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Engineering chloroperoxidase for activity and stability

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Abstract

Random mutagenesis of *Caldariomyces fumago* was carried out to produce a chloroperoxidase (CPO) with enhanced activity in mixtures of aqueous buffers and organic cosolvents. A mutant CPO with a 3.4-fold increased activity in 40% aqueous *tert*-butyl alcohol was obtained.

A CPO-surfactant conjugate, prepared by colyophilisation, mediated the oxidation of thioanisole to its (*R*)-sulfoxide by TBHP in water-saturated isooctane. Copolymerisation of CPO and a toluenediisocyanate prepolymer resulted in a stable preparation that mediated the oxidation of indole to 2-oxindole in a range of organic solvents. The highest yield was obtained in 1-octanol.

Keywords: Chloroperoxidase; Random mutagenesis; Sulfoxidation; Organic solvents; Immobilisation

1. Introduction

Chloroperoxidase (CPO) from *Caldariomyces fumago* has emerged in recent years as an outstanding biocatalyst for oxygen transfer reactions [1]. The choice of reaction medium is rather limited, however, although the very limited solubility of organic reactants in aqueous medium would make the use of organic solvents an attractive option. Aqueous mixtures of acetone and *tert*-butyl alcohol have been used as a partial solution, but CPO reacts slower in, e.g. 30% aqueous *tert*-butyl alcohol than in an aqueous medium [2].

Random mutagenesis has, via directed evolution, been used to develop a CPO mutant [3] that resisted the suicidal inactivation by allylbenzene [4]. Hence, we reasoned that random mutagenesis could be an effective approach to optimise CPO for activity in 40% aqueous *tert*-butyl alcohol. In the present paper, we report the promising initial results.

As an alternative approach, we have investigated the stabilisation of the native enzyme in completely organic media as a surfactant complex or by covalent immobilisation in a polyurethane (PUR) foam.

2. Materials and methods

2.1. Chemicals

The CPO from *C. fumago* (E.C. 1.11.1.10) was cultured and isolated as described in the literature

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[5]. The crude enzyme was purified by precipitation with acetone (vide infra) and concentrated (Centriprep-30 concentrator; Amicon; 1800 min^{-1}; 4°C). The resulting 35.5 mg/ml solution of CPO, with R_z 0.94 (R_z = purity standard = A_{400}/A_{280}; R_z is 1.44 for pure enzyme) and an activity of 27 kU/ml (standard MCD assay [6]), was used in the experiments with covalent attachment to PUR foam and colyophilisation with sodium octadecylsulfonate (SOS). The purification of the mutants is described below.

The foamable hydrophilic prepolymer Hypol™ 3000 (a water-activated derivative of toluene-2,6-diisocyanate) was kindly donated by Hampshire Chemical (Middlesbourough, UK). Brij 52 (diethylene glycol monocetyl ether) and thioanisole were purchased from Aldrich Chemical. Racemic thioanisole sulfoxide was prepared by chemical oxidation [7].

2.2. Analysis and equipment

Samples for monitoring the oxidation of thioanisole and indole in aqueous solution were quenched with a saturated sodium sulfite solution (reactions in aqueous or aqueous–organic medium) or tributyl phosphine (reactions in organic medium) and diluted with methanol. After centrifugation, the samples were analyzed on reversed phase HPLC as described [2].

Samples for analyzing the enantioselectivity of the oxidation of thioanisole were quenched as described above, diluted with a hexane/isopropyl alcohol mixture of 75:25 (v/v) and dried over Na_2SO_4. After centrifugation, the samples were analyzed by chiral HPLC as described [2].

2.3. Random mutagenesis and activity assay

Second generation random mutants, produced by random mutagenesis [3], were grown in 24-well plates. Aliquots of the culture medium (10 μl) were transferred to 96-well microplates containing the reaction medium. Each well contained 200 μl of the reaction medium consisting of a 0.25 mM indole solution in either a 0.1 M citrate buffer pH 5.0 or a 40:60 mixture (v/v) of this buffer and *tert*-butyl alcohol. The reaction was initiated by the addition of oxidant (2 μl, either 25 mM hydrogen peroxide of 1.5 M *tert*-butyl hydroperoxide). The progress of the reaction was monitored at 250 nm in a microplate reader.

Seven selected mutants as well as the wild type (WT) were grown on 500 ml scale for 2 weeks and purified as descibed by Zong [8]. To the filtered medium (400 ml), cold acetone (170 ml; 30% v/v; −20°C) was slowly added at 4°C, followed by incubation at −20°C for 1 h. The precipitated carbohydrate polymers were removed by centrifugation (4400 min^{-1}; 20 min; 0°C). To the supernatant cold acetone (765 ml; final concentration 70%, v/v; −20°C) was slowly added; CPO precipitated overnight at −20°C. The supernatant was decanted and the precipitated CPO was dried for 10 min and dissolved in citrate buffer (50 ml, 50 mM; pH 5.0). The CPO solution was dialysed three times against the citrate buffer (2 l, 50 mM; pH 5.0) and concentrated to a final volume of 5 ml (Centriprep-30 concentrator; Amicon; 1800 min^{-1}; 4°C).

A solution of indole (0.375 mM) in 3.4 ml of either a 0.1 M citrate buffer pH 5.0 or a 40:60 mixture (v/v) of this buffer and *tert*-butyl alcohol was prepared in a 4 ml quartz cuvette. Enzyme solution (10 μl) was added and mixed and the reaction was initiated by the addition of hydrogen peroxide solution (50 μl; 35 mM). The progress of the reaction was monitored at 276 nm.

2.4. Immobilisation of CPO

The covalent entrapment of CPO was essentially carried out as described in the literature [9,10]. The foams were stored for at least 2 h before use. The foam cylinder was cut into round slices of 3 mm thickness (d = 25 mm), and washed and squeezed three times in buffer (50 ml; 50 mM citrate pH 5.0). Wash solvents were analyzed for protein concentration and enzyme activity. The resulting preparation contained 16.4-mg protein/g dry foam. The foam slices with immobilised enzyme were stored in buffer (250 ml) at 4°C. For activity analysis and application in organic solvent slices were frozen in liquid nitrogen, crushed, and dried overnight over P_2O_5.

To prepare the CPO–SOS conjugate, the surfactant (600 mg) was dissolved in citrate buffer (60 ml,

5 mM, pH 5.0). A solution of 1.5 ml enzyme was added; the mixture was frozen in liquid nitrogen and lyophilised.

2.5. General oxidation procedure using immobilised CPO

Reactions in aqueous *tert*-butyl alcohol were carried out as described [2]. Reactions in non-water-miscable solvents were carried out as follows: the reactant was dissolved in the solvent which had been preequilibrated with aqueous citrate buffer (0.1 M, pH 5.0), immobilised CPO was added and the mixture was stirred at room temperature. After 15 min, an appropriate amount of a 5 M TBHP solution in decane was added. The course of the reaction was monitored by HPLC. Full details will be published elsewhere.

3. Results

3.1. Random mutagenesis of CPO

Second generation mutants produced by random mutagenesis [3] were cultured in 24-well plates. Aliquots of the recombinant CPO secreted into the culture medium were directly assayed for activity in the oxidation of indole (Fig. 1) in aqueous buffer as well as in 40% (v/v) aqueous *tert*-butyl alcohol. Because the enzyme concentrations were unknown, the relative activity (the ratio of the activities in 40% *tert*-butyl alcohol and in aqueous buffer) was compared with that of WT CPO. Over 100 mutants were screened for enhanced activity in 40% (v/v) aqueous *tert*-butyl alcohol and seven promising ones were selected for further analysis.

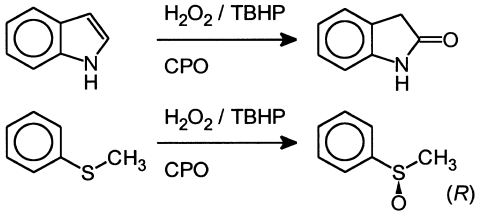

Fig. 1. Oxidation of indole and thioanisole.

Table 1
Oxidation of indole mediated by random mutants of CPO

Mutant	Concentration (mg ml^{-1})	Activity relative to WT	
		Aqueous	40% *tert*-BuOH
WT	13.4	1.0	1.0
1*C*2	3.4	1.1	1.4
2*B*1	0.8	0.4	0.7
2*B*3	2.3	1.0	2.0
2*C*1	0.5	0.2	0.4
2*D*4	4.8	0.6	0.4
3*B*4	1.0	0.9	3.4
3*C*4	0.8	1.0	1.1

The selected mutants were grown on 500 ml scale and purified by acetone precipitation followed by dialysis. The activity of the purified enzyme samples in the oxidation of indole was measured spectrophotometrically in aqueous buffer as well as in 40% (v/v) aqueous *tert*-butyl alcohol. The measured activities (relative to WT) are presented in Table 1.

Three mutants showed an enhanced activity in 40% aqueous *tert*-butyl alcohol combined with a negligible loss of activity in aqueous buffer. The maximum increase in activity was 3.4-fold, which indicates that random mutagenesis is a useful tool for improving the catalytic capabilities of CPO.

3.2. Purification of CPO

As a spin-off from the above study, we have investigated the purification of CPO in some more detail. We found that the purification method of Zong [8] — precipitation with acetone, followed by dialysis and membrane filtration — gave a good result ($R_z \sim 1.2$) at the enzyme concentrations in the culture liquid > 75 mg/l. Below this concentration, the purity of the final preparation decreased dramatically with decreasing concentration (Fig. 2).

3.3. A CPO surfactant conjugate

A conjugate of CPO and SOS was prepared by lyophilisation [11]. CPO–SOS mediated the enantioselective oxidation of thioanisole (Fig. 1) by *tert*-butyl hydroperoxide (TBHP) in a range of organic solvents, although the conjugate did not dissolve in any of these. The (*R*)-sulfoxide was preferentially

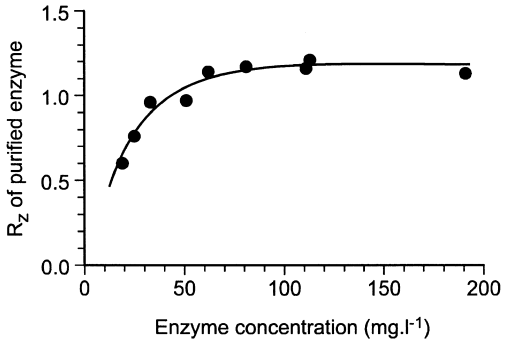

Fig. 2. Effect of the concentration of CPO in the culture medium on the purity of the final preparation.

formed in all cases, although the *ee* was generally rather low due to a significant background reaction (data not shown). In isooctane solution, thioanisole sulfoxide (70% *R*) was formed at an initial turnover frequency of 146 min^{-1}. However, after 2 h, the reaction had come to a premature standstill (Fig. 3); the addition of a second portion of TBHP resulted in further reaction but gave racemic sulfoxide. These results indicate that the lifetime of the catalyst is only 1–2 h in this medium. When hydrogen peroxide was used as the oxidant, a vigorous evolution of oxygen was observed, combined with the formation of a small amount of racemic sulfoxide.

The catalyst lifetime could be prolonged considerably by the addition of α-tocopherol (vitamin E), a radical scavenger. The initial turnover frequency increased to 302 min^{-1}, the total turnover number of

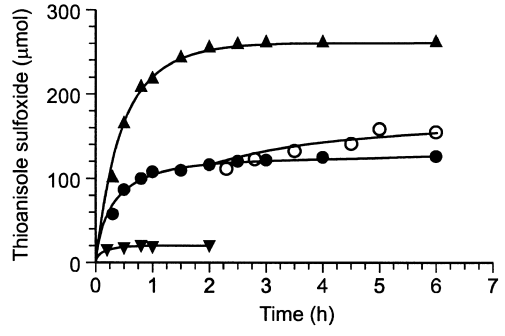

Fig. 3. Oxidation of thioanisole in isooctane mediated by CPO–SOS; ▼: H_2O_2; ●: TBHP; ○: second portion of TBHP; ▲: TBHP and α-tocopherol. Reaction conditions: 500 μmol thioanisole, 1.25 mmol peroxide, 25 mg CPO–SOS in 5 ml water-saturated isooctane at room temperature.

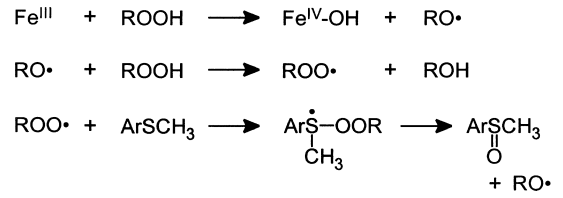

Fig. 4. Proposed mechanism of the non-stereoselective oxidation of thioanisole.

the catalyst doubled and the resulting sulfoxide was nearly enantiopure (94% *ee*). These results strongly suggest that the deactivation of CPO–SOS as well as the non-enantioselective oxidation of thioanisole are due to radical reactions. A plausible explanation is that the resting iron(III) state of the enzyme reacts with TBHP in a one-electron transfer process to form *tert*-butyloxy radicals. Reaction of the latter with TBHP gives *tert*-butylperoxy radicals, which are able to oxidise thioanisole with the generation of *tert*-butyloxy radicals (Fig. 4).

3.4. CPO covalently entrapped in PUR foam

We have shown [12] that glycosylated enzymes, such as CPO (18% sugar content [13]), are eminently suited for covalent immobilisation in PUR foam. CPO–PUR (16.4-mg protein/g dry foam) was prepared according to a published procedure [9,10]. After drying of the preparation over P_2O_5, its activity in the oxidation of indole, in 40% aqueous *tert*-butyl alcohol medium, was unchanged (see Fig. 5). Hence,

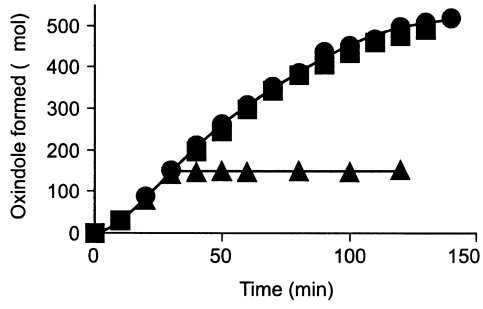

Fig. 5. Oxidation of indole catalysed by CPO–PUR in 40% aqueous *tert*-butyl alcohol; ●: CPO–PUR as synthesised (moist); ■: CPO–PUR dried over P_2O_5; ▲: moist CPO–PUR removed after 30 min.

Table 2
CPO–PUR catalysed oxidation of indole in organic media[a]

Solvent	Initial turnover frequency (min^{-1})	Total turnover number (10^3)
Isooctane	264	13
Toluene	126	8
Dichloromethane	70	10
1-Octanol	64	19
Ethyl acetate	56	5
tert-Butyl methyl ether	17	3

[a]Reaction conditions: 0.7 mmol indole, 1.4 mmol TBHP, 40 mg CPO–PUR (0.56 mg CPO) in 7 ml solvent saturated with 0.1 M citrate buffer pH 5 at room temperature.

this preparation is very stable. Moreover, no leaching of CPO took place, because when the catalyst was removed from the reaction mixture by filtration, the reaction came to a complete standstill (Fig. 5). CPO–PUR also mediated the oxidation of thioanisole to its (*R*)-sulfoxide (> 99% *ee*) by hydrogen peroxide in 50% aqueous *tert*-butyl alcohol as well as by TBHP in water-saturated isooctane.

The effect of the solvent on the CPO–PUR mediated oxidation of indole was investigated. In non-polar solvents, such as isooctane and toluene, the initial reaction rate was high (see Table 2) but the reaction soon came to a standstill, presumably because of the deactivation of CPO. The highest yield (19.10^3 catalyst turnovers) was obtained in 1-octanol.

4. Conclusions

Random mutagenesis of CPO resulted in a mutant with significantly enhanced activity in 40% aqueous *tert*-butyl alcohol. CPO maintained its catalytic activity in water-saturated organic media as a conjugate with SOS as well as upon covalent entrapment in a PUR matrix.

Acknowledgements

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