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Chloroperoxidase catalyzed oxidation of Cbz-ethanolamine to Cbz-glycinal

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ABSTRACT

Chloroperoxidase catalyzed oxidation of Cbz-ethanolamine to Cbz-glycinal was performed using hydrogen peroxide and *tert*-butyl hydroperoxide as oxidants. Frequency of the addition of the oxidants in aqueous medium was optimized. *Tert*-butyl hydroperoxide resulted as more appropriate oxidant when added at the rate of 3 mM/h, while the high catalase activity of CPO towards hydrogen peroxide limited the application of this peroxide as oxidant. Looking for benefits in terms of reaction productivity, reaction medium engineering was further studied. Oxidation of Cbz-ethanolamine was investigated in the presence of miscible (acetone, acetonitrile, dioxane) and immiscible (ethyl acetate) organic solvents. Dioxane at 5% (v/v) was the most appropriate medium as a concentration of 47.6 mM of Cbz-glycinal was produced, which was almost 6-fold higher than the value obtained in aqueous reaction (7.8 mM).

1. Introduction

Chloroperoxidase (CPO; EC 1.11.1.10) from the fungus *Caldariomyces fumago* is one of the most promising enzymes from the group of heme peroxidases due to its broad substrate specificity as well as its expressing peroxidase, halogenase, catalase and oxygen insertion activities [1,2]. CPO uses peroxides as electron acceptors and does not require any cofactor for its activity. CPO catalyzed reactions can be divided in two main classes: reactions of halogenation catalyzed by acidic form of CPO (pH < 3) and non-halogenating reactions catalyzed by more neutral form of CPO (pH = 5–6) [1,3,4]. Within the class of non-halogenating reactions CPO was found to catalyze enantioselective sulfoxidation [5], epoxidation [6,7], benzylic hydroxylation [8], oxidation of amino into nitroso group [9] and oxidation of primary alcohols to aldehydes [10–12]. Catalase activity of CPO takes place in the absence of halide ion and organic substrate and results in the disproportionation of H_2O_2 to molecular oxygen and water [13]. Despite of this, the main shortcoming of CPO and all heme peroxidases is their low operational stability caused by rapid irreversible inactivation in the presence of peroxides [14,15].

The selective oxidation of alcohols to corresponding aldehydes is a preparative method of great importance in organic chemistry. Conventional methods of organic synthesis are not easily performed for this transformation due to the difficulties to prevent the further oxidation of resulting aldehydes to carboxylic acids. Reported methods for the synthesis of aldehydes from alcohols, such as Swern, Jones and Dess-Martin oxidations have several disadvantages: they use harmful compounds for the synthesis, are environmentally unsuitable due to the large amount of waste produced, and in many cases have high costs of oxidizing agents and auxiliaries [16]. Use of transition metals as catalysts is restricted by their high costs, commercial unavailability and potential toxicity [16]. Therefore, the oxidation of alcohols to aldehydes is advantageous to be performed via enzymatic biocatalysis.

α-Amino aldehydes belong to the group of one of the most synthetically versatile aldehydes due to the presence of both N-protected amino and carbonyl groups [17,18]. These compounds are widely applied for the synthesis of important pharmaceuticals and natural products [19–21] and as transition state analogue inhibitors for various proteases [22,23]. A great number of syntheses of natural products, starting from N-protected α-amino aldehydes, involve aldol condensation as the key step. Hence, they can be used as substrates of DHAP-dependent aldolases obtaining aminopolyols, which can be directly cyclated to iminocyclitols, compounds with a great therapeutic potential [24,25].

Enzymatic synthesis of α-amino aldehydes catalyzed by horse liver alcohol dehydrogenase was previously accomplished by Andersson et al. [26]. However α-amino aldehydes were obtained as aldehyde semicarbazones, requiring an additional step for regeneration of the free aldehydes. Another disadvantage of this approach is the necessity of expensive cofactor NAD^+ required for the activity of the enzyme, or effective system for its regeneration. Taking advantage of its capacity for the oxidation of primary

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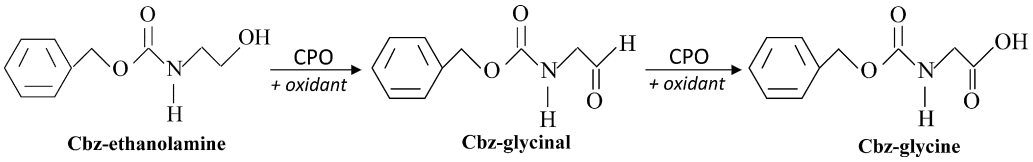

Fig. 1. Scheme of the reaction of Cbz-ethanolamine oxidation catalyzed by CPO.

alcohols, chloroperoxidase from *C. fumago* can be considered an alternative for the enzymatic synthesis of α-amino aldehydes without cofactor requirements [12]. As branched-chain alcohols were not substrates for the enzyme due to steric constraints [11], ethanolamine was selected as precursor of α-amino aldehydes.

The aim of this work is the enzymatic oxidation of ethanolamine containing carbobenzoxy protective group (Cbz-ethanolamine) to yield the amino aldehyde Cbz-glycinal using the enzyme CPO (Fig. 1). The influence of nature and addition frequency of peroxide as well as reaction medium was studied in order to increase amino aldehyde productivity while preserving enzymatic activity.

2. Materials and methods

2.1. Materials

CPO from *C. fumago* was obtained from Chirazyme Labs (Greenville, NC, USA) as a solution of partially purified enzyme (11.6 mg protein/mL). Specific activity of purified enzyme was 1400 U/mg (Rz = 0.9). Monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexanedione), Cbz-ethanolamine (benzyl-N-(2-hydroxyethyl)-carbamate, 98%), Cbz-glycine, hydrogen peroxide (30% (w/w) in water) and *tert*-butyl hydroperoxide (70% (w/w) in water) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cbz-glycinal (benzyl (2-oxoethyl)-carbamate) was purchased from Sunshine Chemlab, Inc. (Downingtown, PA, USA). All the other reagents and solvents were obtained from various commercial suppliers and were of the highest available purity and of analytical grade.

2.2. Chloroperoxidase activity assay

The optical assay for the measurement of CPO chlorination activity is based on the decrease in absorbance for a solution of monochlorodimedone, MCD (ε_{278} = 12,200 M^{-1} cm^{-1}) as it is converted to dichlorodimedone, DCD (ε_{278} = 120 M^{-1} cm^{-1}), by CPO according to the method described by Hager and Morris [27]. The assay mixture contained 100 mM potassium phosphate buffer pH 2.75, 20 mM KCl, 2 mM hydrogen peroxide, 0.16 mM MCD and sample with CPO. The absorbance was measured at 25 °C at a wavelength of 278 nm in UV–visible Spectrophotometer Cary 50 (Varian, Palo Alto, CA, USA) using quartz cuvettes. One unit of CPO is defined as the amount of enzyme required to catalyze the conversion of 1 μmol of MCD to DCD per minute using 100 mM potassium phosphate buffer pH 2.75 at 25 °C.

2.3. Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by CPO

For reactions performed in aqueous medium 20 mM Cbz-ethanolamine was dissolved in 100 mM acetate buffer at pH 5.0 in a final volume of 10 mL. For reactions in biphasic or cosolvent systems between 150 and 500 mM Cbz-ethanolamine was dissolved in the same concentration of the buffer containing between 5 and 15% of a water miscible organic solvent (acetone, acetonitrile, dioxane) or 50% of water immiscible organic solvent (ethyl acetate). Between 500 and 1100 U/mL of CPO were added to the reaction medium. The reaction was started by adding hydrogen peroxide or *tert*-butyl hydroperoxide continuously, in two different manners: (i) manual addition of one pulse (10–200 μL) per hour during the first 8 h of the reaction which was stopped and restarted after 24 h in case that CPO activity was detected; (ii) non-stop addition at a rate of 18 μL/h by using a single-syringe automatic microburette (Crison Instruments, Spain). Concentration of peroxide stock solutions varied between 1.5 and 6.7 M in order to reach peroxide addition rates within the range of 1.5–12 mM/h. Reaction samples were withdrawn periodically and analyzed by liquid chromatography in order to quantify Cbz-ethanolamine and Cbz-glycinal concentrations and to determine the activity of CPO. Each measurement was carried out in duplicate.

Pseudo half-life of CPO was determined experimentally and represents the time at which CPO activity decays to the half of the activity initially added. Conversion and yields of the products were defined respectively as the percentage of the consumed Cbz-ethanolamine or produced products, respect to initially added concentration of Cbz-ethanolamine. These values were additionally corrected by the dilution factor which was calculated for each set of measurements taking into account the volume of the peroxide added in pulses and the volume of the reaction medium withdrawn for the analysis. Initial reaction rate represents the rate of the Cbz-glycinal production during the period of linear change in concentration. Volumetric productivity was defined as the concentration of Cbz-glycinal produced per time unit during the overall reaction time.

2.4. Cbz-ethanolamine, Cbz-glycinal and Cbz-glycine quantification

Concentrations of Cbz-ethanolamine, Cbz-glycinal and Cbz-glycine were estimated by analytical HPLC Dionex UltiMate 3000 with UltiMate 3000 Variable Wavelength Detection (Sunnyvale, USA) employing a reversed-phase column X Bridge C18, 5 μm, 4.6 mm × 250 mm from Waters (Wexford, Ireland). The solvent system consisted of solvent A, composed of 0.1%, v/v trifluoroacetic acid (TFA) in H_2O, and solvent B, composed of 0.095%, v/v TFA in H_2O/CH_3CN 1:4. The solvents were eluted at a flow rate of 1 mL/min using a gradient from 20% B to 36% B over 24 min and peaks were detected at 200 nm and 30 °C. Quantitative analysis of products was performed from peak areas by the external standard method by means of a prior calibration with samples of known concentration.

3. Results and discussion

3.1. Reaction in aqueous medium

3.1.1. Choice of the oxidant and optimization of the addition frequency

As indicated in Section 1, the reactions of oxidation of alcohols to corresponding aldehydes catalyzed by CPO occur via direct oxygen transfer reaction using peroxide (typically hydrogen peroxide and *tert*-butyl hydroperoxide) as electron acceptor, although an excess of peroxide is likely to cause enzyme inactivation [28]. Trying to combine a high reaction rate with a minimal loss of enzymatic activity, the continuous addition of peroxide was proposed by

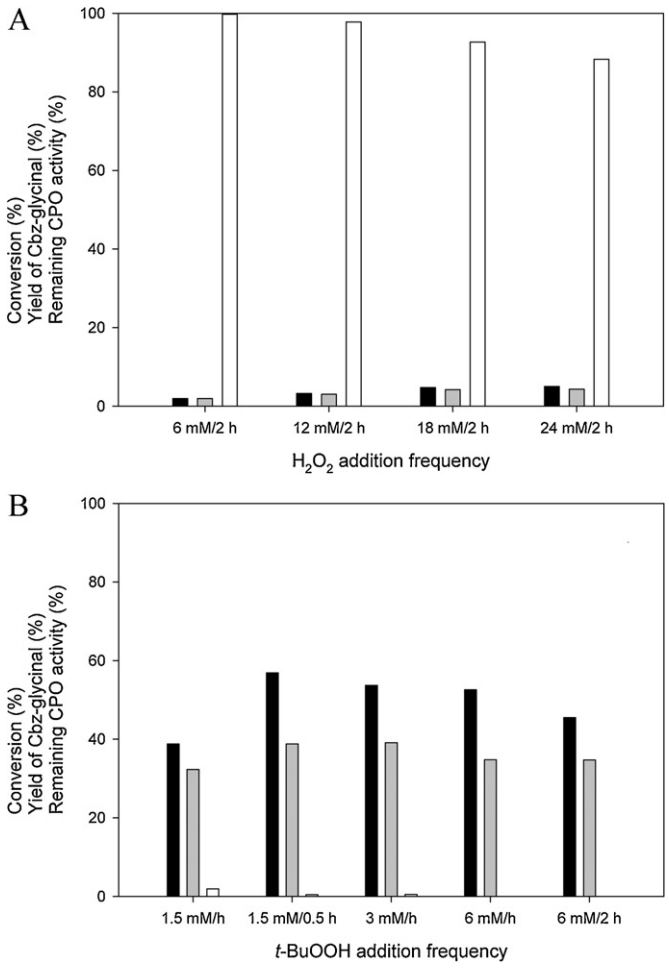

Fig. 2. Effect of peroxide addition frequency on the oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO: Cbz-ethanolamine conversion (%, black bars), yield of Cbz-glycinal (%, grey bars) and remaining CPO activity (%, white bars) using H_2O_2 (A) or *t*-BuOOH (B) as oxidant. The reaction medium contained 20 mM of Cbz-ethanolamine and 500 U/mL of CPO in 100 mM acetate buffer pH 5.0. All the experiments were performed at room temperature (20–23 °C) for 24 h.

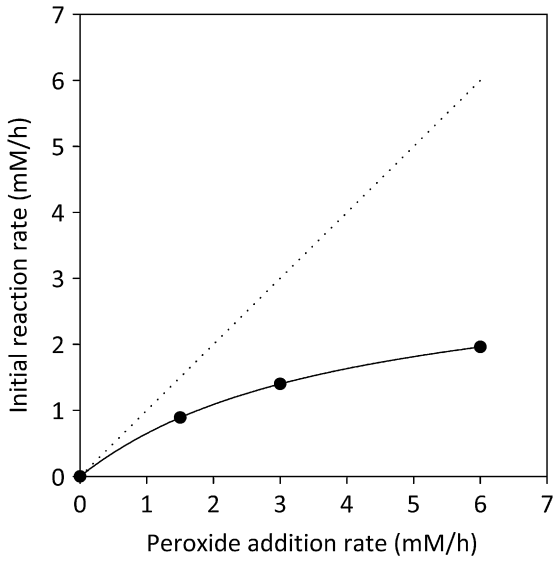

Fig. 3. Variation of initial reaction rates of the Cbz-ethanolamine oxidation catalyzed by CPO using different *tert*-butyl hydroperoxide addition rates. Dot line represents the values where initial reaction rate is equal to peroxide addition rate. The reaction medium contained 20 mM of Cbz-ethanolamine and 500 U/mL of CPO in 100 mM acetate buffer pH 5.0. All the experiments were performed at room temperature (20–23 °C) for 24 h.

other authors [29,30]. The selection of the oxidant for the reaction of Cbz-ethanolamine to Cbz-glycinal using CPO as well as the continuous addition rate were studied trying to maximize Cbz-glycinal production and minimize peroxide dependent inactivation of CPO.

When hydrogen peroxide was used as an oxidant for the CPO catalyzed oxidation of Cbz-ethanolamine to Cbz-glycinal, very low conversions were obtained (Fig. 2A). Nevertheless, the enzyme remained almost completely stable during the reaction time (24 h), retaining around 90–100% of its initial activity depending on the concentration of H_2O_2 added in pulses. This could be explained by a very high catalase activity of CPO towards H_2O_2 which totally decomposes the peroxide added releasing molecular oxygen and water [31]. On the other hand, when *t*-BuOOH was added in pulses (Fig. 2B), the conversion of the Cbz-ethanolamine to Cbz-glycinal was accomplished although a rapid enzyme inactivation was observed resulting in loss of complete enzymatic activity in less than 24 h. This is an expected result as there is evidence that the enzyme has a very low catalase activity towards this oxidant [31]. The percentage of conversion was around 50% in all cases and apart from Cbz-glycinal as a main product, the amino acid Cbz-glycine was produced (6.5–10.8% of Cbz-glycine yield), as the result of the further CPO catalyzed oxidation of amino aldehyde.

CPO requires 1 mol of peroxide to oxidize 1 mol of Cbz-ethanolamine to Cbz-glycinal [1,4]. If peroxide is added at very low rate, the reaction is limited by peroxide and it proceeds under non-optimal conditions. Otherwise, when the rate of peroxide addition significantly exceeds the reaction rate, accumulation of peroxide leads to fast CPO inactivation. The half life of enzyme can be significantly prolonged if the peroxide addition rate is adjusted to the reaction rate. Fig. 3 represents the dependence of initial reaction rate and peroxide addition rate for experiments represented in Fig. 2B. When adding *t*-BuOOH at 3 mM/h the initial reaction rate was calculated as 1.4 mM/h, which is considerably lower than the maximal value that could be reached (3 mM/h). Trying to adjust peroxide addition to reaction rate, the oxidation was performed by adding 1.5 mM/h of *t*-BuOOH, but even lower initial reaction rate was observed (0.89 mM/h) and CPO did not result to be more stable than for the addition of 3 mM/h of *t*-BuOOH. Therefore, lower yields of product were obtained. On the other side, when 6 mM/h of *t*-BuOOH were added, a slightly higher initial reaction rate was achieved (1.96 mM/h), but it was not beneficial in terms of stability of CPO, and consequently, final reaction yield.

3.1.2. Reaction with repeated addition of CPO

Possible increment of the conversion and product yield was studied by means of the repeated addition of CPO in pulses at the moments when rapid decrease of the CPO activity was detected as a result of accumulation of peroxide in the reaction medium. For comparison, a parallel reaction was performed, containing the same amount of CPO as the overall amount added in the whole time of the first reaction. The results are shown in Table 1.

Increment of CPO activity from 500 U/mL (third set of bars in Fig. 2B) to 1100 U/mL (reaction I in Table 1) applied in identical reaction conditions resulted in increased conversions, from 53.7 to 81.7%. However, this benefit was reflected more in the increment of the production of Cbz-glycine than the one of Cbz-glycinal. The yield of Cbz-glycinal reached its maximum at 8 h (Fig. 4) with volumetric productivity of 1.1 mM/h. The accumulation of aminoaldehyde accelerated its further conversion to amino acid, resulting in lower yield of Cbz-glycinal and 2-fold lower ratio Cbz-glycinal/Cbz-glycine at 29 h than that at 8 h. No improvement was observed when CPO was added in some pulses along the reaction (reaction II in Table 1).

Table 1
Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO using *tert*-butyl hydroperoxide as oxidant in addition rate of 3 mM/h. In the reaction I 1100 U/mL of CPO were added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM acetate buffer pH 5.0. In the reaction II the initial activity of CPO was 500 U/mL and addition of 150 U/mL was effected in 4 pulses each 2 h. Both experiments were performed at room temperature (20–23 °C). The end of the reaction was considered when no CPO activity was detected.

	CPO activity (U/mL)	Reaction time (h)	Conversion (%)	Yield of Cbz-glycinal (%)	Yield of Cbz-glycine (%)	Volumetric productivity (mM/h)	Cbz-glycinal/ Cbz-glycine
Reaction I	1100	8	69.0	45.0	24.0	1.1	1.9
		29	81.7	39.6	38.9	0.3	1.0
Reaction II	500 + 4 pulses of 150/2 h	8	64.4	44.1	20.3	1.1	2.2
		29	82.5	39.1	38.1	0.3	1.0

3.2. Biphasic reaction

Poor solubility of Cbz-ethanolamine in aqueous medium is one of the main reasons for relatively low productivity of the synthesis of Cbz-glycinal catalyzed by CPO. Biphasic medium permits working with higher concentrations of organic substrates, which can increase the reaction rate and yield higher productivities [32]. CPO was previously shown by Kiljunen and Kanerva [11] to successfully catalyze the oxidation of alcohols to aldehydes using a biphasic medium of hexane or ethyl acetate in a concentration of 50% (v/v) in sodium acetate buffer at pH 5.0.

The solubilities of Cbz-ethanolamine in 50% (v/v) hexane:buffer and 50% (v/v) ethyl acetate:buffer were evaluated and resulted to be around 50 and 500 mM respectively, while the one in aqueous medium is around 20 mM. Based on this, 50% (v/v) ethyl acetate was chosen as suitable medium for the purpose of oxidation of Cbz-ethanolamine. In this system distribution coefficient of Cbz-ethanolamine between the two phases expressed as the ratio of its concentration in organic (ethyl acetate) and aqueous phase was determined using a concentration of 20 mM and resulted to be around 22.0.

CPO stability was studied in three different systems: biphasic medium, aqueous medium and biphasic medium with continuous peroxide addition. Fig. 5 shows that the main part of the CPO deactivation in biphasic medium was caused by the presence of *t*-BuOOH, while the activity decrease in biphasic medium in absence of peroxide hardly reached 30% after 5 h.

In general, organic solvents can affect the stability and catalytic activity of the enzymes by causing conformational changes, by decreasing its conformational flexibility, by altering the chemical reactivity of the substrate, by changing the access of the substrate to the active site of the enzyme and finally, in biphasic media, by interfacial inactivation [33,34]. Hence, the observed activity loss could be due to the contact of the enzyme with the aqueous–organic interface [35,36], which causes structural rearrangement in the enzyme molecule and consequently partial unfolding [37]. On the other hand, although some authors described that *t*-BuOOH is partitioned between the phases [11], we observed that its concentration nearby the enzyme is still high enough to cause its fast deactivation.

A reaction was performed using a substrate concentration of 500 mM, which ensures a concentration close to the value of solubility in the aqueous phase, where the enzyme is entirely dissolved. Even though a very low yield of Cbz-glycinal was obtained (3.0% after 28 h), the concentration of the aldehyde produced was around 2 fold improved, having 7.8 mM of Cbz-glycinal produced in the reaction performed in aqueous medium and 15 mM in the biphasic one. On the other side, the initial reaction rate value of 0.66 mM/h was much lower than the one in aqueous reaction (1.4 mM/h) due to the partition of *t*-BuOOH between the phases and thus a decrease in concentration in the aqueous phase. Concerning enzymatic activity, pseudo half life of the enzyme was improved from 4 h for the reaction performed in aqueous medium to 8 h for the reaction in biphasic system. This fact points out that the increase in activity loss caused by the interface of the biphasic system is overcompensated by the activity preservation due to a decrease in *t*-BuOOH concentration nearby the enzyme.

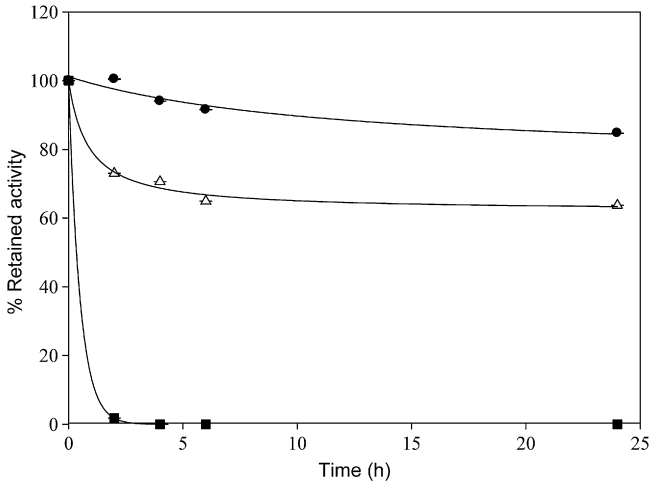

Fig. 5. Stability of soluble CPO in aqueous (●) and biphasic medium (△), in the absence of *t*-BuOOH and substrate, and in biphasic medium (■) with the addition of 3 mM/h of *t*-BuOOH in pulses in the absence of the substrate. Aqueous medium consisted in 100 mM acetate buffer at pH 5, while the biphasic medium contained ethyl-acetate/100 mM acetate buffer pH 5 (1/1). Experiments were performed at room temperature (20–23 °C) with shaker agitation. Data points represent the average of duplicate measurements; error bars represent the standard deviation.

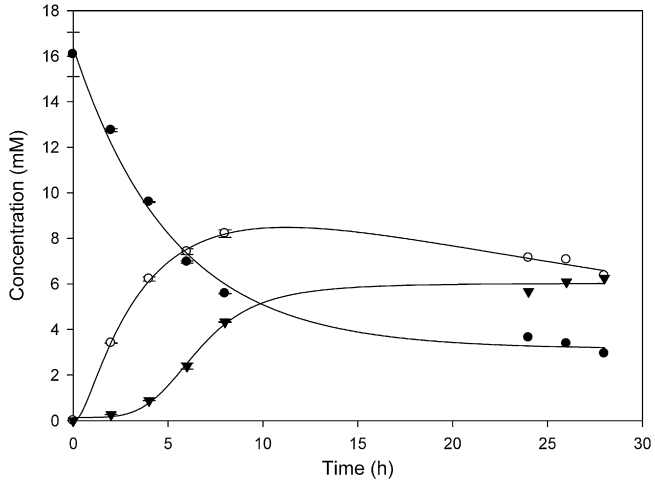

Fig. 4. Time course for oxidation of Cbz-ethanolamine (●) to Cbz-glycinal (○) and Cbz-glycine (▼) catalyzed by CPO using *tert*-butyl hydroperoxide as oxidant in addition rate of 3 mM/h at the temperature of 20–23 °C. 1100 U/mL of CPO were added to the reaction mixture that contained 16 mM of Cbz-ethanolamine in 100 mM acetate buffer pH 5.0. Data points represent the average of duplicate measurements; error bars represent the standard deviation.

Table 2
Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO using *tert*-butyl hydroperoxide as oxidant with addition rate of 3 mM/h in the presence of organic cosolvents in 100 mM acetate buffer pH 5 at room temperature (20–23 °C), with initially added 500 U/mL of CPO. Concentration of Cbz-ethanolamine in all cases was 150 mM. The end of the reaction was considered when no CPO activity was detected.

Cosolvent	Concentration (%, v/v)	Reaction time (h)	Pseudo half life, $t_{1/2}$ (h)	Conversion (%)	Yield of Cbz-glycinal (%)	Yield of Cbz-glycine (%)	Initial reaction rate (mM Cbz-glycinal/h)
Acetone	15	25	6	10.8	10.8	0.0	1.5
Acetonitrile	15	6	2	2.7	2.7	0.0	1.5
	5	25	6	16.0	16.0	0.0	2.4
Dioxane	5	54	30	38.3	31.7	5.1	3.3
	10	48	24	32.1	25.1	3.3	3.1

Experiments with peroxide addition rates of 6 and 12 mM/h were performed and Cbz-glycinal yields of 0.6 and 0.2% respectively were obtained. Apart from increasing the reaction rate, the high addition of peroxide caused a very fast CPO inactivation, being the pseudo half-lives of 2.5 h for the addition rate of 6 mM/h of *t*-BuOOH and 1.8 h for the one of 12 mM/h. Consequently the Cbz-glycinal yield was strongly reduced.

3.3. Cosolvent system

3.3.1. Choice of the cosolvent and the rate of t-BuOOH addition

The use of CPO in the presence of miscible organic solvents was reported by various authors [34,38–40]. In this work, the effect of different water miscible solvents on the stability of CPO was evaluated in preliminary studies in order to establish which solvents could be potentially useful for CPO catalyzed biotransformation. The solvents tested were acetone, acetonitrile and dioxane. In all of the cases studied, the stability of CPO when adding 3 mM/h of *t*-BuOOH was reduced compared to the one in pure buffer (pseudo half lives within the ranges of 1.5–3 h for cosolvent mixtures used in concentration within the range of 5–15% (v/v) and 4 h for pure buffer), except for the dioxane used in concentration of 5% (v/v), when the stability of CPO was the same as in pure buffer. Generally, higher cosolvent content results in faster CPO activity loss.

The use of cosolvents enabled the work with higher concentrations of substrate. The reaction of oxidation of Cbz-ethanolamine was performed in the presence of the selected cosolvents using substrate concentration of 150 mM which represents a value 7.5-fold higher than the one used in aqueous medium (Table 2). As result of the increased substrate concentration in the reaction medium, the initial reaction rates of the oxidation of Cbz-ethanolamine increased compared to that of aqueous medium, and higher reaction rates resulted in higher pseudo half lives. Diminished accumulation of peroxide due to the higher reaction rate can be the reason for the improved values of pseudo half life of CPO compared to the pseudo half life of 4 h for the aqueous medium reaction in the same conditions. Exception was the reaction in presence of 15% (v/v) acetonitrile in which a very fast deactivation of enzyme was caused by the dissolved solvent molecules. Accordingly with the preliminary studies of the stability of CPO in the presence of the cosolvents, when the reaction was performed in 5% (v/v) of dioxane the pseudo half life of CPO was almost 8-fold higher than the one in aqueous reaction.

In presence of 5% dioxane the Cbz-glycinal yield was 31.7%, which represents a value slightly lower than that in aqueous medium (39%), although the synthesized product concentration was almost 6-fold higher (47.6 mM in 5% of dioxane compared to 7.8 mM in aqueous medium). Improvement in the reaction productivity was achieved also in all the other reaction media studied, except for the one containing 15% (v/v) acetonitrile in which very low conversion was obtained due to a very fast inactivation of the enzyme. Hence, 16, 24 and 38 mM were the concentrations of the product at the end of the reactions containing 15% acetone, 5% acetonitrile and 10% dioxane, respectively.

For reactions performed in dioxane, the initial reaction rate reached 3 mM/h, which corresponds to the value of peroxide addition rate. This fact points out that the peroxide addition could be limiting the reaction rate. To avoid this possibility, the increase of the addition rate was studied. For the reaction with 5% (v/v) of dioxane, when the addition of *t*-BuOOH was augmented to 6 mM/h, the initial reaction rate of Cbz-glycinal production was equal to this value, the corresponding activity retention after the first 4 h was 96.7%, and the final yields of Cbz-glycinal and Cbz-glycine obtained were similar to those reached when using 3 mM/h (Table 3). Further increase of the peroxide addition to 12 mM/h could no longer be considered as optimal since the initial reaction rate was lower than this value; the accumulation of the unconsumed peroxide caused the deactivation of 52% of the initial CPO activity in the first 4 h and consequently lower yields of product.

3.3.2. Optimization of the temperature using dioxane as a cosolvent

Effect of the temperature on the performance of the oxidation of Cbz-ethanolamine catalyzed by CPO might be important. In order to accomplish the successful performance, the temperature should be chosen in that way that it could be more beneficial for the increase of reaction rate than for thermal and peroxide dependent inactivation of the enzyme. The reaction in presence of 5% (v/v) of dioxane with constant addition of 3 mM/h of peroxide was chosen as a model reaction, and the effect of temperature was studied at 11, 20 and 37 °C (Table 4). At 11 °C the reaction of oxidation has a very low rate comparing to the one at 20 °C and to the peroxide addition rate, hence CPO is deactivated due to the accumulation of the peroxide in the reaction medium. Despite the lower enzymatic activity and conversion, the ratio of Cbz-glycinal and Cbz-glycine produced was improved to 13.3 comparing to the value of 8.1

Table 3
Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO at different rates of addition of *t*-BuOOH in presence of 5% dioxane in 100 mM acetate buffer pH 5.0 at room temperature (20–23 °C), with initially added 500 U/mL of CPO. Concentration of Cbz-ethanolamine in all cases was 150 mM. The end of the reaction was considered when no CPO activity was detected.

Addition of *t*-BuOOH	Reaction time (h)	Pseudo half life $t_{1/2}$ (h)	Conversion (%)	Yield of Cbz-glycinal (%)	Yield of Cbz-glycine (%)	Initial reaction rate (mM glycinal/h)
Pulses 3 mM/h	54	30	38.3	31.7	5.1	3.2
Pulses 6 mM/h	32	8	34.5	30.8	5.1	6.2
Pulses 12 mM/h	26	4	29.3	25.1	4.2	10.2

Table 4
Effect of the temperature on the oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO. *tert*-Butyl hydroperoxide was used as oxidant in addition rate of 3 mM/h via a microburette in the presence of 5% of dioxane in 100 mM acetate buffer pH 5, with initially added 500 U/mL of CPO. Concentration of ethanolamine in all cases was 150 mM. The end of the reaction was considered when no CPO activity was detected.

T (°C)	Reaction time (h)	Pseudo half life, $t_{1/2}$ (h)	Conversion (%)	Yield of Cbz-glycinal (%)	Yield of Cbz-glycine (%)	Initial reaction rate (mM Cbz-glycinal/h)
11	28	13	21.5	21.2	1.6	0.9
20	24	15	26.2	25.1	3.1	3.1
37	8	3.5	6.6	6.6	0.0	2.0

for 20 °C. This suggests that at lower temperatures, synthesis of amino acid was more delayed than the one of amino aldehyde. At 37 °C rapid thermal deactivation was detected, causing low reaction rates, therefore the slow consumption of *t*-BuOOH, which causes the additional deactivation of CPO. The temperature of 20 °C can be considered as optimal, since at this temperature thermal deactivation is not appreciable, and it allows good rate of the reaction of synthesis and the highest Cbz-glycinal yield minimizing the deactivation of CPO by peroxide.

4. Conclusions

Oxidation of Cbz-ethanolamine to Cbz-glycinal was performed successfully using *tert*-butyl hydroperoxide as an oxidant. Different reaction media were analyzed looking for the way to increase substrate concentration favouring reaction rate and Cbz-glycinal productivity. Even though cosolvents and biphasic medium generally affect enzymatic activity, the adjustment between reaction kinetics and peroxide addition rate avoided the peroxide excess in the reaction medium and, consequently, enzymatic stability and reaction productivity were importantly improved.

Cbz-glycinal yields were limited by the subsequent oxidation reaction of the aldehyde towards Cbz-glycine, and by the strong decrease of Cbz-ethanolamine conversion rate along the reaction, even though some enzyme remained active, probably due to equilibrium constraints. Both aspects could be overcome by coupling this oxidation reaction with another reaction, in which aminoaldehyde would be immediately consumed as a substrate, in a multienzymatic system.

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