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Efficient immobilization of epoxide hydrolase onto silica gel and use in the enantioselective hydrolysis of racemic *para*-nitrostyrene oxide

Antonella Petri, Patrizia Marconcini, Piero Salvadori*

Dipartimento di Chimica e Chimica Industriale, Università di Pisa,CNR–ICCOM, Sezione di Pisa, Via Risorgimento 35, 56126 Pisa, Italy

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Abstract

Epoxide hydrolase from *Aspergillus niger* (E.C. 3.3.2.3) was immobilized by covalent linking to epoxide-activated silica gel under mild conditions. A very easy procedure allowed to prepare an immobilized biocatalyst with more than 90% retention of the initial enzymatic activity. Immobilized and free enzyme showed very similar behaviour with respect to the effect of pH on activity and stability. One benefit of immobilizing epoxide hydrolase from *A. niger* on silica gel was the enhanced enzyme stability in the presence of 20% DMSO. The kinetic resolution of racemic *para*-nitrostyrene oxide was investigated by using this new immobilized biocatalyst. The enantioselectivity of the enzyme was not altered by the immobilization reaction: both unreacted epoxide and formed diol were obtained with very high ee (99 and 92%, respectively). In addition, the biocatalyst could be easily separated from the reaction mixture and re-used for over nine cycles without any noticeable loss of enzymatic activity or change in the enantioselectivity extent. The activity of immobilized AnEH was retained for several months.

Keywords: *Aspergillus niger*; Epoxide hydrolase; Enzyme immobilization; Enzyme stability; Enantioselectivity

1. Introduction

Enantiomerically pure epoxides and their corresponding vicinal diols are intermediates of high value for the synthesis of bioactive molecules of great interest in different fields as the pharmaceutical or agrochemical industry [1,2]. This explains why organic chemists have devoted many efforts over the past few decades to develop innovative methodologies for preparing such chirons as a single enantiomer. The proposed procedures use either an organometallic-based catalyst [3] or a biological catalyst [4,5], each having advantages and drawbacks. A very promising strategy is the hydrolytic kinetic resolution of epoxides using epoxide hydrolases [6,7] (EHs; E.C. 3.3.2.3). These enzymes are able to achieve, without the use of any cofactors, the enantioselective opening of the oxirane ring of various epoxides to form the corresponding diols, thus leading in one row to the unreacted epoxide and the formed vicinal diol both in enantiomerically enriched form. EHs are ubiquitous in nature being found not only in the mammalian world but also in bacteria, yeast, fungi, plants and insects [6,7]. Recently, EH from *Aspergillus niger* (*A*nEH) has been studied in some detail [8]. This fungal EH, which is now commercially available [9], showed high activity and good enantioselectivity as catalyst in the hydrolysis of various racemic epoxides [10,11]. However, it was found [12] that this enzyme was not very stable under the conditions used for enzymatic reactors. In order to become a versatile and convenient tool on a preparative scale, operational stability and recycling are key prerequisites, which can be achieved with the immobilization of the enzyme. As far as the EHs are concerned, few examples are reported and only two approaches are dealing with AnEH [13,14]. Very recently [14], a paper appeared describing an interesting approach for the immobilization/stabilization of AnEH: a modified commercial epoxy support was used providing a stable and re-usable biocatalyst.

* Corresponding author. Tel.: +39 050 2219273; fax: +39 050 2219260.
E-mail address: psalva@dcci.unipi.it (P. Salvadori).

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In the last few years, we became interested in covalent immobilization of enzymes on activated silica gel as an useful support in enantioselective synthesis applications [15,16]. The use of such modified enzymes is very advantageous since, beside a generally improved operational stability, they can be easily recovered at the end of the reaction and continuously recycled. The enzymes lipase from *Pseudomonas cepacia* [15] and chloroperoxidase from *Caldariomyces fumago* [16] were covalently bound onto epoxide-derivatized silica gel. These preparations showed enhanced stability with respect to the free enzymes and enzymatic activities were retained after several months from their first use. In addition, immobilized enzyme reactors based on these covalently bound enzymes were developed and used in the enantioselective preparation of both the enantiomers of 2-substituted 1,3-propane diol monoacetates [15] and epoxidation of styrene, respectively [17]. Therefore, in order to broaden the scope of this approach, we were interested in the covalent immobilization of the enzyme AnEH. This work describes the preparation and enzymatic properties of silica gel-supported AnEH and its application in the enantioselective hydrolysis of racemic *para*-nitrostyrene oxide in repeated-batch reactions.

2. Materials and methods

2.1. Materials

AnEH was obtained as a solid from Fluka and showed an activity of about 1.125 U/mg against *para*-nitrostyrene oxide (*p*-NSO), as measured by a reported assay [11]. Racemic *para*-nitrostyrene oxide was synthesized in our laboratory from 2-bromo-4′-nitroacetophenone by a previously described method [18]. All the products and solvents used in the present paper were obtained from commercial sources and were of analytical grade or superior. Silica gel (5 mm, specific surface 340 m^2/g) was from Alltech.

2.2. Equipment

Chromatographic experiments were performed with two HPLC systems. One system consisted of a Jasco PU-980 pump with a Rheodyne sample valve (20 μL loop), equipped with a Jasco MD-910 diode array multiwavelength detector. The second equipment was a Jasco PU-880 with a Rheodyne sample valve (20 μL loop) equipped with a Jasco UV-875 UV–vis detector and a Jasco CD-1595 Circular Dichroism detector.

2.3. Methods

2.3.1. Covalent immobilization of AnEH onto epoxide-activated silica gel

Epoxide derivatized silica gel was prepared according to the methods previously reported [16,19]. Four milliliters of 0.1 M phosphate buffer solution, pH 7, containing ammonium sulphate 2 M and 1 mL of AnEH solution (4 mg/mL) were added to 0.1 mg of functionalized silica gel. The amount of activity and the concentration of the enzyme in the supernatant were followed as a function of time to determine the amount of immobilized AnEH. A blank was run in parallel with enzyme alone (without support) to account for enzyme inactivation during prolonged incubations. After 4 h, the solid with the immobilized enzyme was recovered through centrifugation and washed with the same buffer (0.1 M, pH 7), until no enzyme activity was found in the washings. The amount of enzyme bound to the support was 26.40 mg AnEH/g of silica gel. The experiment was carried out in duplicate. The SiO_2–AnEH preparations were stored at 4 °C until use.

2.3.2. Enzyme activity assay

Epoxide hydrolase activity was assayed by measuring the rate of hydrolysis of racemic *p*-NSO. Typically, 50 μL of a solution containing 1 mg/mL of AnEH or SiO_2–AnEH suspension was added to 350 μL of phosphate buffer (0.1 M, pH 7) and the mixture was preincubated at 35 °C for 2 min. Then 100 μL of a 20 mM solution of substrate in DMSO was added to obtain a final concentration of 4 mM in a total volume of 0.5 mL. After 10 min incubation, stirring was stopped and the quantity of formed diol was determined by HPLC as described in 2.3.6. One epoxide hydrolase unit (U) was the amount of enzyme that catalyzed the formation of 1 μmol of diol/min under the above conditions.

2.3.3. Stability of immobilized AnEH

One milliliter of 0.1 M phosphate buffer solution containing 1mg/mL of free AnEH or SiO_2–AnEH was incubated at two different pH values (7 and 8) and 25 °C on a rotational shaker (200 rpm). After different times of incubation, samples were withdrawn and residual activity was assayed under standard conditions for 2 weeks. Each experiment was carried out in triplicate. The effect of the co-solvent DMSO was tested by incubating free or bound enzyme in phosphate buffer (0.1 M, pH 7) containing 20% of DMSO at 25 °C as described above. After different times of incubation, samples were withdrawn and residual activity was assayed under standard conditions for 2 weeks.

2.3.4. Enantioselective hydrolysis of para-nitrostyrene oxide

A stirred batch reactor (total volume 5 mL) was run at 25 °C, with a substrate concentration of 4.5 mM with DMSO (0.2 V ratio) as co-solvent in a 0.1 M phosphate buffer pH 7. The reaction was started by the addition of 250 μL of AnEH solution or SiO_2–AnEH suspension (34 U/L). Samples were withdrawn every 30 min for quantification of substrate and product concentrations and ee.

2.3.5. Repeated-batch reactions

The enantioselective hydrolysis of *p*-NSO was carried out by employing the procedure described above with

SiO_2–AnEH preparation. After 2.5 h of reaction, stirring was stopped and the suspension filtered. The recovered enzyme was washed with phosphate buffer (0.1 M, pH 7) and new substrate solution was added for the next cycle. The filtrate was analyzed by HPLC.

2.3.6. Analytical methods

For the enzyme assay, the quantity of diol formed was determined by HPLC. Forty microliters of the reaction mixture were diluted with 200 μL of methanol containing 0.45 mM of *para*-nitrobenzyl alcohol as an internal standard. After vigorous stirring, the solution was filtered and the concentration of diol was determined by using a reverse phase column (Luna® C-18, 250 mm × 4.6 mm, 5 μm). The mobile phase was a methanol–water mixture (50/50, v/v) and the flow rate was 0.5 mL/min (UV detection at 275 nm). The retention times were 7.8, 10.6 and 18.8 for the diol, internal standard and epoxide, respectively. For the enantioselective reactions, the ee of the remaining *p*-NSO and *para*-nitrostyrene diol (*p*-NSD) were determined by HPLC using chiral columns. An aliquot of the reaction mixture (200 μL) was extracted with dichloromethane, dried over Na_2SO_4, diluted with *n*-hexane and injected on CHIRALPAK® AD and CHIRALCEL® OD-H. The retention times were 21.5 and 23.5 min for the (*R*) and (*S*) enantiomers of *p*-NSO (CHIRALPAK AD 250 mm × 4.6 mm, hexane/IPA 96/4, flow rate 0.5 mL/min) and 19.5 and 22 min for the (*R*) and (*S*) enantiomers of *p*-NSD (CHIRALCEL® OD-H 250 mm × 4.6 mm, hexane/IPA 85/15, flow rate 0.5 mL/min). The enantiomers were detected by a UV–vis detector (λ = 275 nm) and by a Circular Dichroism detector (λ = 275 nm).

3. Results and discussion

3.1. Immobilization of AnEH onto epoxide-activated silica gel

There are a number of methods available for the immobilization of enzymes and in some cases the obtained biocatalyst shows enzymatic properties that are very close to those of the free enzyme. Activated silica gel has been successfully used in the covalent immobilization of proteins and enzymes and the resulting preparations have been widely utilized as chiral selectors and in bioreactors [20–23]. In general, this apporach involves two steps, i.e. (i) activation of the carrier with a reactive spacer group and (ii) enzyme or protein attachment via functional groups belonging to aminoacid residues. The covalent attachment to an insoluble matrice enables the formation of stable linkages thus inhibiting leakage almost completely. Nevertheless, the main concern is to carry out the binding on residues, which are not involved in the catalytic mechanism in order to prevent loss of activity of the immobilized enzyme. Among the several examples reported, it has been shown that the epoxide matrix obtained by derivatization of silica gel with epoxy groups is stable and reacts with enzymes and proteins under mild conditions.

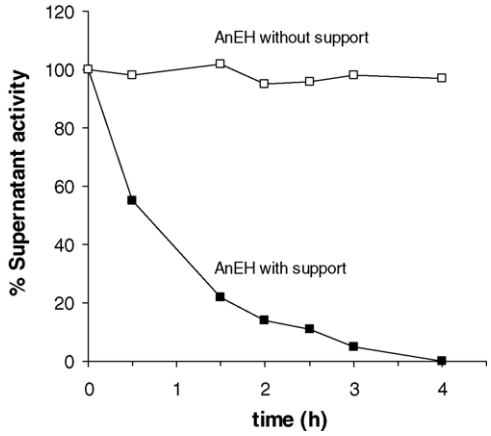


Fig. 1. AnEH immobilization onto epoxide-activated silica gel. AnEH was incubated in 0.1 M phosphate buffer pH 7 at 25 °C: (■) with epoxide-activated silica support; (□) without silica support. The enzymatic activity in the supernatant was monitored periodically with the assay described in Section 2.

Thus, immobilized AnEH was prepared as already reported [16] by adding an enzyme solution to epoxide-derivatized silica gel support. The amount of activity in the supernatant was followed as a function of time and dropped to nearly zero in around 4–5 h (Fig. 1). An enzyme blank was run without support and it was observed that the activity remained at the initial level (around 100%). The experiment was repeated three times and immobilization yield (protein immobilized versus protein contacted) was in the range 65–70%. The retention of activity (activity measured versus activity expected) of the immobilized biocatalyst was very high (89–98%). The preparations were stored at 4 °C until use and retained their activity for several months.

3.2. Properties of immobilized AnEH

As one of the scopes of immobilization is the recycling of the enzyme and the conditions used for enzymatic hydrolysis are not necessarily optimal for enzyme stability, the effects of reaction conditions on the stability of soluble and immobilized enzyme were investigated. Thus, enzyme samples were incubated for 2 weeks at room temperature and pHs 7 and 8 (pHs 7 and 8 are typically used in the enzyme activity assay and in batch reactors). At pH 7, soluble and immobilized AnEH showed almost identical activity curves in the time course considered with a slight increase in the activity of SiO_2–AnEH preparation (Fig. 2).

At pH 8, the activity of both preparations decreased much more but there appeared to be an enhancement in the stability of the immobilized enzyme. After 2 weeks, 30% of activity was retained, while the activity of soluble AnEH dropped to less than 5%.

The effect on activity and operational stability of a co-solvent was also considered. Indeed, with water-insoluble

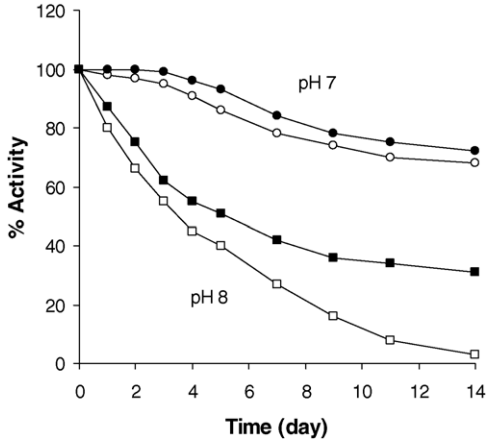


Fig. 2. Effect of storage on the stability of AnEH. Soluble and immobilized AnEH were incubated at 25 °C at different pH values: (◯) AnEH, pH 7; (●) SiO_2–AnEH, pH 7; (□) AnEH, pH 8; (■) SiO_2–AnEH, pH 8. The activities of the enzyme samples were monitored periodically with the assay described in Section 2.

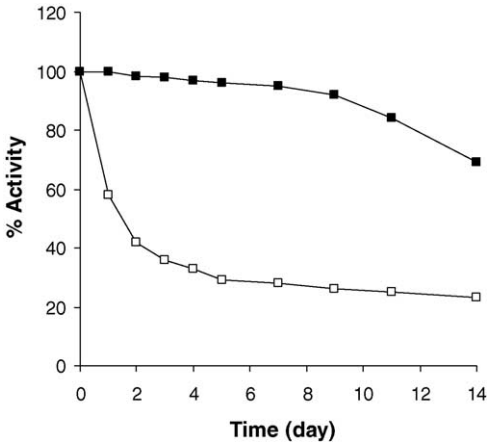


Fig. 3. Effect of storage on the stability of AnEH. Soluble (□) and immobilized (■) AnEH were incubated at 25 °C at pH 7 in 0.1 M phosphate buffer with 20% DMSO. The activities of the enzyme samples were monitored periodically with the assay described in Section 2.

substrates, the addition of an organic solvent is necessary to run the hydrolysis reactions. It has been shown [10] that DMSO is one of the less inhibitory co-solvent among those tested with AnEH. Thus, the influence of 20% DMSO was assayed at room temperature and the results are shown in Fig. 3.

After 24 h, the activity of soluble AnEH is already 60% of the initial activity, while the immobilized enzyme maintained more than 90% activity for nearly 10 days. The activity of immobilized AnEH was still around 70% after 2 weeks and 48% after 3 weeks. So, it can be concluded that covalent immobilization on derivatized silica gel enhanced the stability of AnEH against organic solvent inactivation.

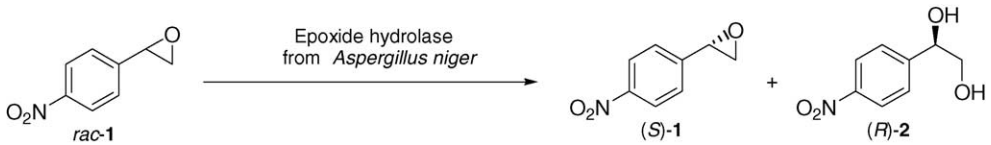
3.3. Enantioselective hydrolysis of p-nitrostyrene oxide

Since it is known that immobilization can alter enzyme specificities, the enantioselectivity of a hydrolysis reaction catalyzed by soluble and immobilized AnEH was also investigated. Racemic *para*-nitrostyrene oxide hydrolysis was selected as model reaction because the substrate is used also in the enzyme activity assay (Scheme 1).

Moreover, this compound is often used because: (a) this is the key chiral synthon implied in the synthesis of β-blocker Nifenalol®; (b) there is no detectable chemical hydrolysis of the epoxide in water; (c) its UV absorbance allows an easy detection after HPLC separation. The kinetic resolution was performed at 4.5 mM substrate concentration by using both the native and immobilized enzyme in bulk. The concentration and ee of the substrate (epoxide) and product (diol) versus time are shown in Fig. 4 (A) and (B).

It is evident that immobilization showed no detectable effect on the enantioselectivity of the reaction at each degree of conversion. The epoxide concentration decreased rapidly from 4.5 mM to around 2.2 mM (50% conversion) in 1.5 h in the kinetic resolution catalyzed by soluble AnEH. At this point, the ee of the remaining (*S*) epoxide was 99%. Meanwhile, the (*R*) diol concentration increased to 2.2 mM (94% ee). Very similar results were obtained with immobilized enzyme: after 3 h (50% conversion), the ee of the remaining epoxide was 98% and the ee of the formed diol was 92%. From these data, an enantiomeric ratio (*E*) of 85 for SiO_2–AnEH was calculated on the basis of the ee of unreacted epoxide and formed diol and of the conversion ratio [24]. This value is in good agreement with that obtained (88) for the native enzyme, confirming that the enantioselectivity was unchanged after the immobilization. The enantiopreference remained always the same with both enzyme preparations.

To check that there was no leaching of the enzyme from the insoluble support under catalytic conditions, the biocatalyst was removed by filtration after 1 h from the reaction mixture. The filtrate was analyzed every 30 min for 3 h and it was observed that the kinetic resolution stopped and no enzymatic activity was present in solution.



Scheme 1. Enantioselective hydrolysis of racemic *para*-nitrostyrene oxide catalyzed by epoxide hydrolase from *Aspergillus niger*.

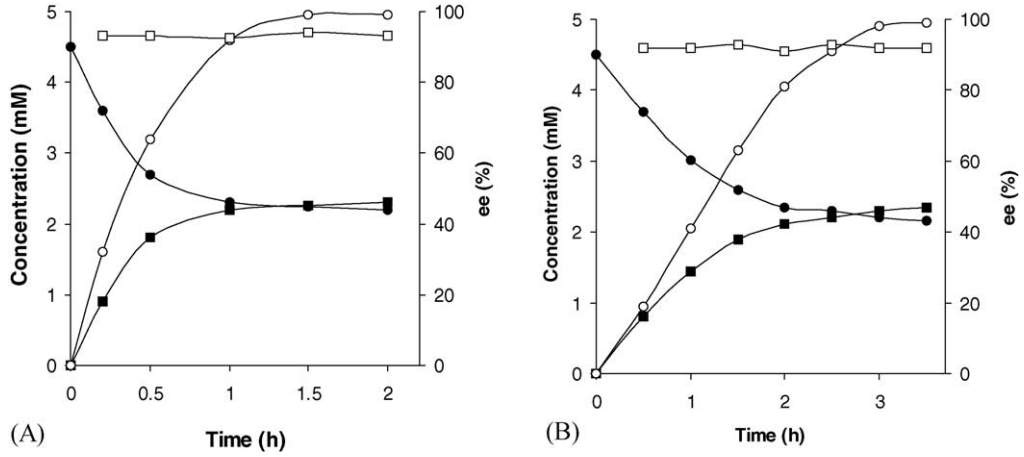


Fig. 4. Kinetics of the hydrolysis of racemic *para*-nitrostyrene oxide catalyzed by (A) soluble AnEH and (B) immobilized AnEH. The curves show the variations in epoxide (●) and diol (■) concentrations and in epoxide (○) and diol (□) enantiomeric excess (ee) with time.

3.4. Re-use of immobilized AnEH

The results obtained in the enantioselective hydrolysis of racemic *para*-nitrostyrene oxide, encouraged us to investigate the reusability of the immobilized enzyme in "repeated-batch" reactions. Indeed, the possibility of separating the biocatalyst from the reaction mixture is one of the most attractive advantages of enzyme immobilization. Therefore, re-use of SiO_2–AnEH for several reaction cycles was affected. Enantioselective hydrolysis of racemic *p*-NSO was performed by using the same sample of the enzyme, which was filtered at the end of each reaction cycle and re-used with fresh substrate solution (Fig. 5).

Fig. 5 shows that the conversion and ee were nearly unchanged after nine cycles. The loss of activity after the first cycle may be related to the first filtration of the support. The enzymatic activity of the biocatalyst recovered after nine cycles was further checked with the standard assay and it was found that nearly the initial activity was recovered.

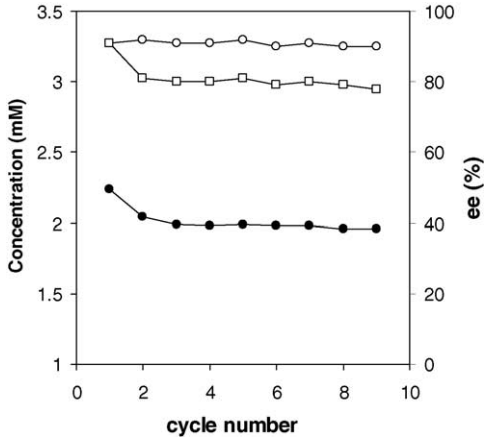


Fig. 5. Re-use of immobilized AnEH in repeated-batch hydrolysis reactions of *p*-nitrostyrene oxide. The curves show the variations in diol concentration (●) and in epoxide (□) and diol (○) enantiomeric excess (ee) for several reaction cycles.

4. Conclusions

The commercially available epoxide hydrolase from *A. niger* was covalently bound to a modified silica matrix without affecting the enzymatic activity. Moreover, the immobilization procedure preserved the enzyme from inactivating process by increasing its stability in the presence of 20% DMSO. It was demonstrated that the catalytic conditions used (substrate concentration, pH, co-solvent) allow the recovery of the enzyme and its re-use for several cycles. The enantioselective enzymatic hydrolysis of racemic *para*-nitrostyrene oxide was also investigated showing that the immobilized enzyme displayed enantioselectivity and reaction rates comparable with those observed with the soluble enzyme. Thus, the described system might be exploited in the preparative synthesis of chiral epoxides and diols and offers excellent potentialities for an industrial scale-up of the enzymatic reaction.

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References

[1] N.S. Finney, Chem. Biol. 5 (1998) R73–R79.
[2] P. Besse, H. Veschambre, Tetrahedron 50 (1994) 8885.
[3] (a) E.N. Jacobsen, M.H. Wu, in: E.N. Jacobsen, A. Pfaltz, H. Yamamoto (Eds.), Comprehensive Asymmetric Catalysis, vol. 2, Springer, 1999, pp. 649–677;
(b) I.E. Markó, J.S. Svenssen, in: E.N. Jacobsen, A. Pfaltz, H. Yamamoto (Eds.), Comprehensive Asymmetric Catalysis, vol. 2, Springer, 1999, pp. 713–789.
[4] R.V.A. Orru, A. Archelas, R. Furstoss, K. Faber, Adv. Biochem. Eng. 63 (1999) 145.
[5] C.A.G.M. Weijers, J.A.M. de Bont, J. Mol. Cat. B: Enzym. 6 (1999) 199.

[6] A. Archelas, R. Furstoss, Curr. Opin. Chem. Biol. 5 (2001) 112.
[7] R.V.A. Orru, K. Faber, Curr. Opin. Chem. Biol. 3 (1999) 16.
[8] C. Morisseau, A. Archelas, C. Guitton, D. Faucher, R. Furstoss, J.C. Baratti, Eur. J. Biochem. 263 (1999) 386.
[9] EH from *Aspergillus niger* used throughout this work was obtained from Fluka.
[10] C. Morisseau, H. Nellaiah, A. Archelas, R. Furstoss, J.C. Baratti, Enzyme Microb. Technol. 20 (1997) 446.
[11] H. Nellaiah, C. Morisseau, A. Archelas, R. Furstoss, J.C. Baratti, Biotechnol. Bioeng. 49 (1996) 70.
[12] K.M. Manoj, A. Archelas, J.C. Baratti, R. Furstoss, Tetrahedron 57 (2001) 695.
[13] S. Karboune, L. Amourache, H. Nellaiah, C. Morisseau, J. Baratti, Biotechnol. Lett. 23 (2001) 1633.
[14] C. Mateo, A. Archelas, R. Fernandez-Lafuente, J.M. Guisan, R. Furstoss, Org. Biomol. Chem. 1 (2003) 2739.
[15] C. Bertucci, A. Petri, G. Felix, B. Perini, P. Salvadori, Tetrahedron: Asymm. 10 (1999) 4455.
[16] A. Petri, T. Gambicorti, P. Salvadori, J. Mol. Catal. B: Enzym. 27 (2004) 103.
[17] T. Gambicorti, Tesi di Laurea, University of Pisa, Italy, 2002.
[18] S. Pedragosa-Moreau, C. Morisseau, J. Zylber, A. Archelas, J. Baratti, R. Furstoss, J. Org. Chem. 61 (1996) 7402.
[19] G. Félix, V. Descorps, Chromatographia 49 (1999) 595.
[20] G. Felix, M. Liu, Bio-Sciences 8 (1989) 2.
[21] I. Marle, A. Karlsson, C. Pettersson, J. Chromatogr. 604 (1992) 185.
[22] G. Massolini, E. Calleri, E. De Lorenzi, M. Pregnolato, M. Terreni, G. Felix, C. Gandini, J. Chromatogr. A 921 (2001) 147.
[23] M. Bartolini, V. Andrisano, I.W. Wainer, J. Chromatogr. A 32 (2003) 715.
[24] J.L.L. Rakels, A.J.J. Straathof, J.J. Heijnen, Enzyme Microb. Technol. 15 (1993) 1051.