Immobilization of Baeyer-Villiger monooxygenases in presence of ionic liquids, a simple approach for enzyme recycling.

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Abstract

Ionic liquids (ILs) are largely used in biocatalysis, mainly as cosolvents of the biocatalyzed reactions, but in the last few years ILs have been used as coating reagents in the immobilization of enzymes. In the present communication, we have developed a set of immobilized Baeyer-Villiger monooxygenases preparations in presence of different ILs. These immobilized biocatalysts have been tested in selective sulfoxidations with the aim of analyzing the activity and selectivity of the novel enzymatic preparations as well as their performance during different reaction cycles.

Keywords

Ionic liquids/ Coating/ Sulfoxidations/ Baeyer-Villiger monooxygenases

Introduction

Biocatalysis, that is, the use of biological systems (purified enzymes, cell free preparations or microorganisms) as catalysts in organic chemistry has gained an increasing interest in the last few years.[1] Biocatalytic processes employ mild and environmentally friendly reaction conditions while they are able to perform organic reactions with exquisite chemo-, regio- and/or enantioselectivities. The advantages of biocatalysis can be observed in oxidation reactions. Thus, oxidative processes catalyzed by enzymes do not require for harsh oxidants and reaction conditions. One example of oxidative biocatalysts are the Baeyer-Villiger monooxygenases (BVMOs).[2] These enzymes are able to perform the Baeyer-Villiger oxidation as well as the oxygenation of different heteroatoms as sulfur, nitrogen, phosphorous or boron, employing molecular oxygen as oxidant, while working at mild reaction conditions (pHs from 6.0 to 9.0;

temperatures around room temperature). Unfortunately, the use of BVMOs presents two main bottlenecks: 1) Requirement of a nicotinamide as a cofactor, which can be solved by using a secondary enzymatic system to regenerate the cofactor; and 2) Difficulty of recovery when they are used as purified enzymes. In order to circumvent this last drawback, the enzymatic immobilization by different methodologies has emerged as a very valuable tool.[3]

Ionic Liquids (ILs) are salts with a melting temperature below the boiling point of water, with negligible vapor pressure and with a high polarity, that present a huge range of applications in organic chemistry, and specifically in biocatalysis.[4] The main use of ILs in biocatalysis has been as cosolvents, in order to create novel reaction media for the enzymatic reactions. But apart for this main usage, many other applications have been developed. Thus, ILs with melting points from 50 to 100ºC can be employed to coat and protect biocatalysts. The corresponding ILs are first heated and melted. Then, enzymes are aggregated and dispersed through this melted liquid. The mixture is subsequently cooled. In general, these enzymatic preparations display better catalytic activities, stabilities and/or enantioselectivities than the free enzymes.

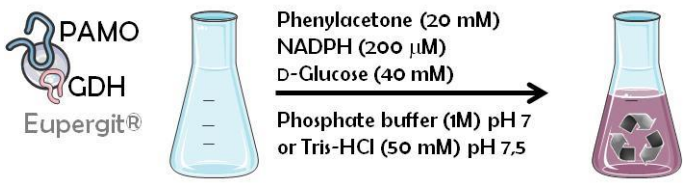
Herein, we have developed a set of enzymatic preparations containing a BVMO with ILs at different conditions. These preparations have been studied in the BVMO-catalyzed sulfoxidation of prochiral sulfides to obtain chiral sulfoxides.

Results and discussion

Immobilization of BVMOs on Eupergit®

Phenylacetone monooxygenase (PAMO)[5] has been coimmobilized together with the NADPH recycling secondary enzymatic system glucose dehydrogenase (GDH) onto solid Eupergit® (Figure 1). This preparation was able to oxidize phenylacetone in mild conditions (30ºC, Tris-HCl 50 mM pH 7.5 or phosphate buffer 1.0 M pH 7.0). Conversions higher than 80% during three consecutive cycles were obtained, while for the fourth cycle part of its activity was lost.

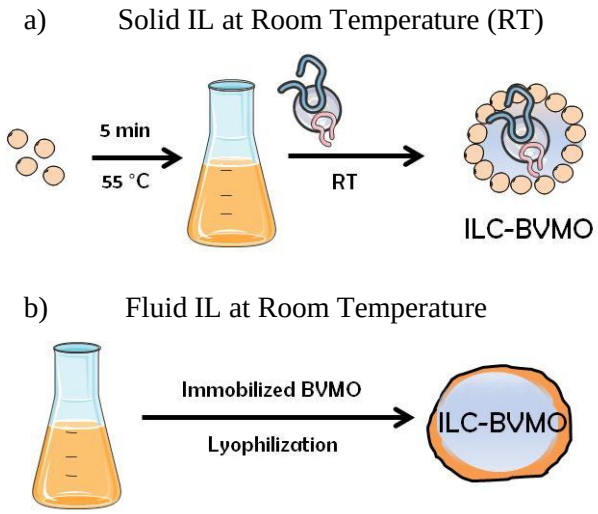
Figure 1. Baeyer-Villiger oxidation catalyzed by immobilized enzymes on Eupergit®.



Coimmobilization of supported BVMOs in the presence of Ionic Liquids

The high tolerance of PAMO to reaction media different from aqueous buffers, such as 70% v/v of ILs, has been previously established.[6] In order to improve the solubility of immobilized PAMO preparation; we have explored the ability of different ILs for the coating. First, we test several solid ILs with melting points close to 55°C (Figure 2a), giving [bmp]PF_6 the best results. Unfortunately, the activity of Ionic Liquid Coated (ILC) PAMO (**I**) was markedly reduced in comparison to isolated enzymes. Even though the 35% of phenylacetone was oxidized to benzyl acetate in the second cycle, we observed significant decreased levels of **I** activity over the time, mainly due to GDH inactivation in the presence of [bmp]PF_6. Then, we decided to perform the coating of a PAMO *E. coli* Cell Free Extract (CFE), which includes the enzyme and the secondary enzymatic system (**II**). For the CFE a second strategy was applied to obtain the immobilized biocatalyst, by using ILs that are fluid at room temperature (Figure 2b), such [thma]$MeSO_4$ and Ammoeng102^{TM}. With this last approach, we observed a two-fold increase in phenylacetone conversion rates compare to **I**, thus aiming to investigate different enzymatic combinations in the presence of these ILs.

Figure 2. Coimmobilization of supported BVMOs in the presence of Ionic Liquids



Immobilization of bifunctional fusion proteins in the presence of Ionic Liquids

In order to create an immobilized self-sufficient BVMO, the fusion protein CRE2-PAMO was expressed and purified as described by Fraaije and co.[7] to be subsequently supported on Eupergit (**III**) and immobilized in the presence of ILs (**IV-V**). Once we confirmed the activity of **IV-V** with its natural substrate phenylacetone (data not shown), the sulfoxidation of substrates poorly soluble in aqueous media such

thioanisole or benzyl methyl sulfide (BzSMe) to obtain the chiral sulfoxides, was analyzed. The same approach was also tested with a bifunctional steroid monooxygenase, CRE2-SMO,[8] thus analyzing the activity and selectivity of the protein supported on Eupergit (**VI**) and immobilized with ILs (**VII**-**VIII**). The results summarized in Table 1 demonstrate for the first time that one IL-containing immobilized BVMOs can be employed as biocatalyst in successive cycles of asymmetric sulfoxidations.

Table 1. Asymmetric oxidation of aromatic sulfides catalysed by immobilized bifunctional CRE2-BVMOs.

Entry	Immobilized biocatalyst (IL)	Sulfide	*c* (%)[a]	*ee* (%)
1	**III**	Thioanisole	58 (1)[a]	38 (*R*)
2	**IV** (Ammoeng102)	Thioanisole	13	16 (*R*)
3	**III** Cycle 1	BzSMe	86 (14)	95 (*S*)
4	**III** Cycle 2	BzSMe	81 (5)	83 (*S*)
5	**IV** (Ammoeng102) Cycle 1	BzSMe	47	77 (*S*)
6	**IV** (Ammoeng102) Cycle 2	BzSMe	26	64 (*S*)
7	**V** ([bmp]PF_6)	BzSMe	89 (4)	90 (*S*)
8	**VI**	Thioanisole	17 (1)	11 (*R*)
9	**VII** (Ammoeng102)	Thioanisole	8 (1)	6 (*R*)
10	**VI** Cycle 1	BzSMe	35	31 (*S*)
11	**VI** Cycle 2	BzSMe	15	14 (*S*)
12	**VII** (Ammoeng102) Cycle 1	BzSMe	11	34 (*S*)
13	**VII** (Ammoeng102) Cycle 2	BzSMe	8	32 (*S*)
14	**VIII** ([bmp]PF_6)	BzSMe	22	19 (*S*)

[a] In brackets the percentage of sulfone overoxidation product measured.

Material and methods

Recombinant BVMOs were obtained as previously described.[7-9] All other reagents and solvents purchased from commercial sources were of the highest quality grade available. Gas chromatography (GC) analyses were performed on a Hewlett Packard 6890 Series II chromatograph. High performance liquid chromatography (HPLC) analyses were carried out with an UV detector at 210 nm using chiral HPLC column. Control experiments in the absence of enzyme resulted in no conversion.

Immobilization of BVMOs on Eupergit® support

BVMO (100 μM) and GDH (25 U, except for bifunctional enzymes) were incubated in the presence of NADPH (5 mg) and 50 g of Eupergit®, yielding a resin-supported biocatalyst. The mixture is allowed to stand for 20 hours at room temperature. Then, we

washed the preparation several times with Tris-HCl 50 mM pH 9.0 and dried on filter plates to obtain 1.0-1.5 g of fine powder. The immobilization efficiency was evaluated by spectrophotometry. In all cases, the remaining enzymatic activity of the wash solution was ≤5%.

Immobilization of BVMOs in the presence of ILs

Ionic liquid [bmp]PF_6 (500 mg) was incubated 5 min at 55ºC before adding the BVMO preparation supported on Eupergit® (100 mg) or the BVMO-containing CFE (30 mg). The mixture is allowed to cool to room temperature and white powder is collected afterwards (Figure 2a). Fluid ILs such as [thma]$MeSO_4$ and Ammoeng102™ were used as lyoprotectants to prevent protein inactivation during freeze-drying cycles. ILs were mixed with immobilized BVMO preparations before freezing in liquid N_2 and lyophilized (Figure 2b). Both resin-supported preparations containing CFE (3 mL) or isolated (bifunctional) BVMOs (300 mg) were mixed with 2% (v/v) of [thma]$MeSO_4$ and/or Ammoeng102™ in 50 mM Tris-HCl pH 7.5 for the coating.

General method for the oxidations catalyzed by immobilized BVMOs

The starting phenylacetone, thioanisole and benzyl methyl sulfide (20 mM) were dissolved in Tris-HCl 50 mM pH 7.5, containing D-glucose or sodium phosphite (40 mM), NADPH (0.2 mM) and the immobilized BVMO (100 mg, 1 U). The mixture was shaken at 250 rpm and 30ºC for the times indicated. The reaction was then stopped, worked up by extraction with EtOAc (3 x 0.5 mL), dried over Na_2SO_4 and analyzed directly by chromatography to determine the conversion and the enantiomeric excesses of the sulfoxidation products

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