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Journal of Molecular Catalysis B: Enzymatic 4 (1998) 101–109

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Regioselective bromohydroxylation of alkenes catalyzed by chloroperoxidase: Advantages of the immobilization of enzyme on talc

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Received 19 April 1997; revised 17 July 1997; accepted 17 July 1997

Abstract

The bromohydroxylation of alkenes catalyzed by chloroperoxidase (CPO) from the mould *Caldariomyces fumago* adsorbed on different types of talc or in reverse micelles was compared to that of the same reaction catalyzed by the free enzyme in buffer. High reactivity was observed in all media, but the reaction was optimized with an enzyme–talc combination that produced the halohydrin with no oxidative by-products in a Markovnikov-type regioselective addition. The reaction was facilitated by the use of this solid and a recyclable biocatalyst, which gave rise to the halohydrin in a quantitative yield. The protective influence of the talc (hydrophilic or hydrophobic) with respect to hydrogen peroxide enabled use of large amounts of oxidizing agent and substrate, opening perspectives for CPO in the synthesis of fine chemicals. © 1998 Elsevier Science B.V.

Keywords: Chloroperoxidase; Talc; Halohydroxylation; Adsorption

1. Introduction

Bio-oxidation reactions are widespread in nature, and they have been the subject of a large number of studies [1,2]. The enzymes involved in such reactions are often heme-based proteins containing a metal atom (Fe, Mn, Cu, etc.) in combination with various types of cofactors [3]. They are thus complex systems that are not readily suited for industrial applications. However, chloroperoxidase (CPO, EC 1.11.1.10) derived from *Caldariomyces fumago* is a hemoprotein whose prosthetic group contains a ferriprotoporphyrin IX, which has the advantage of self-regeneration at the end of the catalytic cycle [4,5]. Although the details of the reaction mechanism have yet to be elucidated, this enzyme has a wide range of applications with a variety of potential substrates using low cost oxidizing agents such as organic or inorganic peroxides [6]. In the presence of halide ions, it can catalyze halogenation [7] and halohydroxylation of alkenes [8], while halide-independent reactions include the epoxidation of alkenes [9], oxidation of sulfides [10], alcohols [11], amines [12] and substituted indoles [13], *N*-dealkylation [14] and linking of aromatic compounds [15] (see Scheme 1). The CPO from *Caldariomyces fumago* is an attractive catalyst for the stereose-

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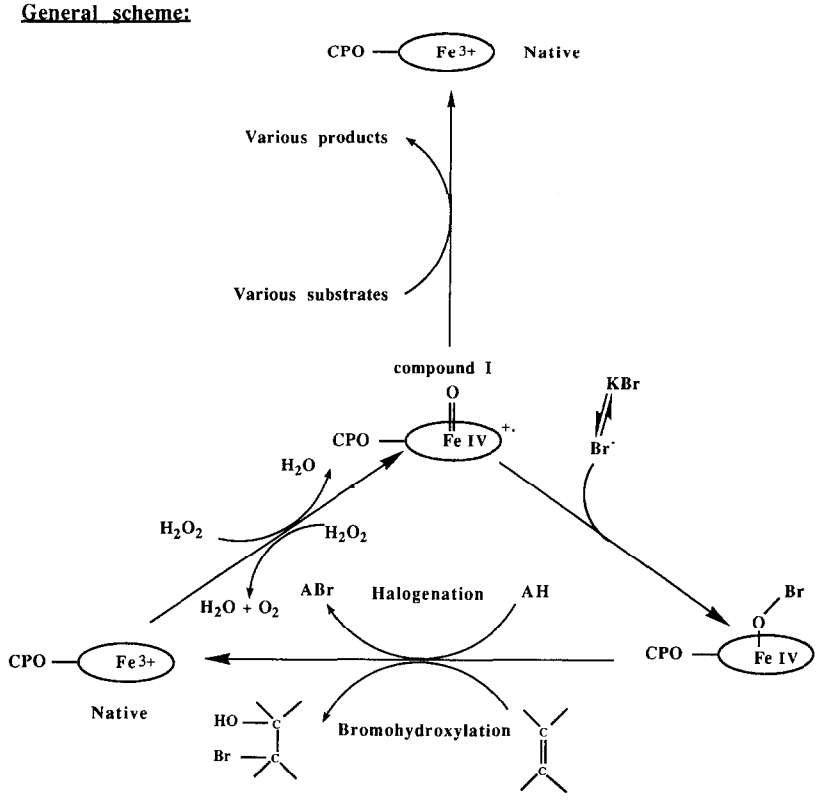
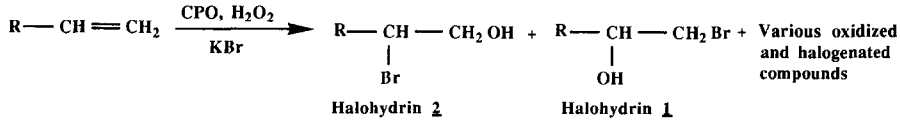
PII S1381-1177(97)00026-X

lective oxidation of sulfides, indoles, and alkenes [10,13,18]. However this enzyme appears to be nonstereoselective in halogenation and halohydroxylation. To our knowledge, only one regioselective enzymatic halogenation reaction is known: the conversion of indole to 7-chloroindole by chloroperoxidase from the bacterium *Pseudomonas pyrrocinia* [16]. The disadvantage of the CPO is inactivated by elevated temperature and it is expensive [17]. Main drawback is the deactivation by high concentrations of hydrogen peroxide. In practice, hydrogen peroxide thus has to be added during the reaction in small amounts [13,18]. Other authors suggested that reactions have been carried out in micellar media [19] or in the presence of organic solvents, which are either miscible or not in aqueous buffer, using either free CPO or the enzyme immobilized on a solid support [20]. We have recently reported the use of talc, a low cost abundant mineral, as a solid support for lipases [21], lipoxygenases [22] and horseradish peroxidase [23]. In view of the potential of the CPO–talc system as a biocatalyst, we studied the





Scheme 1. Competing reactions in the postulated catalytic cycles of chloroperoxidase.

immobilization of CPO on talc and determined optimum conditions for the catalysis of various reactions, including the halogenation of monochlorodimedone and the oxygenation of indole [24]. To extend its application to the synthesis of fine chemicals, we present here our results on the bromohydroxylation of alkenes. We describe the advantages and limits of use of the CPO–talc system compared to that of the free enzyme in both aqueous and microemulsion media (μE).

2. Experimental

2.1. Materials

The chloroperoxidase (CPO, EC 1.11.1.10) from *Caldariomyces Fumago*, lyophilized powder, was from Sigma. One unit of chloroperoxidase catalyzed the conversion of 1.0 μmol of monochlorodimedon to dichlorodimedon at pH 2.75, 25°C in the presence of KCl and H_2O_2. The used enzyme contained one unit per μg.

Styrene, trans β methyl styrene, α methyl styrene, 3(4) methyl styrene, 1-heptene, cis-3-heptene, 1-octene, 1-nonene, allylbenzene were commercial products from Aldrich. Brij 35 (polyoxyethylene 23 lauryl ether) and hydrogen peroxide were obtained from Fluka. Isooctane was supplied by Merck. The various mineral supports were kindly donated by the group Talc Europe (Toulouse). Manufacturer's references were used (Table 2).

2.2. Apparatus

UV spectra was recorded on a Hewlet–Packard HP 8450 spectrophotometer. Temperature and stirring were controlled using the adapted HP 89100 A controller. Identification of the reaction products was made by determining gas-chromatograph mass spectrometer (GC–MS) equipped with an apolar column (CP wax 52 CB). The column temperature was programmed from 70°C to 200°C at a rate of 10 C°/min and then held at 200°C for 10 min. The mass spectrometer operated in electron impact mode at 70 eV.

2.3. Methods

2.3.1. Bromohydroxylation of alkenes

2.3.1.1. By free CPO in phosphate buffer (see Table 1 and Fig. 1). 50 μl of ethyl alcoholic solution of alkenes (1 M) and 375 μl of KBr solution (2×10^{-1} M) in 10 ml of phosphate buffer (10^{-1} M, pH 4.2) were added 2 ml of phosphate buffer containing 25 μg of free CPO. The reaction was started by adding 375 μl solution of H_2O_2 (2×10^{-1} M) at a rate of 7.5 μl/min. After 1 h, the products were extracted by 2 × 25 ml of ether. The organic phases were

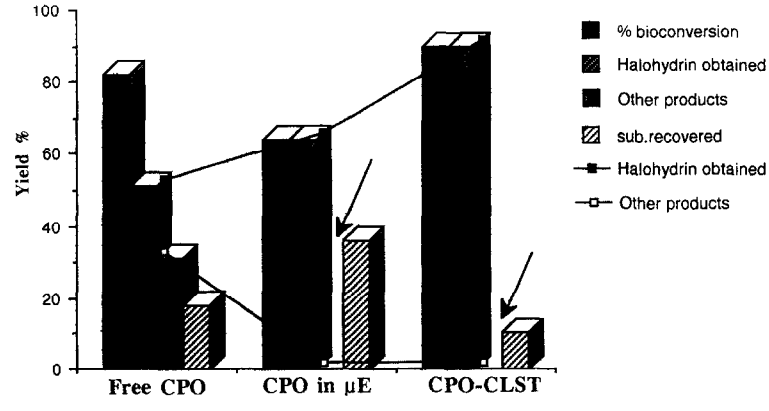


Fig. 1. Bromohydroxylation of styrene at pH 4.2. For details see Section 2.

Table 1
Bromohydroxylation of different alkenes at pH 4.2 [a]

Entry	Substrate	Medium	Bioconversion [b] (%)	Halohydrin **1** (%)	Other products [c] (%)
1	3(4)-methyl styrene	buffer	100	90	10
2		μE	94	94	00
3		talc	95	95	00
4	trans-β-methyl styrene	buffer	100	87	13
5		μE	83	83	00
6		talc	90	90	00
7	α-methyl styrene	buffer	100	87	13
8		μE	90	90	00
9		talc	92	92	00
10	1-heptene	buffer	65	40	25
11		μE	52	52	00
12		talc	48	48	00
13	cis-3-heptene	buffer	30	—	30
14		μE		no reaction	
15		talc		no reaction	
16	1-octene	buffer	77	57	20
17		μE	41	41	00
18		talc	45	45	00
19	1-nonene	buffer		no reaction	
20		μE		no reaction	
21		talc		no reaction	
22	allylbenzene	buffer		no reaction	
23		μE		no reaction	
24		talc		no reaction	

[a] For details see Section 2.
[b] Yield determined at equilibrium.
[c] Total percentage of other products.

dried by anhydrous Na_2SO_4, filtered and evaporated in vacuo. The residual incolore oil was characterized and analyzed by chromatography GC–MS.

2.3.1.2. By free CPO in micellar medium (see Table 1 and Fig. 1). The micellar medium consists in isooctane (81.6%), 1-propanol (16%), Brij 35 (0,4%) and phosphate buffer (2%).

To the suspension of Brij 35 (0,1 g) in isooctane (17.3 g) and 1-propanol (4.2 g) containing 50 μl of organic solution of alkene (1 M) were added 250 μl of aqueous solution of KBr (3×10^{-1} M) and 100 μl solution phosphate buffer of CPO (6 μM). The mixture was stirred vigorously to obtain a clear solution. The enzymatic reaction was started by addition of 250 μl H_2O_2 solution (3×10^{-1} M) at a rate of 25 μl/15 min. After about 3 h, the reversed micellar medium was destroyed by addition of 50 ml of water. The aqueous phase was extracted by 2×25 ml ether. The organic solvents were dried by anhydrous Na_2SO_4, filtered and evaporated in vacuo to give an incolore oil. It was analyzed by chromatography GC–MS.

2.3.1.3. By CPO–CLST in phosphate buffer (see Table 1 and Fig. 1). To 80 mg of CLST were added 20 ml of phosphate buffer (10^{-1} M, pH 4.2) and 150 μl of enzyme solution (12 μM). The mixture was stirred at 15°C for 90 min. It was then centrifuged and the UV spectrum of supernatant recorded. The pellet was washed twice with water (10 ml) to remove any non absorbed enzyme. The amount of enzyme bound to the support was calculated from the UV absorbance by subtracting the absorbance of the supernatant plus washing from the initial ab-

Table 2
Adsorption capacity as a function of the physicochemical properties of various talcs

Talcs samples	Physicochemical properties of talcs [b]			Adsorption ratio [c]	
	B.E.T ($m^2\ g^{-1}$)	d50 (μm)	chlorite %	pH 3	pH 6
15 M00	8.6	3.7	5	8	4
C300	6	3.8	99.5	7	1.5
CLST [a]	10	9	—	2.5	1

[a] Initial mixture of talc and chlorite (70:30) calcined at 1100°C for 1 h.
[b] Abbreviations: B.E.T. = specific surface area; d50 = grain size.
[c] Microgram of enzyme per milligram of support (maximum adsorption).

sorbance of the enzyme solution ($\varepsilon_{403\,nm}$ = 75 300 $M^{-1}\ cm^{-1}$) [4]. The amount of immobilized protein is 25 μg.

To this CPO–CLST pellet was added 20 ml of phosphate buffer (10^{-1} M, pH 4.2), 375 μl of KBr solution (2×10^{-1} M) and 50 μl ethyl alcoholic alkene solution (1 M). The reaction was started by adding 375 μl H_2O_2 solution (2×10^{-1} M). After 3 h, the mixture was centrifuged at 5000 rpm and the products were extracted from the supernatant with 2 × 25 ml ether. The organic phases were dried by anhydrous Na_2SO_4, filtered and evaporated in vacuo. The residual incolore oil was characterized and analyzed by chromatography GC–MS.

2.3.2. Bromohydroxylation of styrene by various CPO–talc pellets

2.3.2.1. Preparation of the CPO–talc pellet. 15 mg mineral support were added to 300 μl of enzyme solution (12 μM) in 5 ml phosphate buffer (10^{-1} M) at a selected pH. The mixture was stirred at 15°C for 90 min. It was then centrifuged and the UV spectrum of the supernatant was recorded. The pellet was washed twice with water (2 ml) to remove any non absorbed enzyme. The amount of enzyme bound to the support was calculated from the UV absorbance by subtracting the absorbance of the supernatant plus washing from the initial absorbance of the enzyme ($\varepsilon_{403\,nm}$ = 75300 $M^{-1}\ cm^{-1}$) [4]. In these conditions the amount of immobilized protein is given in Table 2.

2.3.2.2. Specific activities of various CPO–talc associations (see Fig. 2 and Fig. 3). To a mixture of 50 μl ethyl alcoholic styrene solution (5×10^{-2} M) and 50 μl KBr solution (2×10^{-1} M) in 2.5 ml phosphate buffer (10^{-1} M) at a selected pH containing an amount of CPO–talc pellet equivalent at 1 μg of CPO, were added 100 μl of solution of hydrogen peroxide (10^{-1} M). The enzyme activity was calculated from the disappearance of the substrate at 25°C determined by measuring the absorbance at 246 nm (ε = 7500 $M^{-1}\ cm^{-1}$).

2.3.2.3. Influence of hydrogen peroxide concentration (see Fig. 4). To a mixture of 50 μl ethyl alcoholic styrene solution (5×10^{-2} M) and 50 μl KBr solution (2×10^{-1} M) in 2.5 ml of phosphate buffer (10^{-1} M) at selected pH, containing an amount of CPO–talc equivalent at 1

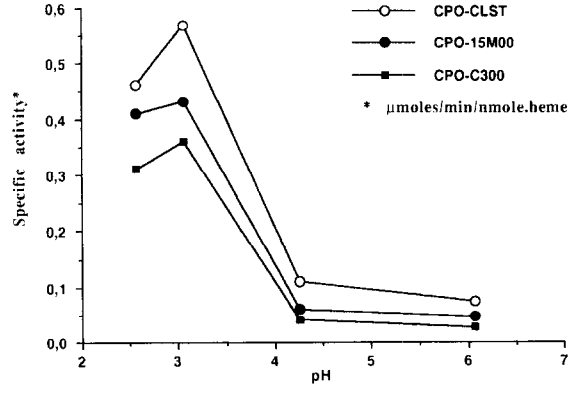


Fig. 2. Specific activities as a function of pH. Styrene is used as substrate: for details see Section 2.

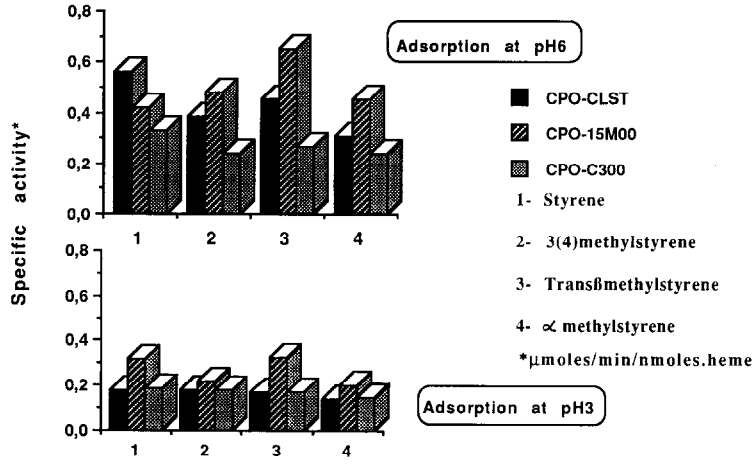


Fig. 3. Specific activities of CPO at pH 3 adsorbed onto different varieties of talc. For details see Section 2.

µg of CPO or 1 µg of free CPO, were added 40 µl hydrogen peroxide solution (10^{-1} M). The rate of disappearance of styrene at 25°C was determined by measuring the absorbance at 246 nm ($\varepsilon = 7500$ M^{-1} cm^{1}). The experience is repeated with addition of 50 µl hydrogen peroxide solution (10^{-1} M). Each point of the curve (Fig. 4) is determined from a novel experience by addition of increasing amounts of solution of hydrogen peroxide: 60, 70, ..., to 120 µl.

2.3.2.4. Influence of increasing amount of styrene (see Fig. 5). To a mixture of 50 µl ethyl alcoholic styrene solution (5×10^{-2} M) and 50 µl KBr solution (2×10^{-1} M) in 2.5 ml of phosphate buffer (10^{-1} M) at selected pH, containing an amount of CPO–talc equivalent at 1 µg of CPO or 1 µg of free CPO, were added 80 µl hydrogen peroxide solution (10^{-1} M). After the disappearance of styrene at 25°C determined

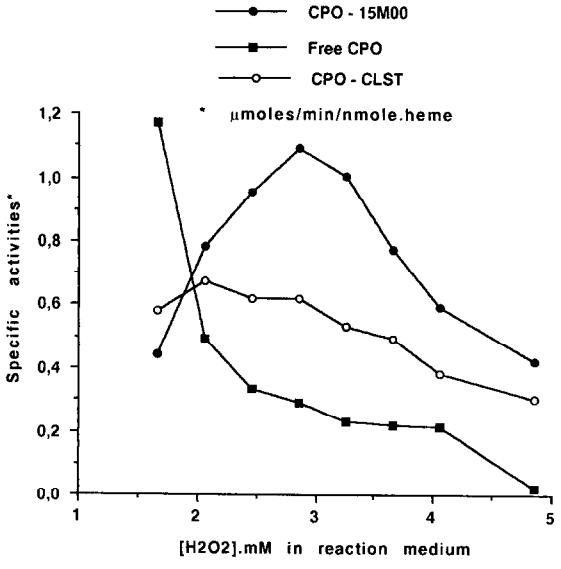


Fig. 4. Influence of the hydrogen peroxide concentration. For details see Section 2.

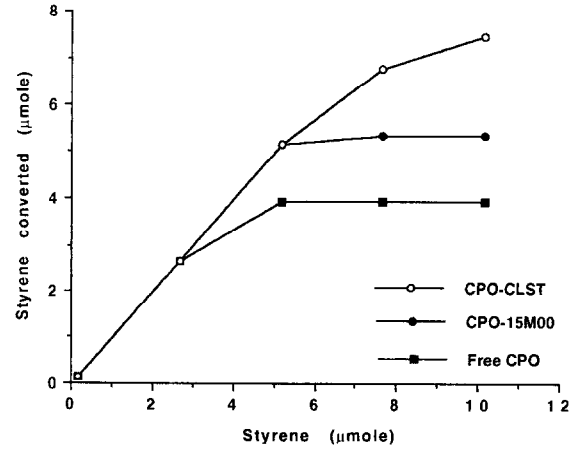


Fig. 5. Influence of increasing amounts of styrene. For details see Section 2.

by measuring the absorbance at 246 nm (ε = 7500 M^{-1} cm^{-1}), an equal aliquot of substrate and KBr solution were added. This procedure was repeated many times (see Fig. 5).

3. Results and discussion

The halohydrins led directly or indirectly (via the epoxide) to a variety of polyfunctional compounds of potential interest [25,26]. The alkene series, which readily undergo addition reactions, represent ideal substrates for biohalohydroxylation [27–31]. However, all reactions present various limitations for biocatalysis, especially the insolubility of the substrates in aqueous media, enzyme denaturation, poor yields and low regioselectivity.

3.1. Interest of the CPO–talc system for bromohydroxylation of styrene

Our previous studies [22,24] showed that immobilization of CPO onto a hydrophilic talc such as CLST (calcined talc, Table 2) [33] produced a solid biocatalyst with high activity for the halogenation of monochlorodimedon. Initially, we compared halohydroxylation using this system to that of the free enzyme in buffer or micellar medium under optimized conditions. According to the literature data [30,31], a pH of 4.2 was used (usual pH of the halohydroxylation by free CPO).

It can be seen from the results summarized in the histogram of Fig. 1 that the CPO–CLST system was the most efficient biocatalyst for bromohydroxylation of styrene. The biotransformation yield (90%) was comparable to that of the free enzyme (82%) and considerably higher than that observed in the micellar medium (65%). The main interest of the CPO–CLST system lay in its regioselectivity, as in contrast to that observed with the free enzyme (51% halohydrin, 31% by-products), it gave rise to 90% halohydrin with no by-products. Furthermore, only the halohydrin **1** (Markovnikov addition) was synthesized, what is not always the rule with the native enzyme [27,28] but in concordance with the formation of HOBr (see Scheme 1).

3.2. Bromohydroxylation of different alkenes

Table 1 lists the results obtained for the bromohydroxylation of other alkenes using the CPO–CLST system. It can be seen that the reactions were regioselective with bioconversion yields as high as those for comparable systems. However, the catalytic potential of the CPO–CLST system was found to depend on the chemical nature of the substrate. The reaction was inhibited by substrates lacking the conjugated system (examples 22–24). However, the presence of a phenyl group and conjugation was not mandatory as 1-heptene and 1-octene gave rise to the corresponding halohydrins in 48% and 45% yield respectively (examples 12 and 18) for CPO–CLST, versus 52% and 41% (examples 11 and 17) for CPO in microemulsion medium. However, the reaction was markedly inhibited for substrates with longer chains (examples 20 and 21), and steric hindrance on unsaturation (examples 14 and 15). These differences in reactivity, which were not readily explicable, were akin to those reported by Hager et al. [32] for the epoxidation of ω-bromoalkenes by CPO. It is of particular interest that since the CPO–CLST biocatalyst is solid and insoluble in the medium, the bromohydrin is readily recovered by simple filtration, which also enables recycling of the catalyst. Although CPO in the microemulsion medium had comparable efficacy, the presence of surfactant hinders purification of the bromohydrin and effectively prevents recycling of the enzyme. The drawback of the CPO–talc system is that being heterogeneous, the reaction takes three times longer than with free enzyme.

3.3. Reaction parameters

We recently showed that the enzymatic activity of CPO–talc systems depended on the pH during immobilization and the chemical compo-

sition of the talc [24]. Talc being an hydrated magnesium phyllosilicate [$Mg_3Si_4O_{10}(OH)_2$] exists in forms with different degrees of hydrophilicity depending on their chlorite content [$(Mg_{6-x-y}Fe_yAl_x)(Si_{4-x}Al_x)O_{10}(OH)_{18}$] [24,33]. A wide range of minerals differing in hydrophile/hydrophobe balance is thus available (Table 2).

3.3.1. pH optimum

Biohalohydroxylations using CPO have been shown to have a pH optimum ranging from 3 to 5 units [26,30,31] which we have guided to work at pH 4.2 previously. It can be seen from the results in Fig. 2 that the CPO–talc systems only possess marked activity at pH 3. However, the adsorption of CPO onto talc must be carried out at pH 6, irrespective of the source of the talc and the nature of the substrate (cf. Fig. 3). In general, we found that the specific activity of the CPO–talc system rose 2–3-fold on raising the pH from 3 to 6 during preparation. These systems are thus efficient catalysts producing almost quantitative yields for somewhat longer reactions times (around 30%) than for the same reactions carried out with the free enzyme. The CPO–talc system had the added advantage of total regioselectivity in favor of the Markovnikov addition irrespective of the nature of the talc. We also found that the CPO–talc system had good catalytic activity over five reaction cycles (filtration and reuse of the catalyst), although the reaction kinetics became markedly slower over subsequent cycles. This is an added advantage for industrial applications where processes are often carried out continuously.

3.3.2. CPO–talc systems: 'Catalase activity'

One disadvantage of CPO is that it is sensitive to H_2O_2. It is inhibited by high concentrations of H_2O_2 and the catalase action is degraded by the peroxide. It can be seen from the results in Fig. 4 that immobilization on talc had a beneficial effect. At identical concentrations of enzyme and substrate, free CPO lost 65% of its specific activity in the presence of 2 mM H_2O_2. Specific activity dropped to zero at 4.8 mM. In contrast, there was little change in the specific activity of CPO–CLST up to 4 mM H_2O_2. The hydrophobic talc 15M00 had an even stronger influence on the CPO, with high specific activity at 2.8 mM H_2O_2, being even less affected by H_2O_2 concentration than the enzyme adsorbed on CLST. This was attributed to the strong protective influence of this hydrophobic talc towards the hydrophilic reactant. In practice, this means that the hydrogen peroxide does not need to be added in small amounts, which effectively precludes utilization in many industrial applications. From a more fundamental standpoint, this could appear as an augmentation of the catalase activity of the enzyme. Under our experimental conditions, we were unable to verify this hypothesis directly by measuring the volume of oxygen produced. On the other hand, we were able to measure the degradation of H_2O_2 from its consumption in the catalytic cycle of bromohydroxylation as a function of increasing amounts of substrate. The results are illustrated in Fig. 5. At 3.2 mM H_2O_2 (8 μmol) (to enable comparison with free CPO, which was blocked at 3.8 μmol substrate), the CPO–CLST system gave rise to a regioselective halohydroxylation up to 7.3 μmol substrate. No bromohydroxylation occurred in the absence of enzyme. This result indicated that the catalase action of CPO was not enhanced by adsorption onto talc and that the CPO–talc system accepts a large quantity of H_2O_2 available to the bromohydroxylation of important amounts of styrene. In the absence of more direct evidence, we suggest that the support protects the enzyme from hydrogen peroxide, conferring a marked improvement in the synthetic capacity of this enzyme.

4. Conclusion

These results support our previous findings on the role of talc–enzyme interactions and the

interest of this support in biotechnological applications. For biohalohydroxylation of derivatives of styrene, immobilization of CPO onto various types of talc, an abundant natural mineral of low cost, overcomes many of the disadvantages of the free enzyme. In comparison with free CPO, excellent regioselectivity was obtained, without formation of oxidative by-products. The bromohydrins were readily isolated by simple filtration due to the insolubility of the CPO–talc system, which can thus be reused. An added advantage for industrial applications is that the CPO–talc combination circumvents the requirement for successive addition of oxidizing agent and enables at least 2-fold higher concentrations of substrate than for the reaction using the free enzyme. On the other hand, the adsorption of the biocatalyst onto talc must be carried out at pH 6, whereas the pH optimum for the bromohydroxylation reaction is around 3. The CPO–talc system thus promises a wide variety of applications and merits further investigation.

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