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Journal of Molecular Catalysis B: Enzymatic 32 (2005) 175–183
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Immobilization of epoxide hydrolase from *Aspergillus niger* onto DEAE-cellulose: enzymatic properties and application for the enantioselective resolution of a racemic epoxide

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Received 7 July 2004; received in revised form 29 October 2004; accepted 1 November 2004
Available online 7 January 2005

Abstract

Recombinant epoxide hydrolase (EH) from *Aspergillus niger* can be a very promising tool for the resolution of various racemic epoxides by enantioselective hydrolysis. The enzyme was successfully immobilized by ionic adsorption onto DEAE-cellulose (99% yield, 70% of retention activity). The temperature for maximal activity (40 °C) and the activation energy (38.8 kJ/mol) were similar for both the immobilized and free EHs, whereas the optimal pH was about one unit less for the immobilized enzyme. Thermal stability was also affected by immobilization; the immobilized enzyme appeared to be slightly less stable than the free one. However, a gram-scale resolution of racemic *para*-chlorostyrene oxide (*p*CSO) was successfully carried out in a repeated batch reactor, operated for seven cycles. Furthermore, using a very high substrate concentration of 2 M (306 g/L), i.e. biphasic conditions, the resolution of 3 g of *p*CSO was also achieved in a repeated batch reactor using approximately 300 mg of immobilized EH, corresponding to less than 3 mg of the enzymatic powder.

Keywords: Epoxide hydrolase; *Aspergillus niger*; Immobilization; Enzymatic resolution; Reactor

1. Introduction

Due to their chemical versatility, enantiopure epoxides are important building blocks for the synthesis of bioactive molecules. Over the last few years, several chemical (transition-metal-based) and biocatalytic approaches have been developed for the synthesis of such chiral epoxides [1]. One of the most promising biocatalytic processes consists of the kinetic resolution of chemically produced racemic mixtures of epoxides by epoxide hydrolases [1–3]. Epoxide hydrolases (EHs) (EC 3.3.2.3) catalyze the addition of a water molecule on the oxiran ring of epoxides leading to the corresponding vicinal diols [4]. EHs are widespread in nature and the mammalian enzymes have been studied in detail [5]. More recently, EHs from microorganisms, which are much more readily available, have attracted a great deal of attention and have been used successfully for the enantioselective resolution of various racemic epoxides [1,3,6]. Numerous reports have been published on the preparation of enantiopure epoxides using this approach [7–11], which proved the feasibility of the method at the preparative scale.

The present authors produced two enantiopure epoxide preparations at very high substrate concentration (up to several 100 g/L) [12,13] by using EH from *Aspergillus niger*. The enzyme was then purified to homogeneity [14], sequenced and cloned into an *E. coli* host [15]. Over-expression of the enzyme was subsequently carried out after cloning the EH gene into an appropriate *Aspergillus* host. However, due to the low stability of this enzyme in soluble form with a typical half-life ranging from 20 to 30 h, immobilization could be a solution to improve its stability, re-use and possible continuous operation in packed-bed reactors. In addition, an important benefit derived from immobilization will be the easy

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doi:10.1016/j.molcatb.2004.11.001

separation of the enzyme from the products, which minimize downstream processing costs. To date, there are only a few reports on the immobilization of EH by adsorption [16,17] and covalent binding [18,19]. Compared to other immobilization techniques, adsorption is simple, inexpensive and not very time consuming. In a preliminary work, *A. niger* EH from the wild strain was efficiently immobilized by simple electrostatic adsorption onto DEAE-cellulose [17] but the low level of enzyme activity prevented the characterization and evaluation of the immobilization procedure from a synthetic point of view. In the present study, EH prepared from the recombinant *A. niger* strain, was found to be a much more active enzyme preparation and further characterized. Factors important for catalytic activity, such as diffusional limitations, pH and temperature of the reaction medium and thermal stability were optimized. Kinetic parameters for the free and immobilized EHs were also determined using *p*-chlorostyrene oxide (*p*CSO) as substrate. The possible reuse of the immobilized EH for successive hydrolyses in batch reactor was examined at both low and high substrate concentrations for the resolution of several grams of racemic *p*CSO.

2. Experimental

2.1. Enzyme preparation

The recombinant *A. niger* Gbcf 79 containing the EH gene [15] was cultivated in a simple medium with only glucose (10 g/L) and corn steep liquor (20 g/L) as nutrients in 7-L fermentors according to the conditions described previously [20]. After 34 h of culture incubation, the mycelia (19.4 g of dry mass) were recovered by vacuum filtration and resuspended in 1.4 L of 10 mM Tris–HCl buffer, pH 7.1, containing 1 mM EDTA, 1 mM cysteine to create a reducing environment as described in the first paper on purification of this enzyme [14], and 0.3 mM phenylmethanesulfonyl fluoride to prevent enzyme inactivation (buffer A). The suspension was homogenized using a mechanical grinder and the cells were disrupted by pressure shock (27 kpsi, Cell Disrupter from Constant System Ltd., Daventry Northants, UK) to liberate the intracellular EH activity. Unbroken cells and cellular debris were precipitated by centrifugation ($8000 \times g$, 20 min) at 4 °C and discarded. The supernatant, containing the EH, was diluted with 1.2 L of buffer A and treated by microfiltration using a membrane with a cut-off of 0.1 μm (Inceltech, PSFH, France) to obtain a clear extract. The resulting microfiltrate, containing the soluble components, was concentrated to 410 mL by tangential flow ultrafiltration through a membrane with a cut-off of 40 kDa (Inceltech, 40UFIB/1/S2, France). The concentrated enzyme solution was then loaded onto a DEAE-sepharose column (2.5 cm × 30 cm, Pharmacia), previously equilibrated with buffer A, and the unbound proteins were removed by washing with 500 mL of buffer A. The adsorbed proteins were subsequently eluted with a linear gradient of KCl ranging from 0 to 0.35 M in a total volume of 924 mL buffer A at a flow rate of 3 mL/min and collected in volumes of 10 mL. EH activity in the eluted fractions was determined using the standard procedure described below. The active fractions were pooled, dialyzed against water and lyophilized. All procedures were carried out at 4 °C.

2.2. Epoxide hydrolase immobilization

The immobilization of *A. niger* EH was carried out by suspending 80 mg wet weight of DEAE-cellulose in 1 mL of EH extract prepared in 10 mM Tris–HCl buffer, pH 7.1. The protein concentration of the EH extract varied from 0.23 to 8.28 mg/mL corresponding to respective loadings of 2.9–101.0 mg protein/g of DEAE-cellulose and respective activities of 46.3–2216 U/g of DEAE-cellulose. The mixture was incubated for 45 min at 4 °C with mild stirring. The support, containing the adsorbed enzyme, was subsequently recovered by centrifugation (1000 rpm, 10 min, room temperature), washed with 1 mL of the same buffer to remove unbound proteins and stored wet at 4 °C.

2.3. Epoxide hydrolase assay

The EH activity was assayed by measuring the rate of hydrolysis of racemic *para*-chlorostyrene oxide (*p*CSO). This epoxide was obtained by epoxidation of the corresponding olefin using *meta*-chloroperoxybenzoic acid. Volumes of 200 μL of the enzyme preparations at appropriate dilutions (ranging from 0.4 to 2 μg of the free EH and from 0.03 to 0.1 mg of the immobilized EH) in 10 mM Tris–HCl buffer (pH 7.1) were pre-incubated at 27 °C for 2 min. The hydrolytic reaction was started by the addition of 200 μL of an 8 mM solution of racemic *p*CSO in 10 mM Tris–HCl buffer, pH 7.1, containing 2% (v/v) of dimethylformamide. The mixture was incubated at 27 °C under agitation using a magnetic stirrer at 750 rpm for 10 min and then stopped by the addition of a mixture composed of 200 μL acetonitrile and 600 μL hexane. The reaction mixture was then shaken vigorously to extract the remaining substrate and centrifuged (15,000 rpm, 3 min, at room temperature). The concentration of formed diol, located in the lower aqueous layer, was determined by HPLC analysis using a Supelcosil LC-18 column as described previously [12]. One EH unit (U) was defined as the amount of biocatalyst that catalyzed the formation of 1 μmol of diol per minute. Protein concentration was assayed according to the method of Lowry using bovine serum albumin as standard.

2.4. Effect of pH on EH activity

The effect of pH on EH activity was investigated at seven different pHs ranging from 4 to 9 at 25 °C and with *p*CSO as substrate as described above for the EH assay.

2.5. Effect of temperature on EH activity and stability

The activities of immobilized and free EHs were assayed at 13 different temperatures ranging from 0 to 70 °C as described previously. The effect of temperature on EH stability was studied at 4, 15, 27, 37 and 47 °C. Both immobilized (10 mg protein/g wet support) and free (1.12 mg protein/mL) preparations were incubated in Tris–HCl buffer (10 mM, pH 7.1) at the desired temperature in a water bath. At various time intervals, samples (200 μL) were withdrawn and assayed for EH activity using the standard assay conditions. Inactivation kinetics were obtained by plotting the remaining activities versus time.

2.6. Determination of kinetics parameters

The rate of racemic *p*CSO hydrolysis was measured at substrate concentration ranging from 0.5 to 8 mM. A protein loading of 10 mg protein/g wet support was used for the immobilized EH. The kinetic parameters, K_{mapp} and V_{max}, were estimated by non-linear regression using a Sigma plot program (Jandel Scientific, Germany). The catalytic constants, k_{cat}, were calculated from the V_{max} using the enzyme concentration values of 5.73×10^{-2} μmol/g of support for the immobilized EH and 1.52×10^{-3} μmol/mL for the free EH. The molecular mass of the *A. niger* EH was 45 kDa [14]. Due to the heterogeneous nature of the reaction medium containing the immobilized EH, the phenomenon of substrate partitioning may have affected the substrate concentration inside the biocatalyst so that the term 'apparent' was subsequently used for the Michaelis constant, K_{mapp}, and the maximal velocity, V_{max}.

2.7. Enzymatic reactor

A batch reactor with a total volume of 30 mL was run at 4 °C using a racemic *p*CSO as substrate at a concentration of 4 mM in Tris–HCl buffer (10 mM, pH 7.1) containing 1% of dimethylformamide. The reaction was started by the addition of 68 mg of immobilized EH, prepared by loading 10 mg of protein onto 1 g of wet support, and then stirred (750 rpm) for 112 min. At different time intervals, aliquots of 100 μL were withdrawn and mixed with 100 μL of methanol to stop the reaction. The remaining epoxide was extracted with 150 μL isooctane containing *meta*-bromoacetophenone as an internal standard and analyzed by chiral GC (col. CP-Chirasil-Dex CB, 125 °C, (*R*) 12.4 min and (*S*) 13.0 min) to determine the degree of conversion and the enantiomeric excess [12]. The formed diol was extracted with 2 mL ethyl acetate after saturation of the aqueous phase with NaCl, the quantification of its enantiomeric excess was performed after derivatization into its acetonide which was analyzed by chiral GC (col. CP-Chirasil-Dex CB, 140 °C, (*R*) 13.8 min and (*S*) 15.0 min) [12]. The *E* value of the enantioselective resolution was calculated with Sih's equation [21] using the degree of conversion and the enantiomeric excess of the remaining epoxide.

2.8. Repeated use of the immobilized EH

The operational stability of the immobilized EH at a low substrate concentration (4 mM) was investigated by assaying 45 mg of the immobilized EH in successive batch reactors with total volumes of 20 mL using the above-defined conditions. When the reaction reached 50% conversion, the immobilized EH was removed from the reaction medium by centrifugation (1500 rpm, 10 min, at room temperature) and rinsed with 2 mL Tris–HCl buffer (10 mM, pH 7.1). For the next cycle, the immobilized EH was reintroduced into 20 mL of a fresh aqueous solution containing 4 mM *rac*-*p*CSO and its activity was measured as described above.

The recycling of the immobilized EH in a biphasic batch reaction, containing a high substrate concentration of 2 M, i.e. 306 g/L, was investigated. A series of seven consecutive hydrolytic reactions, containing 612 mg *rac*-*p*CSO in 2.0 mL of Tris–HCl buffer (10 mM, pH 7.1) and 287 mg of immobilized EH, were performed. The reaction was conducted at 4 °C with gentle magnetic stirring (750 rpm). When the enantiomeric excess of the remaining epoxide reached a value of approximately 98%, the solid support was recovered by centrifugation (1500 rpm, 10 min, at room temperature) and rinsed twice with 2 mL of isooctane to extract any substrate or product eventually adsorbed onto the matrix. For the next cycle, the immobilized enzyme was re-suspended in fresh substrate solution and processed for successive experiments in the same manner.

3. Results and discussion

3.1. Enzyme preparation

In previous immobilization work [17], we had used an enzyme preparation, obtained from the wild type fungus, that exhibited a low specific activity of approximately 0.3 U/mg with estimated EH content less than 0.3%. In order to eliminate possible interference from the contaminating proteins, a more purified EH preparation was used in this work where the EH activity produced by the recombinant of *A. niger* Gbcf 79 strain was found to be 10–15 times higher than that observed earlier with the wild type strain [21]. After cultivation, the mycelia were recovered and subjected to pressure shock to liberate the intracellular EH which was further purified by microfiltration and ultrafiltration thereby resulting in an extract with a very high activity yield (85%) and a 2.6-fold purification factor (Table 1). The extract was then subjected to ion-exchange chromatography that increased the purification factor by approximately 11 with a 51% activity yield. The enzyme preparation was then lyophilized resulting in a slight decrease in both its activity and yield. Overall, the specific activity of the EH preparation was in the range of

Table 1
Partial purification of the epoxide hydrolase from *A. niger*

Fractions	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude extract	9638	2.7	100	1
Microfiltration	8342	4.6	87	1.7
Ultrafiltration	8183	6.9	85	2.6
DEAE-sepharose	4909	28.9	51	10.7
Lyophilization	3229	23.9	33.5	8.9

Data are given for a 5 L culture in fermentor leading to 19.4 g of mycelium (dcw).

20–24 U/mg protein. Compared to the specific activity of the purified enzyme (unpublished results), the amount of pure EH in this partially purified preparation was estimated to be at 27% and 100 times higher than that of the previously used preparation [12]. The lyophilized powder was stored at 4 °C with no detectable loss of activity for at least 2 months (<3%).

3.2. Immobilization

Based on previous results published in the literature [16] and our research [17], the DEAE-cellulose support was selected for EH immobilization. Since the isoelectric point of the purified EH was 4.5 [15], a 10 mM Tris–HCl, pH 7.1 buffer was used for immobilization. Different amounts of the EH preparation were mixed with wet DEAE-cellulose to obtain final loadings ranging from 2.9 to 101.0 mg protein/g support (Table 2) and the activities of supernatants were assayed with time. The kinetics of adsorption (data not shown) were relatively fast so that equilibrium was reached within 10 min for the lowest enzyme amounts and with all of the EH activity immobilized. At higher protein loadings, adsorption was slower requiring 20–35 min for the two highest concentrations tested. These longer times could be due to limitations of the protein diffusion rate through the stagnant water layer and inside the support; as a precaution, an incubation time of 45 min was routinely used in all experiments.

Table 2 shows that the immobilization yield of EH activity was near 100% for the 10 experiments carried out at the lowest enzyme loading. For the two highest concentrations, the immobilization yield of activity decreased. Contrary to this, the protein immobilization yield was 70–80% for the eight lowest protein amounts and decreased at higher protein loadings. These differences between the immobilization yields for protein and EH activity suggest that EH adsorbed preferentially onto this support compared to the other proteins present in the enzymatic preparation. The ratio of the specific activity in solution before and after adsorption can be used as a measure of the adsorption selectivity [22]. The adsorption of *A. niger* EH onto DEAE-cellulose gave a high adsorption selectivity factor of about 84 at the 10 lowest enzyme loading. This high selectivity could be explained by the higher affinity of EH for the support compared to the other proteins present in the enzyme preparation, which could be due to the low isoelectric point of EH and/or the weak interactions between

Table 2
Immobilization of different amounts of EH preparation onto DEAE-cellulose

Protein contacted[a] (mg/g)	Protein immobilized[b] (mg/g)	Protein immobilization yield[c] (%)	EH activity immobilized contacted[d] (U/g)	EH activity immobilized theoretical[e] (U/g)	EH immobilization (%)	Activity immobilized yield[f,g] (U/g)	Retention of activity[h] (%)
2.9	2.4	80.5	46.3	46.2	99.7	33.4 ± 2.2	72.5
5.0	3.6	77.4	92.3	92.3	100.0	67.8 ± 2.0	73.4
7.0	5.0	74.5	121.2	120.0	99.0	85.1 ± 8.6	70.9
9.9	7.7	77.5	184.6	184.6	100.0	126.9 ± 28.3	68.8
13.8	10.7	77.5	256.2	256.0	99.9	172.3 ± 14.1	67.3
19.6	15.2	77.6	363.2	363.2	100.0	186.3 ± 4.0	51.3
22.1	17.0	77.1	357.1	357.1	100.0	193.3 ± 2.2	54.1
35.9	25.5	71.0	579.9	579.8	100.0	226.8 ± 4.0	39.1
47.0	29.0	61.7	698.1	700.0	100.2	235.1 ± 1.2	33.6
51.2	35.6	69.5	827.2	826.7	100.0	249.0 ± 0.24	30.1
61.4	39.2	63.8	1176.3	1124.8	95.6	255.1 ± 12.5	22.7
101.0	50.0	49.5	2216.0	1678.0	75.7	273.5 ± 21.9	16.3

[a] Calculated from the protein content of the solution used.
[b] Estimated from the difference between protein contacted and protein recovered in supernatant and washing of the immobilization procedure.
[c] Calculated from protein immobilized and protein contacted.
[d] Calculated from the EH activity of the solution used.
[e] Calculated from the difference between activity contacted and activity recovered in supernatant and washing of the immobilization procedure.
[f] Calculated from activity immobilized and activity contacted.
[g] EH activity assayed using the immobilized enzyme preparation, data are average of four independent determinations and standard deviations are given.
[h] Calculated from the assayed activity of the immobilized preparation and that of the theoretical activity of the immobilized EH.

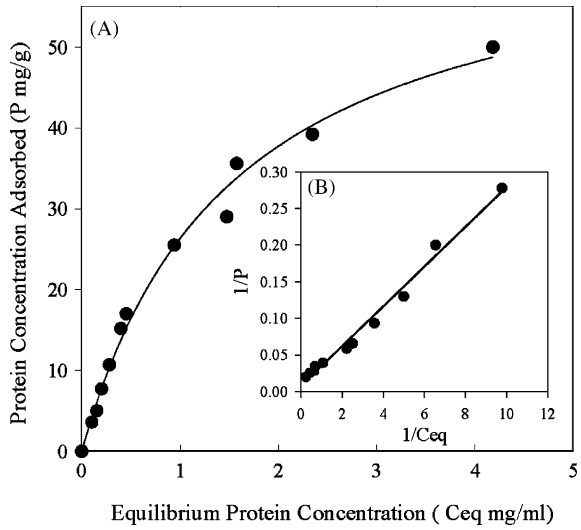

Fig. 1. (A) Adsorption isotherm of protein on DEAE-cellulose from a solution of crude EH preparation from *A. niger*. (B) Linear representation of Langmuir isotherm model.

the other proteins and the support. These findings indicate that immobilization of the enzyme preparation onto this specific support could also be used to increase its purification. However, the retention of activity was approximately 70% for the five lowest amounts of protein immobilized. These values are in the same range as those reported for immobilized EH [16,17,19]. These observed results could be explained by the occurrence of conformational changes in the enzyme during immobilization, steric hindrance at the active sites, or micro-environmental effects such as substrate partitioning. At higher EH loadings, the retention activity decreased significantly to around 16% which was likely due to the formation of a dense protein layer generated a steric and/or diffusional barriers, thus limiting further access of the substrate to the enzyme active site (see Section 3.4).

3.3. Adsorption isotherms

The adsorption of protein onto a solid surface is mainly governed by the surface area of the carrier, the size of the enzyme molecule and the adsorption conditions [22,23]. When ion exchange resins are used, the specificity and the strength of the interaction, i.e. ionic attraction, are also important factors. In general, the adsorption isotherms of protein molecules onto carriers show a fast increase followed by a plateau corresponding to monolayer formation [22]. Fig. 1A shows that saturation of the DEAE-cellulose support with different amounts of EH preparation was not reached. However, Fig. 1B (linear representation of $1/P$ versus $1/C_{eq}$) indicates that the adsorption isotherm of our enzymatic extract on DEAE-cellulose obeys well the Langmuir adsorption model, which assumes an energetically homogeneous support surface with identical adsorption sites throughout [22,24,25]. The maximum amount of protein adsorbed per gram of support and the Langmuir constant, K_L, estimated from non-linear regression of data to the Langmuir model, were 66.5 mg/g and 1.5 mg/mL, respectively. These results showed that a relatively good loading capacity and affinity between DEAE-cellulose and the proteins in the crude EH enzymatic extract was obtained.

3.4. Diffusional limitations

Since the reaction mixture containing the immobilized enzymes was heterogeneous consisting of a solid support loaded with proteins suspended in an aqueous phase containing the substrate, reaction kinetics took into account the mass transfer of substrate molecules from the aqueous phase to the enzyme molecule, i.e. the occurrence of external mass transfer in the stagnant layer around the solid particles and internal mass transfer inside the support [26].

The enzymatic reaction rate at different relative flow rates between the liquid and solid phases was measured to investigate the occurrence of external diffusional limitations. Increasing the fluid velocity by changing the agitation rate of the assays was used to decrease the width of the stagnant film of liquid at the support surface and to lower the external mass transfer limitations. At agitation rates between 250 and 1250 rpm (not shown), the reaction rate was almost constant at 141.1 ± 7.9 U/g wet support, whereas without agitation, the reaction rate was reduced to 51.1 ± 5.8 U/g wet support due certainly to the occurrence of external diffusional limitations. An agitation speed of 750 rpm was therefore selected for the following experiments.

The occurrence of internal diffusional limitations was investigated by measuring the reaction rates using different amounts of immobilized EH ranging from 2.35 to 50.00 mg protein/g wet support. The results indicate (Fig. 2) that the activity of the immobilized EH increased linearly with the amount of immobilized protein up to 10.7 mg/g wet support corresponding to an activity of 172 U/g wet support. These findings indicate the absence of internal diffusional limitations as indicated by the proportionality between EH activity and immobilized protein. At higher loadings, the activity increased to a lower extent due the occurrence of internal diffusional limitations. The maximum reaction rate of 249 U/g wet support obtained at the plateau represents the extent to which the substrate could be carried from the support surface to enzyme molecules located inside the support particles. All subsequent experiments were therefore run with a protein loading of around 10 mg/g.

3.5. Effect of pH on activity

The effect of pH on the activity of immobilized and free EHs was performed in the pH range of 4.0–9.0. The activity profile of the immobilized EH (Fig. 3) shifted towards the more acidic pH values and showed an optimum pH of 6.5, retaining more than 80% of its maximum activity in the pH range of 5.0–7.5. The maximum activity of the free EH was obtained at pH 7.5, with more than 83% of this maximum

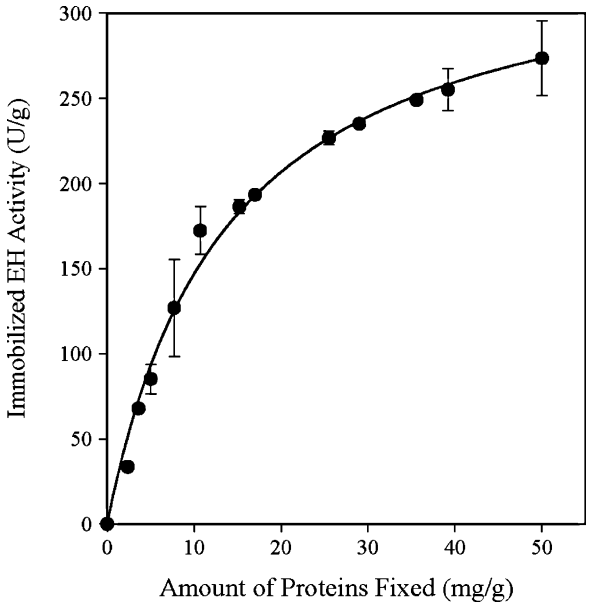

Fig. 2. Enzymatic activity of the solid support with different loads of enzyme. Different amounts of proteins from a crude EH preparation were immobilized on the DEAE-cellulose. The hydrolytic activity was assayed under standard conditions using *rac*-*p*CSO as substrate.

activity maintained in the pH range of 6.5–9.0. These differences in the behaviors of the free and immobilized EHs could be explained by the poly-cationic nature of a support like DEAE-cellulose that attracts more OH^- ions around the immobilized enzyme, thus making the pH of enzyme's microenvironment higher than the rest of the bulk of the solution. The immobilized enzyme therefore requires a lower pH for optimal activity than the free enzyme.

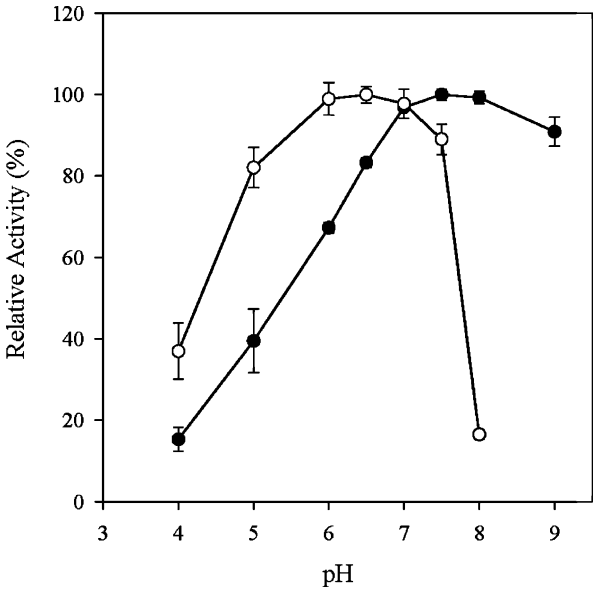

Fig. 3. Effect of pH on the activity of free (●) and immobilized (○) EHs from *A. niger*. The activities were assayed on *rac*-*p*CSO at different pH values under standard conditions. Results are average of three determinations of activity with a standard deviation of less than 10%.

3.6. Effect of temperature on activity

The free and immobilized EH activities were investigated at extensive temperatures ranging from 0 to 70 °C. Temperature profile of free and immobilized enzyme were practically identical with a sharp maximum at 40 °C. For both preparations, the initial reaction rate of *p*CSO hydrolysis increased by a factor of seven at temperatures ranging from 0 to 40 °C and then decreased sharply at higher temperatures. However, immobilization did not change the optimal temperature of 40 °C at which the reaction rate was maximal. Similar trends have been reported for *Citrullus vulgaris* urease [29] and *A. niger* EH [17] immobilized on DEAE-cellulose.

From the experimental data, activation energies of 35.4 and 38.8 kJ/mol were determined for the free and immobilized EHs, respectively. These values were not significantly different, suggesting that the limiting step for biocatalysis was the same for both preparations. These results also confirm previous findings indicating that diffusion limitations had no effect on the reaction rates of *p*CSO hydrolysis by immobilized EH with the conditions used.

3.7. Temperature stability

The thermal stability of the free and immobilized EHs at different temperatures was investigated. Thermal inactivation of the free and immobilized EHs followed first-order kinetics (not shown). The first-order inactivation constants, k_i, estimated from the slopes of the semi-logarithmic plots of all data, are given in Table 3. The k_i values of the immobilized EH were higher than those of the free EH indicating that immobilization slightly decreased enzyme stability in the range of temperatures investigated. These unexpected results suggest that the conformational structure of the enzyme was affected by immobilization onto DEAE-cellulose. In contrast, the *Nocardia* EH immobilized onto DEAE-cellulose [16] and the *A. niger* EH immobilized onto Eupergit C/EDA [19] showed that the immobilized EH was more stable than the free one.

Half-life times at different temperatures were also estimated from the inactivation kinetics (Table 3). Higher temperatures induced a remarkable loss in stability for both preparations where the half-life decreased significantly from 330 and 157 h at 4 °C to 4.3 and 0.3 h at 47 °C for the free and

Table 3
Inactivation constants and half-life times of free and immobilized *A. niger* EH

Temperature (°C)	Inactivation constant (h^{-1})		Half-life (h)	
	Free EH	Immobilized EH	Free EH	Immobilized EH
4	0.002	0.004	330	157
15	0.004	0.015	183	45
27	0.006	0.078	115	9
37	0.046	0.270	16.5	2.5
47	0.162	2.520	4.3	0.3

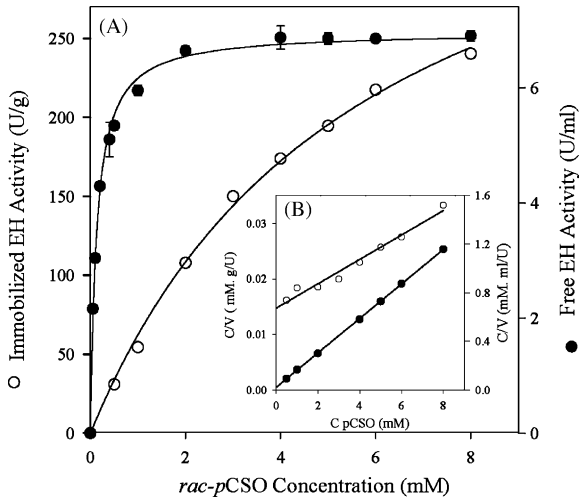

Fig. 4. (A) Effect of *rac-p*CSO concentration on the activity of free (●) and immobilized (○) EH from *A. niger*. The activities were assayed for different *rac-p*CSO concentrations under standard conditions. Results are average of three determinations of activity with a standard deviation of less than 10%. (B) Hanes–Woolf plots.

immobilized EHs, respectively. These findings indicate that the kinetics of EH inactivation are highly influenced by temperature. A quantitative estimation of the effect of temperature was obtained by calculating the activation energy of thermal inactivation from the plot of the logarithm of the inactivation constant, k_i, versus the reciprocal of the absolute temperature (not shown). The activation energies of the free and immobilized EHs were 94.6 and 98.2 kJ/mol, respectively. These high values confirmed the high sensitivity of the free and immobilized EHs to thermal inactivation. However, these values were lower to those obtained for EHs from wild type *A. niger* (177 kJ/mol) [30] and *Rhodosporidium toruloides* (167 kJ/mol) [31].

3.8. Kinetic parameters

The kinetic parameters of *rac-p*CSO hydrolysis were determined for both the free and immobilized EHs from *A. niger*. For the free EH, the rate of *p*CSO hydrolysis versus substrate concentration gave a hyperbolic curve, suggesting Michaelis–Menten kinetics (Fig. 4A). These results were confirmed by the linearity of the Hanes–Woolf plot (Fig. 4B). For the immobilized EH, no plateau was observed in the range of substrate concentrations investigated (Fig. 4A) and concentrations higher than 8 mM could not be investigated due to the insolubility of the substrate in the reaction mixture; however, the Hanes–Woolf plot was linear, indicating that the kinetics of the immobilized EH followed the Michaelis–Menten model. Table 4 shows the kinetic parameters estimated by non-linear regression. The K_{mapp} and k_{cat} of the EH increased by approximately 68 and 2 times after immobilization onto DEAE-cellulose, respectively. Consequently, the specificity constant, k_{cat}/K_{mapp}, of the immobilized EH was 31 times lower than that obtained for the free

Table 4
Kinetic parameters for the free and immobilized *A. niger* EH on *p*CSO

Parameter	Free EH	Immobilized EH
K_{mapp} (mM)[a]	0.13 ± 0.01	8.1 ± 0.8
V_{max} (U/mg)[b]	26.9 ± 0.3	54.4 ± 4.9
k_{cat} (s^{-1})[c]	76.3	153.0
k_{cat}/K_{mapp} (s^{-1} mM^{-1})	587.0	18.9

[a] K_{mapp} is an apparent parameter because of a possible partition effect of the substrate concentration between macro- and micro-environment of immobilized EH.

[b] V_{max} is expressed per milligram of proteins for the free and immobilized EH.

[c] The catalytic constant was calculated from the maximum reaction rate using the relation, $V_{max} = k_{cat} E_0$ with $E_0 = 5.73 \times 10^{-2}$ μmol/g of carrier for the immobilized EH and $E_0 = 1.52 \times 10^{-3}$ μmol/mL for the free EH. The specific activity for the pure EH was 90 U/mg of the proteins. The molecular mass was 45 kDa.

EH. The increase in the K_{mapp} value upon immobilization could be due to several effects including (i) hydrophilic character of the support on the partitioning of the substrate [32]; (ii) conformational change in the tertiary structure of EH; (iii) steric hindrance of the active sites of the enzyme by the support thereby rendering them less accessible for the substrate. The increase in the k_{cat} value could be explained by a preferential adsorption of the EH from a crude enzyme preparation onto the support resulting in an apparent increased activity in the immobilized preparation and/or a conformational change of the tertiary structure of EH.

3.9. Application of the immobilized EH on the resolution of racemic pCSO

The resolution of *rac-p*CSO by the enzymatic extract obtained from the wild *A. niger* strain was previously reported [12] where the EH exhibited a high enantioselectivity towards the hydrolysis of the (*R*)-enantiomer, thus leading to the recovery of the slow-reacting antipode (*S*)-*p*CSO. The reaction using the immobilized EH was monitored over time with a substrate concentration of 4 mM at 4 °C (Fig. 5). The results show that the concentration of the (*R*)-epoxide decreased rapidly whereas that of the (*S*)-epoxide remained almost constant. Based on these data, an enantiomeric ratio (*E*) of 76 was calculated for the immobilized EH while the enantiomeric ratio (*E*) of the free enzyme was determined to be 90 using the same conditions. Similarly, the immobilized EH hydrolyzed the (*R*)-epoxide with retention of the configuration of the corresponding diol showing a preferential attack at the less substituted carbon atom. Therefore, immobilization of EH onto DEAE-cellulose only slightly affected its enantioselectivity and regioselectivity compared to the free EH.

3.10. Repeated use of the immobilized EH

Recycling of the immobilized biocatalyst was investigated by performing a series of consecutive catalytic runs. The enzymatic activity of the recovered biocatalyst was measured

Table 5
Re-use of immobilized EH onto DEAE-cellulose for several reaction cycles in a low concentration reactor (4 mM) and biphasic high concentration reactor (2 M)

	Number of re-use						
	1	2	3	4	5	6	7
Low concentration (4 mM)							
Relative activity (%)	100.0	98.0	96.4	88.4	91.6	87.6	81.4
High concentration (2 M)							
ee of (*S*)-epoxide (%)	99.0	98.0	98.0	98.0	97.5	92.0	90.0
Time (h)	4	6	10	15	21	25	30

in a low concentration batch reactor (4 mM at 4 °C) after each resolution cycle, for seven successive batch reactions. As shown in Table 5, the immobilized EH retained more than 90% of its initial activity over the first five cycles whereas after seven cycles, a decrease of 18.6% in relative activity was obtained. Taking into account that a small loss of biocatalyst occurred during small scale filtration to recovery the immobilized EH after each cycle, the immobilized EH resolved *rac-p*CSO with good operational stability in a repeated batch reactor. However, from a preparative point of view, this system was limited by the low substrate concentration of 4 mM in the water phase. Consequently, the immobilized EH was recycled successively in biphasic batch reactions containing a high concentration of substrate (2 M, i.e. 306 g/L). At the beginning, the heterogeneous reaction system consisted of three phases including the support loaded with EH, the water phase saturated with the substrate and the pure substrate. Under these experimental conditions, the resolution of 612 mg of *rac-p*CSO was carried out in about 4 h of reaction for the first cycle using 287 mg of immobilized EH (10 mg protein/g wet support) after which the immobilized EH was washed with 4 mL of isooctane to extract the remaining epoxide and the formed diol, and then re-suspended again in a fresh reaction solution. As shown in Table 5, during the first five cycles, the ee of the remaining epoxide reached a value higher than 97% within the reaction times ranging from 4 to 21 h where at this stage, 3 g of *rac-p*CSO were resolved under very good conditions by only about 3 mg of immobilized protein corresponding to about 1 mg of pure EH. After seven cycles, the ee of the remaining epoxide still reached a value of 90% in about 30 h of reaction time, which could be due to the progressive denaturation of enzyme. These results clearly indicated that immobilization of EH led to an advantageous use of the biocatalyst in several successive biphasic batch reactions and at very high substrate concentrations of 2 M. This point is important from a practical point of view because it allows a decrease of the biocatalyst cost, particularly in large-scale industrial applications.

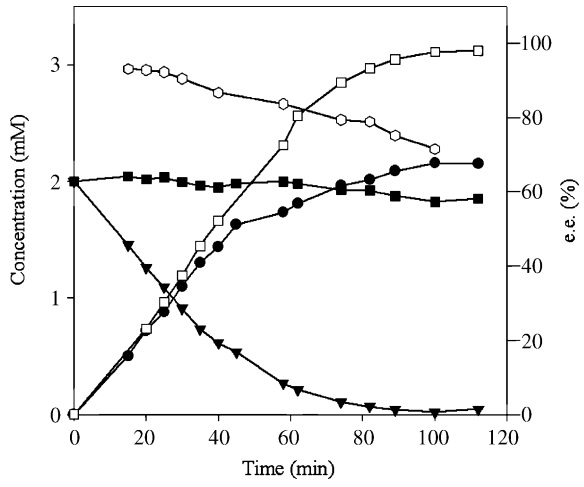

Fig. 5. Kinetics resolution of *rac-p*CSO (4 mM) catalyzed by the immobilized EH from *A. niger*. Time course of concentration of (*R*)-epoxide (▼), (*S*)-epoxide (■), formed diol (●) and of enantiomeric excess (ee) of epoxide (□), diol (○).

4. Conclusion

EH from *A. niger* was successfully immobilized onto DEAE-cellulose and the resulting preparation showed enzymatic properties that were slightly different from those of the free EH in terms of optimal pH and kinetic parameters. From a preparative point of view, a gram-scale resolution of racemic *para*-chlorostyrene oxide using a repeated batch reactor was carried out where the enzyme preparation was re-used for seven cycles. With a very high (2 M, 306 g/L) concentration (i.e. substrate/aqueous phase ratio), the resolution of 3 g of *p*CSO was achieved in a repeated batch reactor using about 300 mg of immobilized EH corresponding to less than 3 mg of enzymatic powder. The recycling properties of the immobilized EH demonstrated its superior potential for practical applications in the preparation of various interesting chiral building blocks in enantiopure form.

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