98
5	88	≥98
6	89	≥98
7	85	≥98
8	75	≥98

[a]The conditions are the same as those described in the footnote to Table III, but with 60 m*M* AA instead of H_2O_2. Also, the reactor was continuously bubbled with air.

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