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Immobilized *Aspergillus niger* epoxide hydrolases: Cost-effective biocatalysts for the prepation of enantiopure styrene oxide, propylene oxide and epichlorohydrin

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ABSTRACT

This study aimed to prepare robust immobilized epoxide hydrolase (EH) preparations for asymmetric hydrolysis of racemic epoxides such as styrene oxide, propylene oxide and epichlorohydrin. For this purpose, *Aspergillus niger* EH was immobilized onto Lewatit® VP OC 1600 support by adsorption, modified Florisil® and Eupergit® C supports by covalently. The suitability of the supports was examined for protein binding capacity and rate of racemic styrene oxide hydrolysis. The protein-activity recovery yields were 75–85%, 82–78% and 90–75%, respectively for EH immobilized onto Lewatit® VP OC 1600, modified Florisil® and Eupergit® C supports. All *A. niger* EH preparations catalyzed preferentially hydrolysis of (*R*)-epoxides. Although enantiomeric excess values of all the tested epoxides were 99%, the highest enantiopure epoxide yields were obtained as 48% for (*S*)-styrene oxide by the immobilized EHs onto modified Florisil® and Eupergit® C. The highest diol yield was obtained as 78% for 3-chloro-1,2-propanediol, however, its enantiomeric excess value was 28.2%. Enantioselectivity of *A. niger* EH was improved with the preparation of mentioned immobilized forms. The highest enantioselectivity value was obtained as 95 toward styrene oxide by *A. niger* EH immobilized onto modified Florisil®. The results of reusability studies show that the immobilized EH preparations offer feasible potentials for the preparation of enantiopure epoxides than that of free form.

1. Introduction

Enantiopure epoxides as well as their corresponding diols are attractive synthons in the preparation of beta-adrenergic receptor blocking agents, anticancer and anti-obesity drugs, nematocides and ferroelectric liquid crystals with respect to versatile reactivity of oxirane ring which can be opened by various nucleophiles to prepare a multitude of more elaborate intermediates [1–6]. Since the demand has been increased for the preparation of these chiral compounds with sustainable methods with regard to legal pressures for the production of safer drugs, biocatalytic methods have been rapidly applied for the syntheses of enantiopure epoxides in organic chemistry [7–9]. Epoxide hydrolases (EHs, EC 3.3.2.3) catalyze addition of a water molecule to oxirane ring leading to formation of corresponding vicinal diol (Fig. 1) [2,10,11]. EHs were reported to be cofactor-independent enzymes and found in all living organisms [12,13]. Besides, the exhibitions of high regio-, stereo- and enantio-selectivity make EHs unique biocatalysts in the preparation of enantiopure epoxides as well as corresponding 1,2-diols via kinetic resolution of racemic epoxides [14–17]. One of them, recombinant *Aspergillus niger* EH, was used the preparation of various enantiopure epoxides and their corresponding vicinal diols with high yield and enantiopurity. Notwithstanding their mentioned excellent properties, EHs have some drawbacks in industrial applications such as deficiency of operational and storage stability, high price and denaturation at high temperatures. Therefore, the immobilizations of EHs have been increasing interest to overcome these drawbacks. Up to now, *A. niger* EH has been previously immobilized onto polypropylene and Eupergit® [18], epoxy-activated supports [19], DEAE-cellulose [20], epoxide-activated silica gel [21], Accurel EP 100 [22] and modified Eupergit® C 250 L [23]. The results of these studies showed that the immobilized EHs were as effective biocatalysts as free forms and more stable EH preparations were often obtained upon immobilization depending on the used immobilization protocol [19,23,24]. However, pursuing a preparation of immobilized EH having enhanced thermal stability, operational stability and enantioselectivity has always been a goal to obtain a robust EH preparation.

In the present study, we aimed to assess the potential uses of Lewatit® VP OC 1600, modified Florisil® and Eupergit® C for the immobilization of *A. niger* EH. The optimum pHs, temperatures and kinetic parameters of free and immobilized EHs were determined for racemic styrene oxide hydrolysis. All the EH preparations were tested for the asymmetric hydrolysis of racemic styrene oxide,

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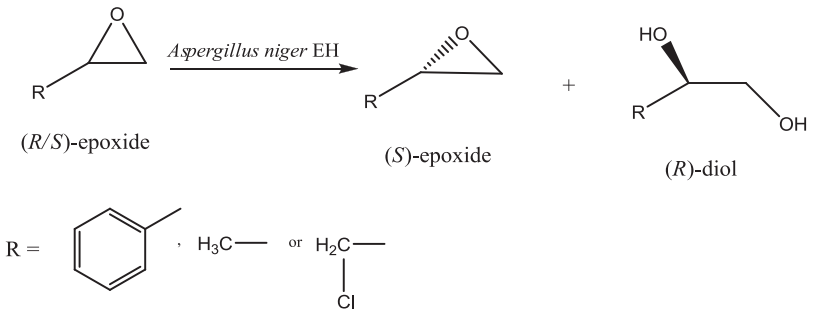

Fig. 1. Asymmetric hydrolysis of Racemic styrene oxide, propylene oxide and epichlorohydrin by *A. niger* EH.

propylene oxide and epichlorohydrin to obtain enantiopure form of the mentioned epoxides and their corresponding vicinal diols.

2. Materials and methods

2.1. Materials

Lewatit® VP OC 1600 (divinylbenzene-crosslinked poly(methylmethacrylate) resin, particle size 315–1000 μm, pore size 150 Å and surface area 130 $m^2 g^{-1}$) was kindly provided by Lanxess (Deutschland GmbH). Epoxide hydrolase (1.7 U mg solid^{-1}, *A. niger* sp., recombinant from *A. niger*), racemic styrene oxide ((*R*/*S*)-SO) and (*S*)-styrene oxide ((*S*)-SO) were obtained from Fluka. Eupergit® C (particle size 150–250 μm, a macroporous copolymer prepared by copolymerization of N,N′-methylene-bis-meth-acrylamide, glycidyl methacrylate, allyl glycidyl ether and methacrylamide, oxirane content ≥600 μmol g^{-1} oxirane), glutaraldehyde (aqueous solution, 50%, w/w), (3-aminopropyl)trietoxysilane (3-APTES), ethylenediamine (EDA), racemic propylene oxide ((*R*/*S*)-PO), epichlorohydrin ((*R*/*S*)-ECH), 1,2-propanediol ((*R*/*S*)-PD), 1-phenyl-1,2-ethanediol ((*R*/*S*)-PED), 3-chloro-1,2-propanediol ((*R*/*S*)-CPD), (*S*)-propylene oxide ((*S*)-PO), (*S*)-epichlorohydrin ((*S*)-ECH), (*R*)-1-phenyl-1,2-ethanediol ((*R*)-PED), (*R*)-1,2-propanediol ((*R*)-PD), (*R*)-3-chloro-1,2-propanediol ((*R*)-CPD), dimethyl sulfoxide (DMSO) and ninhydrin reagent (2% solution) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Florisil® (magnesium silicate, composition: SiO_2: 85%, MgO: 15%; particle size 150–250 μm, pore size 6–8 nm, specific surface area 170–300 $m^2 g^{-1}$ and pH value 9.0–10.0), acetonitrile and triethylamine were supplied from Merck (Darmstadt, Germany).

2.2. Methods

2.2.1. Modification of Florisil® and Eupergit® C supports

Before *A. niger* immobilization, Florisil® was silanized with 3-APTES and activated with glutaraldehyde according to Alptekin et al. [25] and Eupergit® C was treated with EDA and activated with glutaraldehyde according to Yildirim et al. [23]. The free primary amine (—NH_2) group amounts onto Florisil® and Eupergit® C supports after 3-APTES and EDA treatments were measured using ninhydrin reagent and glutaraldehyde activation [25].

2.2.2. Immobilizations of A. niger epoxide hydrolase

To 2 g of Lewatit® VP OC 1600, 8 mL of EH solution (1 mg mL^{-1}) prepared in a phosphate buffer (50 mM, pH 7.0) was added. The immobilization mixture was slowly agitated in a water bath at 5 °C for 4 h.

To 2 g of modified Florisil® support, 8 mL of a phosphate buffer (50 mM, pH = 7.0) containing 1 mg mL^{-1} EH was loaded [25]. The mixture was slowly shaken at 5 °C during 2 h immobilization time. After that, the mixture was filtrated to collect the resulting immobilized EH preparations.

In case of using modified Eupergit® C as immobilization support, to 2 g of the support 8 mL of EH solution (1 mg mL^{-1}) prepared in phosphate buffer (100 mM, pH = 7.0) was added [23]. The immobilization reaction was continued for 2 h at 5 °C.

At the end of related immobilized times, the mixtures were filtrated to collect the immobilized EH preparations for each immobilization study followed by washing with their immobilization buffers (50 mM, pH = 7.0) until no protein could be detected in the filtrates. The protein method of Lowry was used to determine the amounts of enzyme protein bound onto Lewatit® VP OC 1600, modified Florisil® and Eupergit® C supports [26]. The all immobilized EH preparations were kept at 5 °C for 12 h to eliminate inter-particle water and then stored at 5 °C until it was used.

The immobilized EH preparations were mentioned as EHIL (EH immobilized onto Lewatit® VP OC 1600), EHIF (EH immobilized onto modified Florisil®) and EHIE (EH immobilized onto modified Eupergit® C) throughout the text.

2.2.3. Epoxide hydrolase assay

The activity measurements of all EH preparations were performed according to Petri et al. [21]. To 350 μL of phosphate buffer (100 mM, pH = 7.0), 50 μL of free EH solution (1 mg mL^{-1}) or 10 mg of immobilized EH was added. The reaction was initiated with 100 μL (*R*/*S*)-SO addition (100 mM in DMSO). After 10 min, 100 μL of sample was withdrawn and analyzed on C18 column (Phenomenex Luna, 4.6 mm × 250 mm) by high-performance liquid chromatography (HPLC) at 220 nm. The mobile phase was a mixture of methanol/water (50/50%, v/v) at a flow rate of 0.5 mL min^{-1}. One μmol of PED produced per mg protein of EH in 1 min under the assay contiditions was defined as 1 unit.

2.2.4. Characterization of free and immobilized epoxide hydrolases

2.2.4.1. Effect of pH. To optimize pH, the activities of all EH preparations were determined at different buffer solutions such as 100 mM acetate (pH = 5.0, 5.5) citrate (pH = 6.0) and phosphate (pH = 6.5–8.0) buffers.

2.2.4.2. Effect of temperature. The activities of free and immobilized EHs were measured at 20–60 °C to determine optimal temperature for EH activity.

2.2.4.3. Kinetic parameters. The free and immobilized EH activities were determined at different (*R*/*S*)-SO concentrations (1–20 mM) under the optimum conditions of each EH preparation. Michealis–Menten constants (K_m) and maximum velocity (V_{max})