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Journal of Molecular Catalysis B: Enzymatic 9 (2000) 163–172

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Chloroperoxidase-catalysed oxidation of alcohols to aldehydes

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Received 15 July 1999; accepted 30 August 1999

Abstract

Chloroperoxidase (CPO) catalyses the oxidation of primary alcohols (hexan-1-ol, hexen-1-ols, epoxyhexan-1-ols and 3-phenylglycidol) selectively to the aldehyde in biphasic systems of hexane or ethyl acetate and a buffer (pH 5.0). The *cis* to *trans* isomerization in the case of *cis*-2-hexenal can be avoided by working at low water contents or in organic solvents saturated with water. In the case of epoxyalcohols, oxidation to the aldehyde proceeds enantioselectively. Hydrogen peroxide and *tert*-butyl hydroperoxide have been used as an oxidant.

Keywords: Chloroperoxidase; Alcohol oxidation; Two-phase system

1. Introduction

Chloroperoxidase (CPO; EC 1.11.1.10) from the fungus *Caldariomyces fumago* was first isolated in crystalline form and its physical properties were described over 30 years ago [1]. Today, CPO is recognized as a versatile and most promising of the heme peroxidase enzymes for synthetic applications [2–5]. This is mainly due to the fact that in addition to halogenation and classical peroxidation reactions, various transformations typical of catalases and cytochrome *P*-450 like monooxygenases are catalysed by CPO. The enzyme resembles cytochrome *P*-450 in possessing an axial thiolate ligation (Cys-29 in the case of CPO) on the heme iron in the place of imidazole nitrogen, which is typical of most heme proteins [6]. The enzyme exists in two active forms: the acidic form was reported to catalyse halide-dependent and the neutral form halide-independent reactions, the transition between the two forms occurring between pH 3 and 5 [2]. The neutral form of CPO shows a broad pH optimum at around pH 5–6 where the enzyme is responsible, e.g., for various halide-independent oxidation reactions such as epoxidation of alkenes, *S*- and *N*-oxidations of sulfides and amines, respectively, as well as propargylic and other hydroxylation reactions [2–5,7–17]. In these reactions, the products are usually produced in highly enantioselective manners whenever a chiral centre is created in the product.

In the halide-independent oxidation reactions, CPO employs hydrogen peroxide as the natural source of oxygen in direct oxygen transfers to its substrates via an iron(IV) oxo porphyrin

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1381-1177/00/$ - see front matter
PII: S1381-1177(99)00093-4

radical cation (compound I). The main mechanistic difference between CPO and monooxygenases is that the latter require reduction with a cofactor and oxidation with molecular oxygen in the formation compound I [4,5,14,18]. Interestingly, CPO was previously reported to catalyse also the aerobic oxidation of various substrates with the combination of molecular oxygen as a primary oxidant and dihydroxyfumaric acid or ascorbic acid as a sacrificial reductant [13,14]. All the same, a reaction mechanism was proposed which involves the initial formation of hydrogen peroxide for the selective oxidation of the substrate [14]. In many synthetic applications of the enzyme, *tert*-butyl hydroperoxide is now replacing hydrogen peroxide as an oxygen donor. The use of *tert*-butyl hydroperoxide is reasonable because *tert*-butyl alcohol, the product from *tert*-butyl hydroperoxide, was previously shown to exert a stabilizing effect on CPO [18]. Moreover, CPO is deactivated by hydrogen peroxide and accordingly its concentration is critical for enzymatic oxygen transfers.

Aldehydes have a wide application area in synthetic organic chemistry. Publications on alcohol oxidation by CPO are rare [2,15,19–21]. The CPO-catalysed oxidations of allylic, propargylic and benzylic alcohols with hydrogen peroxide were reported to proceed selectively to the aldehyde stage [15,19]. However, the further oxidation of the aldehyde to the corresponding dicarboxylic acid was detected in the case of 5-hydroxymethylfurfural as a substrate [21]. In these works, possible stereochemical aspects in the product formation were not considered. On the other hand, stereochemistry cannot be neglected in modern synthetic chemistry where the prospects of biocatalysis are mainly seen in the selectivity of the catalyst connected to the possibility in working under mild conditions where stereochemical isomerization is slow or can be avoided. In the present work, the CPO-catalysed oxidation of primary alcohols (**1**)–(**9**) to the aldehydes in general and the stereochemistry accompanied with such reactions in particular have been studied (Fig. 1). Attention has been paid to the effect of organic solvents by performing reactions in homogenous aqueous solutions and in two-phase systems in the presence of hydrogen peroxide and organic peroxides as oxygen donors. For optimization, the oxidation of alcohol (**4**) to 2-hexenal was used as a model reaction. Because *trans*-alkenes are often substantially more stable than their *cis*-isomers the *trans*-isomer is more commonly commercially available. Thus, e.g., from the diastereomeric *cis*/*trans* pair of 2-hexenal the *trans*-isomer is available. Sometimes alkenals are merchandised as a mixture of diastereomers.

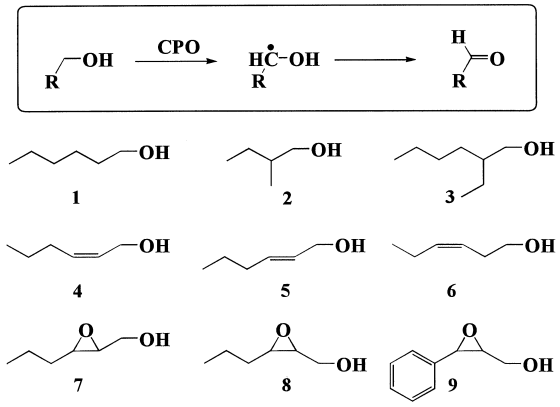

Fig. 1. Oxidation of alcohols (**1**)–(**9**) to the aldehyde.

2. Experimental

2.1. Material and methods

Partially purified CPO from *C. fumago* with a specific activity of 1300–1400 IU/mg was obtained as a solution (6.7 mg protein/ml) from Chirazyme (Urbana, IL, USA). Hydrogen peroxide (30% (w/w) in water), *tert*-butyl hydroperoxide (70% (w/w) in water), cumene hydroperoxide (80% (w/w) in cumene), *tert*-butyl peroxide, alcohols (**1**)–(**6**) and organic solvents were the products of Aldrich. The purities of the alcohols were 92% for (**4**), 96% for (**5**) and 98% for (**6**) the rest being mainly the corre-

sponding diastereomeric *trans*- or *cis*-alcohol. Racemic alcohols (**7**)–(**9**) were prepared from the corresponding allylic alcohols by epoxidation with 36% aqueous hydrogen peroxide in the presence of tungstic acid as a catalyst and characterized as previously described [22,23].

In the case of homogenous aqueous systems, the alcohol and the corresponding aldehyde in the reaction mixture were analyzed by taking samples (50 μl) at intervals followed by extraction with hexane (50 μl). The conversion of the reaction and the *cis*/*trans* ratio of the produced aldehyde or the enantiopurities of the compounds were determined from the hexane extract or directly from the organic layer of a two-phasic system by injecting a sample into GLC. The disappearance of the alcohol and the formation of the aldehyde against decane as an internal standard were followed.

GLC analysis was performed on a Perkin Elmer 8500 gas chromatograph equipped with a flame ionization detector and a CP-Chirasil-Dex CB (25 m) column. Good base-line separations in the chromatograms were recorded only for the enantiomers of (**8**) and for those of the aldehydes corresponding to alcohols (**7**) and (**8**). Thus, chiral GLC allows the enantiomeric excess (ee) determination for those counterparts. ^{1}H NMR spectra were recorded in $CDCl_3$ on a Jeol Lambda 400 Spectrometer using TMS as an internal standard. Chemical shifts are reported in δ ppm. MS spectra were recorded on a VG Analytical 7070E instrument equipped with a VAXstation 3100 M76 computer. Optical rotations were measured on a JASCO Model DIP-360 digital polarimeter at the wavelength of the sodium D-line. The temperature of the thermostated cuvette was maintained at 25°C by means of a water bath. Specific rotations are given in units of 10^{-1} deg cm^2 g^{-1}.

2.2. Oxidation of alcohols (1)–(9): general procedure

In the case of homogeneous systems, alcohol (**4**) (0.03 M) was dissolved in a citrate buffer (2 ml; 100 mM, pH 5.0) or in the same total volume of the buffer containing a known amount of a water soluble organic solvent. CPO (0.79 mg) was added. In the case of two-phasic systems, one of the alcohols (**1**)–(**9**) (final concentration 0.02–2.0 M) was dissolved in a known amount of a water insoluble organic solvent (or that of the mixture of organic solvents) and the enzyme was added in a citrate or acetate buffer (100 mM, pH 5.0) to make the total volume of 2 ml. The reaction was started by adding hydrogen peroxide (4.5 equivalents compared to the amount of an alcohol) or an organic peroxide (1.0–1.7 equivalents compared to the amount of an alcohol) in four to five equal portions during the course of the reaction. The solution was stirred at room temperature (25°C). The reactions were followed by the GLC method. The products were identified directly by analyzing samples taken from the organic layer of a two-phasic system using GLC-MS. Hydrogen peroxide and *tert*-butyl hydroperoxide were aqueous solutions. The water contents given in this work represent the water contents initially present in the reaction mixture.

For determining the absolute configuration of the less reactive alcohol enantiomer (2*S*,3*R*)-(**8**), the GLC chromatograms and the value of specific rotation were compared to those previously obtained for the lipase-catalysed resolution and to the literature data [24,25]. In other cases, the absolute configuration is unknown.

2.3. Gram-scale preparation of cis-2-hexenal

Citrate buffer (3 ml; 100 mM, pH 5.0) and hexane (12 ml) containing *cis*-2-hexen-1-ol (**4**; 0.48 g, 4.8 mmol) were combined and CPO (5.0 mg) was added. The reaction was started by adding *tert*-butyl hydroperoxide (4.8 mmol in four equal portions in 2 h). The reaction mixture was stirred at room temperature for 4 h before the reaction was stopped at 95% conversion by separating the organic phase from the aqueous phase. The aqueous phase was washed with ethyl acetate and hexane. The collected organic

phases were dried over Na_2SO_4 and the solvent was evaporated. GLC-MS gave the molecular peak of 98. The structure of *cis*-2-hexenal (0.34 g, 3.5 mmol) was confirmed by ^{1}H- and ^{13}C NMR spectroscopies. The *cis*/*trans* ratio of the product was 95:5 by the GLC method and 96:4 according to the ^{1}H NMR spectrum.

^{13}C NMR: 13.58 (C-6); 22.39 (C-5); 29.87 (C-4); 130.38 (C-3); 153.46 (C-2) and 191.16 (C-1), ^{1}H NMR: 0.93 (t, 3H, 6-H); 1.20 (m, 2H, 5-H); 2.50–2.60 (m, 2H, 4-H); 5.90–5.95 (m, 1H, 2-H); 6.56–6.62 (m, 1H, 3-H); 10.03 and 10.02 (d, 1H, $J = 8.2$ Hz, 1-H).

2.4. Gram-scale resolution of cis-2,3-epoxyhexanol

Citrate buffer (0.6 ml; 100 mM, pH 5.0) and hexane (12 ml) containing racemic *cis*-2,3-epoxyhexanol (**8**; 0.56 g, 4.8 mmol) were combined and CPO (5.0 mg) was added. The reaction was started by adding *tert*-butyl hydroperoxide (4.8 mmol in four equal portions in 2 h). The reaction mixture was stirred for 24 h at room temperature before the reaction was stopped at 50% conversion by separating the organic phase from the aqueous phase. The aqueous phase was washed with ethyl acetate and hexane. The collected organic phases were dried over Na_2SO_4 and the solvent was evaporated. The resolution products were separated by column chromatography on silica. Elution with hexane:acetone (9:1) gave (2*S*,3*S*)-epoxyhexanal (142 mg, 1.25 mmol; ee 40%, $[\alpha]_D^{25} = +4.3$ (*c* 4.8, $CHCl_3$)). GLC-MS gave the peak $M_r + 1 = 115$. The structure of the aldehyde was confirmed by ^{1}H- and ^{13}C NMR spectroscopies.

^{13}C NMR: 199.18 (C-1); 57.82 (C-2); 56.00 (C-3); 30.02 (C-4); 26.34 (C-5) and 13.66 (C-6). ^{1}H NMR: 0.95 (t, 3H, 6-H); 1.45–1.75 (m, 4H, 4-H and 5-H), 3.25 (m, 1H, 3-H); 3.31 (m, 1H, 2-H); 9.44 and 9.33 (d, 1H, $J = 5.2$ Hz, 1-H).

The unreacted (2*S*,3*R*)-epoxyhexanol (182 mg, 1.57 mmol; ee 40%, $[\alpha]_D^{25} = -2.0$ (*c* 2.9, $CHCl_3$)) was eluted next from the column with hexane:acetone (4:1) ($M_r + 1 = 117$ by GLC-MS). The value of specific rotation is in accordance with $[\alpha]_D^{20}$ (lit.) $= -10.0$ (*c* 0.37, $CHCl_3$) for the (2*S*,3*R*)-alcohol (ee > 92%) [25].

3. Results and discussion

3.1. The nature of an oxidant for alcohol oxidation

The CPO-catalysed oxidation of activated primary alcohols with hydrogen peroxide in phosphate buffers previously showed relatively low conversions, leading at best only to 30%–40% theoretical yields for the aldehyde [19]. Such low yields can be seen as one reason for low interest in producing aldehydes by CPO catalysis. The sensitivity of CPO for hydrogen peroxide was not taken into account in that work. On the other hand, the CPO-catalysed oxidation of 5-hydroxymethylfurfural was shown to proceed to a nearly complete conversion when hydrogen peroxide was added slowly with a H_2O_2-stat [21]. In the present work, attention was paid to the nature and the addition of an oxidant for the CPO-catalysed oxidation of (**4**) in an aqueous citrate buffer as a model reaction. The results are shown in Table 1.

Table 1
Oxidation of *cis*-2-hexen-1-ol by different peroxides in citrate buffer[a]

Oxidant (equiv.)[b]	Time (h)	Conversion (%)	Aldehyde (*cis*/*trans*)
H_2O_2 (4.5)	4	97	86/14
	24	98	51/49
H_2O_2 (4.5)[c]	4	88	89/11
^{t}BuOOH (1.2)	4	99	81/19
	24	99	44/56
^{t}BuOOH (1.7)	4	99	81/19
	24	99	38/62
$PhC(CH_3)_2OOH$ (1.2)	4	5	68/32
	24	10	52/48
^{t}BuOO^{t}Bu (1.2)	4	1	37/63
	24	< 5	28/72

[a] *cis*-2-Hexen-1-ol (0.03 M), the oxidant and CPO (0.79 mg) in citrate buffer (2 ml; 100 mM, pH 5.0) at 25°C.
[b] Total equivalents compared to the alcohol amount; addition in four small portions.
[c] Acetate buffer (2 ml; 100 mM, pH 5.0).

Interestingly, both hydrogen peroxide and *tert*-butyl hydroperoxide, when added in small portions, allowed practically complete oxidation to the aldehyde. Epoxide formation was expected but not observed. On the other hand, extensive *cis* to *trans* isomerization of 2-hexenal in Table 1 is evident with time. Taking the worst case, the *cis*/*trans*-ratio of 92/8 for the produced 2-hexenal in the first (reaction time 20 min) sample changed to that of 38/62 when alcohol (**4**) was oxidized with *tert*-butyl hydroperoxide for 24 h. The other oxidants were ineffective. Chemical oxidation of (**4**) was not observed in the absence of the enzyme when the reaction was followed for 3 days.

3.2. Solvent effects on alcohol oxidation; biphasic systems

In order to suppress the observed *cis* to *trans* isomerization of 2-hexenal, the mechanism of isomerization needs to be considered. A wide variety of catalysts in chemistry have been employed to bring about *cis*/*trans* interconversions, among them free radicals and acids [26]. A radical R-$\dot{C}$HOH (Fig. 1) was previously suggested to be on the reaction pathway from an alcohol to the aldehyde in CPO catalysis [20]. This radical can reversibly add to the carbon–carbon double bond of the aldehyde and cause isomerization. At pH 5.0, the possibility for acid-catalysed isomerization cannot be ruled out either. Thus, α,β-unsaturated carbonyl compounds are easily isomerized by acids through resonance-stabilized enol formation [H–O^+═C–C═C ↔ H–O–C═C–C^+], leading to double bond weakening and rotation around it. Expecting that one or both of these mechanisms explain the isomerization, it is clear that the phenomenon can be avoided by isolating the aldehyde from the aqueous environment immediately after formation. Typical of two-phase

Table 2
Oxidation of *cis*-2-hexen-1-ol by hydrogen peroxide in homogeneous or two-phasic solvent mixtures of a buffer (100 mM, pH 5.0) and organic solvents[a]

Row	Reaction medium (composition)	Time (h)	Conversion (%)	Aldehyde (*cis*/*trans*)
1	Acetone/Citrate (3/7)	4	90	90/10
		24	85	58/42
2	Toluene/Citrate (1/1)	4	40	93/7
		24	70	88/12
3	EtOAc (saturated with citrate)	4	35	94/6
		24	40	93/7
4	EtOAc/Citrate (1/1)	24	50	89/11
5	EtOAc/Acetate (1/1)	4	25	92/8
		24	40	84/16
6	EtOAc/Acetate (2/3)	4	20	93/7
		24	35	92/8
7	EtOAc/Acetate (1/4)	4	15	90/10
		24	55	88/12
8	EtOAc/Citrate/Acetone (2/1/1)	4	25	93/7
		24	45	91/9
9	EtOAc/Citrate/Acetone (2/1.3/0.7)	4	30	93/7
		24	50	91/9
10	EtOAc/Citrate/^{t}BuOH (2/1/1)	4	25	92/8
		24	40	91/9
11	EtOAc/Citrate/^{t}BuOH (2/1.5/0.5)	4	30	92/8
		24	45	91/9
12	EtOAc/Citrate/DMF (2/1/1)	4	5	39/61
		24	5	44/56

[a] *cis*-2-Hexen-1-ol (0.03 M), H_2O_2 (4.5 equiv.) and CPO (0.79 mg) in the solvent system (2 ml) at 25°C.

systems, the enzyme tends to exist in the aqueous phase and hydrophobic organic substrates and/or products predominantly in the organic phase expecting that the latter phase is correctly valid, i.e., an appropriate two-phase system will solve the isomerization problem.

Solvent effects on the CPO-catalysed oxidation of *cis*-2-hexen-1-ol (**4**) with hydrogen peroxide and *tert*-butyl hydroperoxide were studied. The reaction medium consisted from a water-soluble organic solvent (homogenous system), a water-insoluble organic solvent (two-phasic system) or a mixture of organic solvents (two-phasic system) combined with aqueous buffers at pH 5.0. The results are shown in Tables 2 and 3. Acetone in a citrate buffer does not affect the tendency for *cis* to *trans* isomerization compared to the reactions in the neat buffer (Tables 1 and 2; row 1)) On the other hand, the *cis*/*trans* ratios in Tables 2 and 3 clearly indicate that the suppression of isomerization is effective in most two-phase systems studied, low water contents (30% (v/v) or less; Table 2; row 3 and Table 3; rows 2–6, 11 and 12) being most preferable. At higher water contents, (Table 2; rows 2, 4–7 and Table 3; rows 1, 7, 8 and 13) isomerization starts to be seen at longer reaction times. The presence of acetone or *tert*-butyl alcohol in two-phasic systems enhances the solubility of 2-hexenal into the aqueous phase, leading to slight tendency for isomerization in the CPO-catalysed oxidation of (**4**) with *tert*-butyl hydroperoxide (Table 3; rows 9 and 10). The effect of the same cosolvents is negligible when used for oxidation with hydrogen peroxide (Table 2; rows 8–11). The results

Table 3
Oxidation of *cis*-2-hexen-1-ol by *tert*-butyl hydroperoxide in two-phasic solvent mixtures of a buffer (100 mM, pH 5.0) and organic solvents[a]

Row	Reaction medium (composition)	Time (h)	Conversion (%)	Aldehyde (*cis*/*trans*)
1	Toluene/Citrate (1/1)	4	70	93/7
		24	70	91/9
2	EtOAc (saturated with citrate)	4	60	94/6
		24	90	91/9
3	EtOAc/Citrate (49/1)	4	65	90/10
		24	97	92/8
4	EtOAc/Citrate (9/1)	4	60	93/7
		24	99	92/8
5	EtOAc/Citrate (4/1)	4	80	94/6
		24	99	93/7
6	EtOAc/Citrate (7/3)	4	90	93/7
		24	99	92/8
7	EtOAc/Citrate (1/1)	4	96	94/6
		24	99	88/12
8	EtOAc/Acetate (1/1)	4	93	94/6
		24	98	87/13
9	EtOAc/Citrate/Acetone (2/1.5/0.5)	4	60	94/6
		24	99	90/10
10	EtOAc/Citrate/^{t}BuOH (2/1.5/0.5)	4	75	–
		24	99	90/10
11	Hexane (saturated with citrate)	0.22	67	93/7
		24	99	93/7
12	Hexane/Citrate (4/1)	0.22	79	94/6
		4	99	92/8
13	Hexane/Citrate (1/1)	4	99	94/6
		24	99	84/16

[a] *cis*-2-Hexen-1-ol (0.03 M), ^{t}BuOOH (1.7 equiv.) and CPO (0.79 mg) in the solvent system (2 ml) at 25°C.

further indicate that the observed *cis* to *trans* isomerization is not a CPO-catalysed process but rather a process taking place in an aqueous environment by some products (radicals) of CPO catalysis and/or being caused by reaction conditions. The stabilizing effect of *tert*-butyl alcohol for the oxidation of (**4**) by hydrogen peroxide is not clearly seen in the present work (Table 2; rows 10 and 11). The reason may be the high *tert*-butyl alcohol contents in these experiments [18].

The solubility behaviour of hydrogen peroxide and *tert*-butyl hydroperoxide in two-phasic systems causes a distinct difference for the CPO-catalysed oxidation of alcohols. Hydrogen peroxide is mainly dissolved in the water phase whereas *tert*-butyl hydroperoxide is partitioned between the two phases. Accordingly, the most probable reason for the low conversions of Table 2 is the difficulty in controlling the oxidant concentration of the aqueous phase. On the other hand, the results in Table 3 indicate that reactions in the presence of *tert*-butyl hydroperoxide practically proceed to completion in 24 h except in the case of toluene (row 1) as an organic phase. Toluene can also serve as a substrate for CPO [3]. Reactivity (conversion reached at certain time) increases with increasing water content (rows 2–8). Due to the isomerization properties of the aldehyde, it is advantageous, however, to work at low water levels as mentioned above. Hexane is clearly the most favourable organic solvent for the CPO-catalysed oxidation of (**4**), leading to fast oxidations with clean stereochemistry (Table 3; rows 11 and 12).

Even in an appropriate two-phasic system, the substrate alcohol (**4**) and the produced aldehyde can be slightly partitioned to the water phase. The progress for the CPO-catalyzed oxidation of (**4**) by *tert*-butyl hydroperoxide in the mixture of hexane:citrate buffer (4:1) indeed shows that at the end the amount of the aldehyde in the organic phase does not fully correspond to what is expected from the disappearance of (**4**) (Fig. 2; filled signs). This makes the exact kinetic analysis somewhat troublesome. It can be expected, however, that for preparative purposes the aldehyde can be completely extracted from the water phase to organic solvents. The stereochemistry of the enzymatic aldehyde formation (Tables 2–5), on the other hand, can be misleading if the *cis*- and *trans*-isomers are differently partitioned between the two phases. According to the aldehyde obtained through the gram-scale oxidation of (**4**) followed by the separation of the product (Section 2) and to the results obtained by taking samples from the organic phase of the two-phasic system this is not a problem though. We have not studied the possibility that part of the aldehyde, which stays in the water phase can further participate in acetal formation. According to the CPO-catalyzed oxidation of (**4**) in hexane (saturated with a citrate buffer), the substrate is completely transformed to the aldehyde (Fig. 2; open signs).

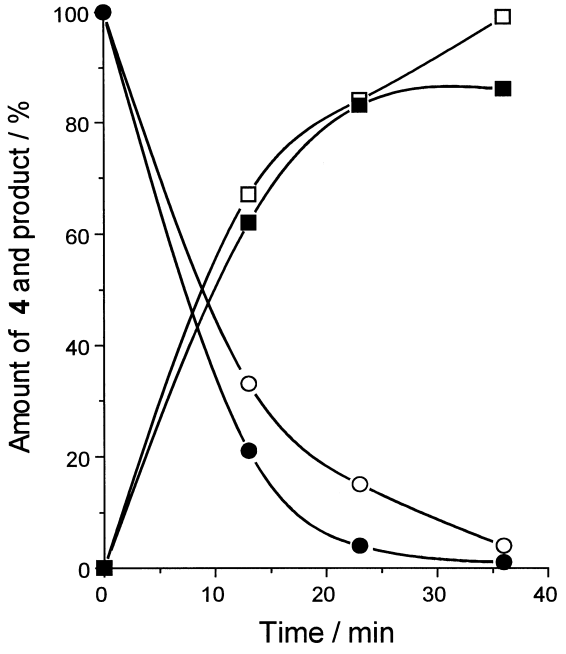

Fig. 2. Progress for the CPO-catalysed oxidation of (**4**; 0.03 M) with *tert*-butyl hydroperoxide (1.7 equiv.): (○) unreacted (**4**) and (□) produced aldehyde in hexane staurated with citrate buffer and (●) unreacted (**4**) and (■) produced aldehyde in hexane:citrate buffer (4:1) for the oxidation of (**4**) in hexane:citrate buffer (4:1).

Table 4
Oxidation of *cis*-2-hexen-1-ol by *tert*-butyl hydroperoxide in a mixture of hexane:citrate buffer (4:1; 100 mM, pH 5.0)[a]

[Substrate] (M)	Time (h)	Conversion (%)	Aldehyde (*cis*/*trans*)
0.03	2.5	93	93/7
0.05	2.5	99	89/11
0.10	2.5	99	89/11
0.20	2.5	70	93/7
	4.0	96	94/6
0.40	2.5	75	92/8
	4.0	93	93/7
1.00	2.0	55	92/8
	5.5	92	91/9
2.00	2.0	35	90/10
	5.0	55	92/8

[a] *cis*-2-Hexen-1-ol, ^{t}BuOOH (1.0 equiv.) and CPO (0.79 mg) in the medium (2 ml) at 25°C.

3.3. Effect of substrate concentration on alcohol oxidation

Substrate or product inhibition and low solubilities of the reagents are often the most serious limitations of enzymatic methods in organic chemistry, allowing the use of only dilute solutions. The use of an appropriate two-phasic system ensures that the substrate and/or products are removed and concentrated in a catalyst-free organic phase. In the present work, this ensures that product isomerization is suppressed. It is also justifiable to assume that alcohol concentrations higher than 0.03 M can be used in the two-phasic systems. That was improved for the CPO-catalysed oxidation of *cis*-2-hexen-1-ol (**4**; 0.03–2.0 M) in the mixture of hexane and a citrate buffer (4:1) (Table 4). Although increasing substrate concentrations cause rate retardations reactions still are relatively fast up to the concentration of 1.0 M (**4**). The *cis*/*trans* ratio is practically unaffected by substrate concentration.

3.4. Reuse of CPO

As already stated, the use of an appropriate two-phasic system ensures that the substrate and/or products are removed and concentrated in a catalyst-free organic phase. This led to an idea that the enzyme could be easily reused by replacing the organic phase with a fresh solution of the substrate and the oxidant after the reaction. In the reuse, however, *tert*-butyl alcohol starts to concentrate in the aqueous phase. Although *tert*-butyl alcohol exerts a stabilizing

Table 5
Oxidation of alcohols (**1**)–(**9**) by *tert*-butyl hydroperoxide in a mixture of hexane:citrate buffer (4:1; 100 mM, pH 5.0)[a]

Alcohol (M)	Hexane:Citrate	Time (h)	Conversion (%)	Product (*cis*/*trans* or ee)
1 (0.02)	(49/1)	5	62	
(0.03)	(4/1)	6.5	81	
(0.02)	(49/1)[b]	5	14	
2 (0.02)	(19/1)	24	0	
3 (0.02)	(1/1)	24	0	
4 (0.4)	(4/1)	5	95	95/5
5 (0.03)	(19/1)	5	99	1/99
6 (0.03)	(4/1)	6	97	99/1
7 (0.02)	(19/1)	5	70	–; ee_{CHO} 15
8 (0.02)	(19/1)	2	50	ee_{OH} 30[c]; ee_{CHO} 40[c]
(0.4)	(19/1)	24	51	ee_{OH} 43; ee_{CHO} 41
(0.02)	(9.5/9.5/1)[d]	24	20	ee_{OH} 25; ee_{CHO} 45
9 (0.03)	(4/1)	21	46	–; –

[a] Alcohol, ^{t}BuOOH (1.7 equiv.) and CPO (0.79 mg) in the medium (2 ml) at 25°C.
[b] Ethyl acetate:citrate.
[c] Absolute configurations (2*S*, 3*R*) for the alcohols and (2*S*, 3*S*) for the aldehyde.
[d] Hexane:ethyl acetate:citrate.

effect on CPO at concentrations up to 30%, it has been shown to be harmful at higher concentrations [18]. Following the above protocol for the oxidation of (**4**) in hexane:citrate buffer (4:1; 0.20 M), CPO preserved its catalytic properties at least in six reuses when after every use *tert*-butyl alcohol was removed from the water phase by extracting with ethyl acetate and hexane. Thus, 50% conversions in 2 h were reached in six reuses the *cis*/*trans* ratios varying between 89/11 and 92/8.

3.5. Substrate specificity of alcohol oxidation

The substrate specificity of CPO for the oxidation of alcohols (**1**)–(**9**) to the aldehydes with *tert*-butyl hydroperoxide in the mixture of hexane or ethyl acetate with a citrate buffer is illustrated in Table 5. The two-phase systems allow the oxidation of hexan-1-ol (**1**) to hexanal. The reaction is critical to the nature of the organic phase, hexane (rows 1 and 2) being usable compared to ethyl acetate (row 3). This result disproves the previous statement that CPO oxidizes only activated primary alcohols, yielding 2%–4% conversions for the oxidation of methanol and ethanol [19]. Branched-chain alcohols (**2**) and (**3**) were not substrates for CPO. This observation is in accordance with the sterically constrained active site of the enzyme. The oxidations of alcohols (**4**)–(**6**), smoothly proceed to the aldehydes. Isomerization was not observed in the case of *trans*-2-hexen-1-ol (**5**) and *cis*-3-hexen-1-ol (**6**) under the reaction conditions. This result suggests the importance of the conjugated double bond system of *cis*-2-hexenal for isomerization. Epoxy alcohols (**7**)–(**9**) have also been oxidized to the aldehydes. This is the first time when enantioselectivity is observed for alcohol oxidations using CPO. The question is about typical enzymatic kinetic resolution reactions where one of the enantiomers reacts faster to the aldehyde than the other. For the oxidation of racemic (**8**), the faster reacting (2*R*,3*S*)-alcohol enantiomer yields the aldehyde with the (2*S*,3*S*) absolute configuration leaving the (2*S*,3*R*)-enantiomer less reactive. Under the reaction conditions, however, enantioselectivity stays relatively low. Moreover, the ee values for the produced aldehyde tend to drop with time. However, the method is promising for the preparation of the less reactive alcohol enantiomers and work on the subject is now in progress in our laboratory.

3.6. Oxidation in gram-scale

Most of the reactions of the present work were studied by following the reactions in small scale without purification of the products. To show the usability of the method, *cis*-2-hexen-1-ol (**4**) and *cis*-2,3-epoxyhexan-1-ol (**8**) were successfully oxidized in gram-scale in hexane:citrate buffers (4:1 and 19:1, respectively). Clearly and as shown in Section 2, *cis*-2-hexenal can be prepared in relatively pure form, containing the acid as a minor impurity according to the GLC method. Evidently, the acid is obtained by slow chemical oxidation of the aldehyde. How long the *cis*-isomer is stable was not tested in this work. For the oxidation of (**8**), enantiomerically enriched counterparts (one as an unreacted alcohol and the other as a produced aldehyde) were separated by the aid of column chromatography. In the reaction mixture, some racemization was observed although the aldehyde well withstood the separation in the column.

4. Conclusions

In the present work, the biphasic systems of hexane or ethyl acetate mixed with acetate or citrate buffers (pH 5.0) have been used for the preparation of aldehydes from the corresponding alkanols, *cis*- and *trans*-alkenols and 2,3-epoxyalcohols by CPO catalysis. As the benefit of the biphasic systems, the *cis* to *trans* isomerization of *cis*-2-alkenals can be slowed down effectively keeping the water level low. On the other hand, in the 1:1 mixtures some isomeriza-

tion is already observed after 1 day. The present work indicates that the stereochemical *cis* to *trans* isomerization is connected to the aqueous environment. Similarly, extensive racemization is evident for the produced epoxyaldehydes with time. Further work for studying isomerization in more detail is in progress in our laboratory.

Another benefit of the biphasic systems is the possibility to use high substrate concentrations up to 2 M. The enzyme was reused several times by replacing the organic phase, which was concentrated with respect to the produced aldehyde with the fresh solution of a substrate. In these systems, the nature of the oxidant is important, *tert*-butyl hydroperoxide being most applicable. It is partitioned between the two phases and it produces *tert*-butyl alcohol, which is known to exert a stabilizing effect on the enzyme [18].

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