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Optimization of oxidative bioconversions catalyzed by phenylacetone monooxygenase from *Thermobifida fusca*

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ABSTRACT

By choosing properly the nature of the reaction medium and its ionic strength, biocatalytic properties of isolated phenylacetone monooxygenase from *Thermobifida fusca* can be improved, achieving the best results when working in Tris or phosphate buffers presenting moderate ionic strengths. The use of different enzymatic cofactor regenerating systems has been studied, resulting in the highest activities by using glucose or glucose-6-phosphate dehydrogenase. The cofactor concentration, key parameter when oxidizing with isolated Baeyer–Villiger monooxygenases, was optimized, being demonstrated that PAMO can perform its biocatalytic activity with the highest TTNs with low requirement of nicotinamide cofactor (2 μM).

1. Introduction

An interesting set of enzymes to apply for oxidative biocatalysis are the Baeyer–Villiger monooxygenases (BVMOs). These redox enzymes have emerged as useful tools for catalyzing chemo-, regio- and/or enantioselective oxidations difficult to achieve by the conventional chemical methods [1–4].

One particularly interesting BVMO is phenylacetone monooxygenase from *Thermobifida fusca* (PAMO, EC 1.14.13.92), which was recently cloned, overexpressed and structurally characterized [5,6]. This thermostable flavoprotein catalyzes the Baeyer–Villiger reaction of carbonylic compounds, as well as heteroatom oxidations. In this regard, PAMO was demonstrated to be a very effective and selective biocatalyst for the kinetic resolution of racemic benzylketones in order to obtain the corresponding (*S*)-esters and (*R*)-ketones [7,8]. Moreover, PAMO has been employed as enantioselective sulfoxidation biocatalyst [9].

Biocatalysts are sensitive to the employed reaction conditions. Thus, the manipulation of the physical environment of proteins in order to control the enzymatic activity and/or selectivity has become an attractive target. Besides pH and temperature, the effect of which on PAMO activity and selectivity has been previously shown [8,10], there are other factors that may alter the enzyme properties, such as the nature or the ionic strength of the reaction medium [11–14] in which oxidative processes are developed.

The use of isolated enzymes offers some advantages with respect to whole cells systems, since this practise steers clear of the enzymatic co-metabolism of substrates, diminishes the substrate/product toxicity and avoids the problems for transporting these compounds in and out of the cells [15]. However, isolated oxidoreductases require the presence of cofactors as a source of electrons to perform their catalytic activity. Due to the prohibitive cost associated with stoichiometric use of nicotinamide cofactors and the problems derived from inhibitory effects on the enzymes, an effective method for recycling NAD(P)H is required. While chemical, electrochemical or photochemical approaches have been proposed for that purpose [16–19], the enzymatic regeneration systems are still the method of choice when performing BVMO-catalyzed reactions. These enzymatic methods reduce technological complications and are highly selective yielding only the 1,4-NAD(P)H isomer. Dehydrogenases are often used for oxidizing a sacrificial co-substrate in the reaction medium in order to regenerate NAD(P)H. Although other types of enzymes have been recently described, such hydrogenases [20], their application is not widespread. In case of reactions catalyzed by isolated NADPH-dependent BVMOs, glucose-6-phosphate dehydrogenase (G6PDH) from *Leuconostoc mesenteroides* [21] has been widely employed. Nevertheless, there are other alternatives, such as glucose dehydrogenase (GDH) from *Bacillus* sp. [22], alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH) [23] and phosphite dehydrogenase (PTDH) from *Pseudomonas stutzeri* [24,25]. The latter

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enzyme has been recently fused to different BVMOs for obtaining self-sufficient redox biocatalysts, the so-called CRE-BVMOs (CRE: *Coenzyme Regeneration Enzyme*) [26]. A second generation of these bifunctional enzymes (CRE2-BVMO) includes a polyhistidine tag and a thermostable mutant of PTDH [27], for optimizing the purification process and the efficiency of the biocatalytic system [28].

For the large-scale application of cofactor-dependent enzymatic synthesis, not only a proper regeneration system is required, but also the optimization of the cofactor amount is essential. The utilization of a cofactor can be expressed by its total turnover number (TTN), moles of product formed per mol of cofactor used for the reaction, and by the turnover frequency (TOF), referred as the TTN per unit of time. Higher TTN and TOF values ensure more effective biotransformations. It has been established that TTNs higher than 10^3 may be sufficient to make a process economically viable [29].

In this study the influence of the reaction medium and the type of cofactor regenerating system on the biocatalytic properties of isolated PAMO has been analyzed. The optimization of Baeyer–Villiger oxidations and sulfoxidations catalyzed by PAMO has been carried out by comparing different enzymatic cofactor regeneration systems and by optimizing the amount of NADPH required during the enzymatic process in order to ensure more effective oxidative processes. This has revealed that for an optimal PAMO-based biocatalytic process, it is crucial to select a proper reaction medium and cofactor regeneration system.

2. Experimental

2.1. General materials and methods

Recombinant histidine-tagged phenylacetone monooxygenase (PAMO) [5], phosphite dehydrogenase (PTDH E175A/A176R) [25] and bifunctional CRE2-PAMO fusion protein [28] were purified as previously described. One unit of PAMO was defined as the amount of enzyme that oxidizes 1.0 μmol of phenylacetone to benzyl acetate per minute at pH 8.0 and 30 °C in the presence of NADPH. D-Glucose-6-phosphate dehydrogenase (G6PDH 640 U/mg) from *L. mesenteroides* and alcohol dehydrogenase (TBADH 5.28 U/mg) from *T. brockii* were products from Sigma–Aldrich. D-Glucose dehydrogenase 002 (GDH 30 U/mg) was purchased from Codexis, as well as the sodium salts of the nicotinamide coenzymes $NAD(P)^+$ and NAD(P)H (purity ≥ 99%).

Phenylacetone (**2a)** was purchased from Merck. Benzyl acetate (**2b)**, thioanisole (**4a**), methyl phenyl sulfoxide (**4b**), D-glucose, D-glucose-6-phosphate and sodium phosphite were supplied by Sigma–Aldrich. (±)-3-Phenylbutan-2-one [(±)-**1a**] was prepared according to the literature, using methyl iodide and NaOH in a biphasic medium (46% yield) [30]. 3-Methyl-4-phenylbutan-2-one [(±)-**3a**] were obtained according to the literature with 30% yield, by Heck arylation of 3-methyl-3-buten-2-ol, using iodobenzene in presence of palladium chloride, tetra-*n*-butylammonium bromide and $NaHCO_3$ [31]. 3-Methyl-3-buten-2-ol was prepared by addition of methyl magnesium iodide to a methacrolein solution in diethyl ether. Esters (±)-**1b** and (±)-**3b** were prepared by chemical acylation of commercial (±)-1-phenylethanol or (±)-1-phenyl-2-propanol, respectively (yields higher than 80%). All others solvents and reagents used were of highest quality grade available. Compounds **1a** [32], **3a** [33], **1b** [34] and **3b** [35] exhibit physical and spectral properties in accord with those reported.

Absolute configuration of (*S*)-**1b** and (*S*)-**3b** was established by comparison with an authentic sample prepared from chemical acylation of the corresponding commercial chiral alcohol. Absolute configuration of sulfoxide (*S*)-**4b** was established by comparison of the HPLC chromatograms with the patterns described in previous experiments for the known configurations.

Flash chromatography was performed using Merck silica gel 60 (230–400 mesh). ^{1}H NMR, ^{13}C NMR and DEPT spectra were recorded, with TMS (tetramethylsilane) as the internal standard, on a Bruker AC-300-DPX (^{1}H: 300.13 MHz and ^{13}C: 75.4 MHz) spectrometer. ESI^+ using a HP1100 chromatograph mass detector or EI with a Finigan MAT 95 spectrometer was used to record mass spectra. GC analysis were performed on a Hewlett Packard 6890 Series II chromatograph equipped with HP-1 cross-linked methyl siloxane column (30 m × 0.32 mm × 0.25 μm, 1.0 bar N_2) for achiral analyses and Restek RtβDEXse (30 m × 0.25 mm × 0.25 μm, 1.0 bar N_2) for chiral determinations. For all the analyses, the injector temperature was 225 °C and the FID temperature was 250 °C. The following temperature programs were employed: (1) 70 °C (5 min), 1 °C min^{-1} 120 °C, t_R (*R*)-**1a**: 44.3 min; t_R (*S*)-**1a**: 46.4 min; t_R (*S*)-**1b**: 42.9 min; t_R (*R*)-**1b**: 50.5 min; (2) 70 °C (7 min), 10 °C min^{-1} 90 °C, t_R **2a**: 6.0 min; t_R **2b**: 7.7 min; and (3) 90 °C (30 min), 5 °C min^{-1} 120 °C, t_R (*R*)-**3a**: 46.9 min; t_R (*S*)-**3a**: 48.5 min; t_R (*S*)-**3b**: 42.7 min; t_R (*R*)-**3b**: 45.8 min. HPLC analyses were developed with a Hewlett Packard 1100 LC liquid chromatograph. The following conditions were used for the determination of the conversion and the enantiomeric excess of **4b**: Chiralcel OD column (0.46 cm × 25 cm), isocratic eluent: *n*-hexane/^{i}PrOH (90:10), 20 °C, flow 1 mL min^{-1}. t_R **4a** 5.3 min; t_R (*R*)-**4b** 11.2 min; t_R (*S*)-**4b** 14.2 min.

2.2. General procedure for the enzymatic oxidations catalyzed by isolated PAMO

Ketones (±)-**1a**, **2a**, (±)-**3a** or sulfide **4a** were dissolved in the corresponding reaction media (different pHs and concentrations, 1.0 mL), containing the cosubstrate (20 mM or 5–70% (v/v) when ^{i}PrOH was employed), the cofactor regeneration enzyme (5.0 units), NADPH (0.2 mM) and PAMO (1 unit). The mixture was shaken at 250 r.p.m. and the selected temperature in a rotatory shaker for the times indicated. The reaction was then stopped, worked up by extraction with EtOAc (3 × 0.5 mL), dried over Na_2SO_4 and analyzed directly by GC or HPLC to determine the conversion and enantiomeric excess in case of compounds (*R*)-**1a**, (*S*)-**1b** and (*S*)-**3a**, (*R*)-**3b**.

2.3. Baeyer–Villiger oxidations or sulfoxidations catalyzed by the fusion protein CRE2-PAMO

Substrates (±)-**1a**, **2a**, (±)-**3a** or **4a** were dissolved in 50 mM Tris/HCl (1.0 mL), containing sodium phosphite (10 mM), NADPH (0.2 mM) and the fusion protein CRE2-PAMO (4.0 U). The mixture was shaken at 250 r.p.m. and the selected temperature in a rotatory shaker for the times indicated. The reaction was then stopped, worked up by extraction with EtOAc (3 × 0.5 mL), dried over Na_2SO_4 and analyzed directly by GC or HPLC to determine the conversion and enantiomeric excesses in case of compounds (*R*)-**1a**, (*S*)-**1b** and (*S*)-**3a**, (*R*)-**3b**.

3. Results and discussion

3.1. PAMO-catalyzed Baeyer–Villiger oxidation of racemic 3-phenylbutan-2-one in different reaction media

The effect of the reaction media on the biocatalytic properties of PAMO when oxidizing (±)-3-phenylbutan-2-one [(±)-**1a**] was analyzed using the coupled enzymatic system glucose-6-phosphate/glucose-6-phosphate dehydrogenase to regenerate the NADPH consumed in the enzymatic oxidation (Scheme 1). All the buffer solutions were of the same concentration (50 mM) and pH (pH 9.0), with the exception of sodium tetraborate (so-called Borax) and sodium bicarbonate ($NaHCO_3$) solutions (pH 9.5). Oxidation of (±)-**1a** in Tris–HCl or phosphate buffer led to a similar