Enzyme-Catalyzed Asymmetric Synthesis: Kinetic Resolution of Racemic Hydroperoxides by Enantioselective Reduction to Alcohols with Horseradish Peroxidase

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Abstract: The horseradish peroxidase (HRP)-catalyzed kinetic resolution of racemic, secondary hydroperoxides in the presence of guaiacol is reported. Both the catalytic efficiency and the stereoselectivity of HRP highly depend on the structure of the hydroperoxides. The enzyme selectively recognizes sterically uncumbered (*R*)-configurated alkyl aryl hydroperoxides, which allows kinetic resolution by means of enantioselective reduction to furnish optically pure (*S*) hydroperoxides and (*R*) alcohols. Poor enzyme recognition is observed with hydroperoxides possessing branched aliphatic chains and none at all with tertiary hydroperoxides. This enzyme-catalyzed reduction can be performed on preparative scale to provide optically pure hydroperoxides conveniently. Since HRP is known to be composed of different isoenzymes, the kinetic resolution was investigated with the isolated isoenzymes A and C, which showed that mostly isoenzyme C catalyzes the asymmetric reduction of the racemic hydroperoxides.

Introduction

The catalytic, asymmetric oxidation of organic substrates is a subject of current interest and intense activity in synthetic organic chemistry.[1] Besides metal-assisted oxidations such as the Sharpless epoxidation of allylic alcohols[2] or Jacobsen epoxidation of non-coordinating olefins,[3] the Sharpless *cis*-dihydroxylation of olefins,[4] or the Kagan sulfoxidation of prochiral sulfides,[5] to mention a few of the most significant achievements in recent years, also several practical methods based on metalloenzyme catalysis have been developed.[6] Thus, chloroperoxidase (CPO) and horseradish peroxidase (HRP) have been successfully employed for oxidation purposes.[7] The heme peroxidases are a superfamily of enzymes which oxidize a variety of structurally diverse substrates by using hydroperoxides as oxidants. For example, CPO catalyzes the regio- and stereoselective halogenation of glycals,[8] the enantioselective epoxidation of disubstituted alkenes,[9] and the stereoselective sulfoxidation of prochiral thioether by racemic 1-arylethyl hydroperoxides. The latter reaction results in (*R*) sulfoxides, (*S*) hydroperoxides, and the corresponding (*R*) alcohols all in optically active form.[8] For HRP, merely the asymmetric sulfoxidation of prochiral sulfides is known.[10]

Recently we have reported on the HRP-catalyzed kinetic resolution of racemic, secondary hydroperoxides.[11] The structure of the hydroperoxide dramatically influences not only the enantioselectivity but also the enantiomer preference of the enzyme to yield hydroperoxides and alcohols up to 99% ee with *R* to 97% ee with *S* configuration. This method allows the convenient preparation of optically active hydroperoxides as potential stereoselective oxidants. In contrast, the preparation of enantiomerically pure hydroperoxides by nonenzymatic means is quite cumbersome.[12] In the present work, we have investigated in detail the catalytic efficiency and the substrate selectivity of HRP on a series of racemic, secondary alkyl aryl hydroperoxides with cyclic, branched, and functionalized alkyl chains (Scheme 1).

Results and Discussion

In order to assess the catalytic efficiency of the enzymatic transformations, we determined the kinetic parameters K_m (Michaelis constant) and k_{cat} (turnover number). The K_m value reflects the affinity of the enzyme for the hydroperoxide, whereas k_{cat} measures the number of substrate molecules turned over per enzyme molecule per minute. The kinetic parameters obtained for the different hydroperoxides in the presence of

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Scheme 1. HRP-Catalyzed Kinetic Resolution of Hydroperoxides in the Presence of Guaiacol

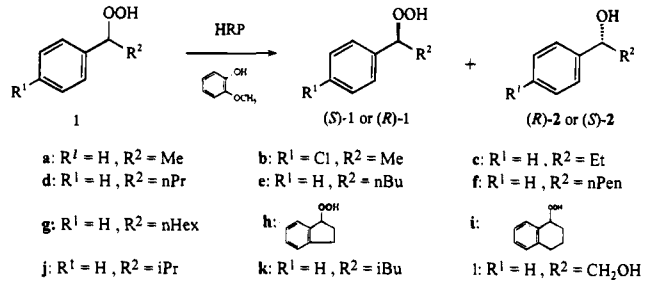


Table 1. Kinetic Parameters for Horseradish Peroxidase-Catalyzed Kinetic Resolution of Hydroperoxides **1** in the Presence of Guaiacol[a]

entry	peroxide	enzyme	K_m[b] [mM]	k_{cat}[c] [min^{-1}]	k_{cat}/K_m [mM^{-1} min^{-1}]
1	H_2O_2	HRP	0.05	4600	92000
2	(1a)	HRP	0.7	568	811
		Isoenzyme A[d]	0.8	116	145
		Isoenzyme C	0.8	1040	1300
3	(1b)	HRP	3.4	376	110
4	(1c)	HRP	26	92	3.54
5	(1d)	HRP	4	2	0.5
6	(1e)	HRP	6	520	86.7
7	(1f)	HRP	4.6	1.6	0.3
8	(1k)	HRP	18	176	9.8
9	(1j)	HRP	6.5	256	39.4
10	(1h)	HRP	4.4	584	132

[a] The kinetic parameters were obtained at a fixed guaiacol concentration of 500 μM; the initial rates were monitored by following the appearance of the guaiacol oxidation product ($\epsilon = 2.66 \times 10^4$ M^{-1} cm^{-1} at 470 nm) in 0.1 M potassium phosphate buffer (pH 6.0); the data were processed with the Duggleby program to give K_m and k_{cat} values. [b] Error limit between 5 and 10% of the stated values. [c] Error limit between 1 and 5% of the stated value. [d] For isoenzyme A (mixture of three acidic isoenzymes) 0.1 M sodium acetate buffer (pH 5) was employed.

guaiacol as electron donor are shown in Table 1. Both the K_m and k_{cat} values depend on the R substituent. The ratio k_{cat}/K_m represents the second-order rate constant for the enzyme–substrate reaction. Except for H_2O_2, of the hydroperoxides **1**, the highest values are obtained for derivative **1a** (Table 1, entry 2). Introduction of functional groups either in the aromatic ring, as in derivative **1b** (Table 1, entry 3), or in the alkyl chain, as in derivative **1j** (Table 1, entry 9), decreases the rate of the reaction significantly.

The homologous series **1a**–**1g** of the *n*-alkyl aryl hydroperoxides (Table 1, entries 4–7) reflects the sensitivity of HRP toward steric demand of the R^2 substituent. A stepwise increase of the alkyl chain dramatically decreases the catalytic efficiency, presumably due to size restrictions at the active site. Thus, it was difficult to determine reliably the K_m values for hydroperoxide **1g** (not listed in Table 1) on account of its extremely low affinity for the enzyme.

Table 2. HRP-Catalyzed Kinetic Resolution of Hydroperoxides **1** in the Presence of Guaiacol[a]

entry	peroxide	ROOH : HRP [mol]	time [min]	enantiomeric excess [%][b]	
				(-)-(*S*)-ROOH (1)	(+)-(*R*)-ROH (2)
1	(1a)	12000 : 1	5	>99	>99
		1850 : 1[c]	70	>99	>99
		3160 : 1[d]	20	>99	>99
2	(1b)	4720 : 1	90	>95	>95
3	(1c)	6000 : 1	150	93	95
4	(1d)	2400 : 1	90	<5	<5
5	(1e)	11200 : 1	90	44	36
6	(1f)	2280 : 1	90	11	16
		4400 : 1[c]	240	0[e]	0
		2570 : 1[d]	260	8	6
7	(1g)	1000 : 1	90	4	8
8	(1h)	10000 : 1	60	>99	*f*
9	(1i)	2400 : 1	180	95	97
				(+)-(*R*)-ROOH (1)	(-)-(*S*)-ROH (2)
10	(1j)	480 : 1	180	15	14
		2640 : 1[c]	1230	0[e]	0
		3090 : 1[d]	1230	9	10
11	(1k)	1000 : 1	960	36.6	47.7
12	(1l)	12000 : 1	120	>95[g]	>95

[a] All reactions were conducted on a 0.06-mmol scale; conversion of the peroxides was 50%, determined photometrically; the absolute configurations of the corresponding alcohols were confirmed by comparison with the authentic sample or literature data. [b] The enantiomeric excess was established by HPLC on a Chiralcel OD column by using the area under each enantiomer peak; in all cases a clean separation of the hydroperoxide and alcohol enantiomers was achieved; detection limit ≤5%. [c] Isoenzyme A. [d] Isoenzyme C. [e] Conversion rate was below 5%. [f] The alcohol was not detected in the HPLC analysis. [g] The sign of the optical rotation for (*R*)-**1k** was negative and for (*S*)-**2k** positive.

Exceptional kinetic parameters were obtained for hydroperoxide **1e** (Table 1, entry 6), for which a rate increase up to 600-fold was observed compared to the lower and higher homologous hydroperoxides **1d** and **1f**. The k_{cat} value of the derivative **1e** is similar to that obtained for phenethyl hydroperoxide **1a** (Table 1, entry 2), although the larger affinity constant (K_m) of the former (Table 1, entry 6) resulted in lower catalytic efficiency in comparison to that of derivative **1a** (811 versus 86.7 in Table 1, entries 2 and 6). The same trend was observed for the bicyclic derivative **1h** (Table 1, entry 10). In such cases, the kinetic resolution must be carried out at high hydroperoxide concentrations to ensure optimal reaction rates. Unfortunately, the kinetic data for the bicyclic hydroperoxide **1i** could not be determined, due to its low water solubility.

In view of the observed trends in the catalytic efficiency (k_{cat}/K_m), the kinetic resolutions were conducted at different substrate concentrations by varying the amounts of HRP to assess optimal reaction conditions (substrate concentration, reaction time) during the kinetic resolution of hydroperoxides **1**. Fortunately, all hydroperoxides do not react at room temperature with guaiacol in the absence of HRP, so that the enzymatic reactions could be conducted at about 20 °C. To limit the hydroperoxide consumption to 50% and thereby avoid overreduction, equimolar amounts of hydroperoxides **1** and guaiacol were used. Conversion rates, absolute configurations, and enantiomeric excess (ee) values are reported in Table 2.

The absolute configurations of alcohols **2** were assigned by

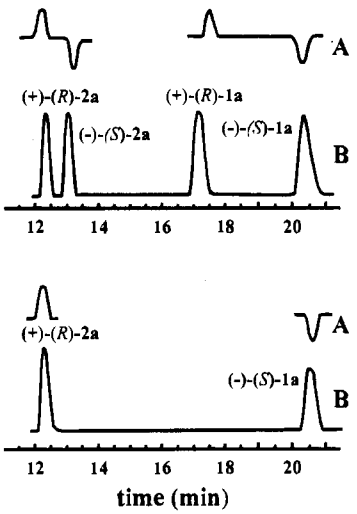


Figure 1. HPLC traces of the HRP-catalyzed reaction of 1-(1-phenyl)-ethyl hydroperoxide (**1a**) with guaiacol. Top: Authentic racemic samples of 1-phenylethanol (**2a**) and hydroperoxide **1a**. Bottom: Reaction products after enzymatic resolution. The polarimetric detection with the ChiraLyser (A) is placed above the UV signals (B) at 220 nm. Column, Daicel-Chiralcel OD, 250 × 4 cm; eluent, *n*-hexane/isopropyl alcohol (90:10); flow rate, 0.6 mL min^{-1}.

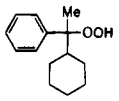
comparison of the HPLC data with those of the authentic substances or according to literature data.[13] The absolute configuration of hydroperoxides **1** was assigned analogously. The enantiomeric excess of hydroperoxides **1** and alcohols **2** was determined from the ratio of areas of the two enantiomers in the HPLC traces (Chiralcel OD column). A representative chromatogram is given in Figure 1 for hydroperoxide **1a** and the corresponding alcohol **2a**. As can be seen from this example, HRP selectively recognized the (+)−(*R*) substrate enantiomer, which yielded consequently the enantiomerically pure (+)-(*R*)-**2a** alcohol and (−)-(*S*)-**1a** hydroperoxide.

The hydroperoxide structure dramatically influenced not only the enantioselectivity but also the enantiomer preference of the enzyme. In the cases of the *n*-alkyl aryl hydroperoxides **1a**−**g** (Table 2, entries 1−7) and both bicyclic derivatives **1h** and **1i** (Table 2, entries 8 and 9), HRP preferentially accepts the (*R*) hydroperoxides as substrates with concurrent formation of (*R*) alcohols, while the (*S*) hydroperoxides are left behind (Table 2, entries 1−9). Interestingly, the stereochemical course is opposite for the branched hydroperoxides **1j** and **1k** (Table 2, entries 10 and 11). For the hydroxy-functionalized hydroperoxide **1l** (Table 2, entry 12) a change in the sequence rule priorities applies and, thus, only the branched hydroperoxides **1j** and **1k** exhibit reversed stereoselection compared to that of **1a**−**g**. Consequently, the HRP-catalyzed kinetic resolution affords (*S*) alcohols **2j**−**l** and leaves behind (*R*) hydroperoxides **1j**−**l** (Table 2, entries 10−12). Presumably the bulky alkyl chains cause distinct binding of the hydroperoxides in the heme pocket of the enzyme and thereby express opposite enantiomer preference.

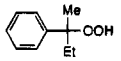
High stereoelectivity was observed for 1-phenethyl hydroperoxide (**1a**) and its *p*-chloro derivative **1b** (Table 2, entries 1 and 2), 1-phenylpropyl hydroperoxide (**1c**) (Table 2, entry 3), the hydroxy-functionalized derivative **1l** (Table 2, entry 12), and the bicyclic hydroperoxides **1h** and **1i** (Table 2, entries 8 and 9). Unfortunately, the corresponding alcohol **2h** could not be detected in the HPLC analysis of the crude reaction mixture. Since a control experiment with authentic racemic alcohol **2h** under the enzymatic reaction conditions established that it was completely recovered on workup, we suspect that alcohol **2h** suffers further transformation during enzyme catalysis, but the details of the chemical fate are not known.

Replacement of the ethyl group by a longer alkyl chain as in hydroperoxides **1d**−**f** (Table 2, entries 4−7) dramatically decreased the degree of asymmetric induction. Except for phenylpentyl hydroperoxide (**1e**), poor or even no resolution was observed (Table 2, entry 5). This probably tallies with the kinetic parametes, since the derivatives **1d** and **1f** are poor substrates for horseradish peroxidase (Table 1, entries 5 and 7), while for hydroperoxide **1e** a higher catalytic efficiency operates (Table 1, entry 6). These results clearly define the limitation of HRP-catalyzed kinetic resolution of racemic hydroperoxides. Therefore, the physiologically relevant long chain fatty acid hydroperoxides, e.g. 15-hydroperoxy-(*5Z,8Z,11Z,13E*)-eicosatetraenoic acid of the arachidonic acid cascade, cannot be resolved by this method.

Branching of the alkyl substituent as in derivatives **1j** and **1k** (Table 2, entries 10 and 11) results in only moderate enantioselectivities even at 50% conversion. Moreover, the position of the branching point in the alkyl chain exhibits significant differences in the ee values. Thus, α branching, i.e. right next to the stereogenic center as in hydroperoxide **1j**, reduces the enantioselectivity much more effectively than β branching in **1k** (Table 2, entries 10 and 11). As already alluded to above, this speaks for rather sensitive steric effects in the enantiomer recognition by the HRP enzyme. Attempts to resolve tertiary hydroperoxides such as **3a** and **3b** by HRP



3a



3b

failed,[11b] which underlines the sensitivity of HRP toward steric effects and marks the limitation of this enzymatic method for the preparation of optically active hydroperoxides. Recently it was reported that such tertiary hydroperoxides can be resolved by the Sharpless reaction, but in low enantiomeric excess (ee 1.2−29%).[14]

Two other enzymatic methods were reported for the kinetic resolution of hydroperoxides, namely the lipase-catalyzed acylation of hydroperoxides[15] and the CPO-catalyzed asymmetric sulfoxidation in the presence of racemic hydroperoxides.[8] A comparison of these methods with the HRP-catalyzed reduction by using the hydroperoxide **1i** as a model substrate revealed that HRP is the most effective biocatalyst for the preparation of enantiomerically pure hydroperoxides.[11b]

Since HRP is known to be composed of different isoenzymes,[16] it was of interest to assess the enantioselectivity for the individual enzyme constituents. Generally one can distinguish between two groups of isoenzymes according to their isoelectric point (pI), namely the acidic and the basic ones, which possess different amino acid compositions and sequences.[17] Studies on structure−reactivity relationships revealed that isoenzymes show differences in substrate specificity.[16] Indeed, isoelectric focusing of the enzyme preparation used in the present study confirmed that the HRP (Sigma, RZ 2.0)

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consists of the acidic and basic isoenzymes A and C, with the basic isoenzyme C as the major component. To assess which of the isoenzymes is responsible for the kinetic resolution, the HRP powder was further purified by ion exchange chromatography on (carboxymethyl)cellulose. The two groups of isoenzymes were successfully separated by this method. Isoenzyme C with an isoelectric point between pH 7 and 8 was obtained in electrophoretically homogeneous form, whereas the acidic protein fraction contained three isoenzymes A with isoelectric points ranging from pH 3.5 to 5.5.

Kinetic resolution studies of hydroperoxides **1** were carried out with these isoenzyme fractions, wherein the phenethyl hydroperoxide (**1a**) was chosen as model substrate to establish their catalytic efficiency. The results indicate that mostly isoenzyme C is responsible for the kinetic resolution of racemic hydroperoxides as the turnover number is 100-fold higher compared to isoenzyme A (Table 1, entry 2). Selectivity studies support this reactivity trend in that isoenzyme A only catalyzes the conversion of phenethyl hydroperoxide (**1a**) as shown in Table 2 (entry 1); all other substrates were too unreactive, as illustrated for derivatives **1f** and **1j** (Table 2, entries 6 and 10). On the contrary, isoenzyme C reduces all the hydroperoxides examined so far with a similar selectivity as the crude Sigma HRP powder (Table 2, entries 1, 6, and 10). Although the isoenzymes A and C differ in catalytic efficiency, the sense of stereoselection is identical (Table 2, entry 1).

In summary, we have shown that it is mostly the isoenzyme C which catalyzes the asymmetric reduction of racemic hydroperoxides **1**. Kinetic and stereochemical studies establish that both the catalytic efficiency and the stereoselectivity of HRP highly depend on the structure of the hydroperoxide substrate, i.e. with branched aliphatic chains poor enzyme recognition was observed and with tertiary hydroperoxides none at all. Moreover, catalytic efficiency plays a major role in dictating the stereochemical course of the reaction. Thus, the HRP enzyme selectively recognizes sterically unencumbered (*R*)-configurated alkyl aryl hydroperoxides **1** and enantioselectively reduces them to the corresponding alcohols **2**. As a bonus, optically active hydroperoxides **1** remain, which are to date difficult to acquire enantiomerically pure by non-enzymatic means. Moreover, the HRP enzyme is much less substrate specific than any of the previously employed enzymes[11b] and accepts a variety of hydroperoxides to perform highly enantioselective asymmetric transformation.

Experimental Section

Materials and Methods. Horseradish peroxidase (HRP) was purchased from Sigma (RZ 2.0). The literature known racemic hydroperoxides[19] were prepared by a modified procedure reported earlier.[20] Hydroperoxides **1a**,[15] **1c**,[15] and **1i**[11b] and alcohols **2**[21] are known in optically active form. For the enantiomerically enriched hydroperoxides **1b**, **1e**, **1h**, **1k**, and **1l** the specific rotation $[\alpha]^{20}_D$ was determined after purification by silica gel chromatography (pentane–diethyl ether 7:3) on a Perkin-Elmer polarimeter by using methanol as eluent. The $[\alpha]^{20}_D$ values are the following: **1b**, −82 (c = 0.12, MeOH); **1e**, −34 (c = 0.21, MeOH); **1h**, −86 (c = 0.18, MeOH); **1k**, +32 (c = 0.34, MeOH); and **1l**, −72 (c = 0.37, MeOH). Enantiomeric excesses and signs of optical rotation for hydroperoxides **1** and alcohols **2** were determined by HPLC analysis on a Chiralcel OD column (0.46 × 25 cm) with a 90:10 *n*-hexane–isopropyl alcohol mixture as eluent at a 0.6 mL/min flow rate. A photodiode array detector, coupled with a polarimetric Chiralyser detector, was employed to assess the configuration of the enantiomer formed.

Purification of Horseradish Peroxidase. The commercial horseradish peroxidase powder from Sigma was further purified according to the procedure by Shannon *et al.*[16] Prior to purification, the HRP was characterized by isoelectric focusing. A 60-mg portion of the enzyme was dissolved in a small volume (5 mL) of 5 mM sodium acetate buffer (pH 4.7) and dialyzed against a large excess of the same buffer for 24 h. Chromatography was performed on a Servacel (carboxymethyl)cellulose CM52 column (2.6 × 25 cm), which had previously been equilibrated with a 5 mM sodium acetate buffer (pH 4.7). Initial elution with the same buffer, after loading the column with the crude enzyme preparation, yielded a mixture of three anionic isoperoxidases. The cationic isoenzymes, which remained on the column, were eluted with a linear gradient, formed by mixing 5 mM acetate buffer with 0.05 M NaCl. The fractions with the isoenzymes were lyophilized, dissolved in a small volume (5 mL) of potassium phosphate buffer (pH 6), dialyzed against the same buffer, and used as obtained. The cationic fraction (isoenzyme C) was found to be of homogeneous composition on electrophoretic analysis, whereas the acidic fraction contained three different isoenzymes (isoenzymes A). Isoenzymes A and C were stored at −20 °C.

Isoelectric Focusing (IEF). Flat bed isoelectric focusing (IEF, Desaga) was carried out at 4 °C for 4 h at the final setting of 1500 V and 3 mA on a polyacrylamide gel plate (Sevalyte Precotes, Serva) of a pH range 3−10 in a Desaphor chamber. Proteins were detected by staining with Coomassie Brilliant Blue G-250; the pH was determined by pI markers purchased from Serva.

Peroxidase Assay. Peroxidase activity was measured at pH 6.0 and 25 °C according to the method of Chance and Maehly[22] by using hydrogen peroxide as oxygen donor and guaiacol as substrate.

Determination of Kinetic Parameters for HRP. To determine the kinetic parameters K_m and k_{cat} for hydroperoxides **1** (0 to 35 mM), a 0.1 M stock solution of guaiacol in either potassium phosphate buffer (0.1 M, pH 6 for isoenzyme C) or sodium acetate buffer (0.1 M, pH 5 for isoenzyme A) was prepared. In each assay, the guaiacol concentration was adjusted to 0.5 mM by diluting 100 μL of the stock solution to a final volume of 2 mL, which contained the required amount of hydroperoxide, and the sample was placed into a quartz cuvette. After efficient mixing, 50 μg of enzyme was injected to start the reaction. The appearance of the guaiacol oxidation product, which has an absorption maximum at 470 nm (ϵ = 2.66 × 10^4 M^{-1} cm^{-1}), was monitored for 3 min to calculate the initial rate. Once all the initial rates were acquired, the data were processed with the Duggleby program on a personal computer to give the K_m and k_{cat} values.

General Procedure for the Kinetic Resolution of Hydroperoxides 1 with HRP. In a typical reaction, 0.3−0.06 mmol racemic hydroperoxide and equimolar amounts of guaiacol were dissolved in 2−5 mL of 0.1 M phosphate buffer solution (pH 6) and subsequently HRP (25−125 μmol) was added. The reaction progress was followed photometrically[23] and terminated at a conversion rate of 50%. The mixture was extracted with diethyl ether (3 × 3 mL) and the combined organic layers were dried over Na_2SO_4. The solvent was removed under vacuum (20 °C/17 Torr) and the products were isolated from the crude mixture by silica gel chromatography with a 7:3 petroleum ether–diethyl ether mixture as eluent. The enantiomeric excess of hydroperoxides **1** and alcohols **2** was determined as described above.

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