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Enzymatic transformations.† Immobilized *A. niger* epoxide hydrolase as a novel biocatalytic tool for repeated-batch hydrolytic kinetic resolution of epoxides

Cesar Mateo,[a] Alain Archelas,[a] Roberto Fernandez-Lafuente,[b] Jose Manuel Guisan[b] and Roland Furstoss*[a]

[a] *Groupe Biocatalyse et Chimie Fine, UMR CNRS 6111, Université de la Méditerranée, Faculté des Sciences de Luminy, Case 901, 163 avenue de Luminy, 13288 Marseille Cedex 9, France. E-mail: furstoss@luminy.univ-mrs.fr; Fax: +33 491 82 91 45; Tel: +33 491 82 91 55*

[b] *Departamento de Biocatalisis, Instituto de Catalisis, CSIC, Campus Universidad Autonoma, Cantoblanco, 28049, Madrid, Spain*

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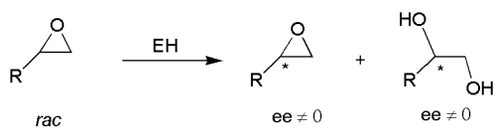
Studies aimed at immobilization of the *Aspergillus niger* epoxide hydrolase were performed. The use of conventional approaches, *i.e.* of commercially available supports and classical methodologies, only led to low stabilisation and unsatisfactory enzymatic activity recovery. Therefore, a new strategy based on the use of a "second generation" type of epoxy-activated supports allowing multi-point covalent immobilization, *i.e.* Eupergit C, partially modified with ethylene diamine (Eupergit C/EDA), and of an adequate experimental procedure was set up. This allowed us to prepare an immobilized biocatalyst with 70% retention of the initial enzymatic activity and a stabilisation factor of about 30. Interestingly, this biocatalyst also led to a noticeable increase of the *E* value for the resolution of two test substrates, styrene oxide **1** and *p*-chlorostyrene oxide **2**. This was improved from about 25 to 56 and from 40 to 100, respectively. A typical repeated batch experiment indicated that the thus immobilized enzyme could be re-used for over 12 cycles without any noticeable loss of enzymatic activity or change in enantioselectivity. This therefore opens the way for the use of an 'heterogeneous catalysis' methodology for achieving the preparation of various enantiopure epoxides *via* biocatalysed hydrolytic kinetic resolution.

Introduction

Due to the world wide ever increasing demand for enantiopure pharmaceuticals or, more generally, bioactive compounds, the necessity of developing the so-called 'chirotechnology area' is nowadays beyond discussion. In this context, numerous approaches have been focused on the preparation of enantiopure epoxides, a class of highly versatile, and therefore highly valuable, chiral intermediates. The most famous methods in this area are the so-called Sharpless, Katsuki or Jacobsen oxidation of double bonds,[1] or the Jacobsen hydrolytic kinetic resolution approach.[2] However, these very elegant methods still suffer from limited substrate selectivity, from the fact that they are using potentially toxic heavy-metal based catalysts and/or only exhibit low to moderate turnover frequencies. Also, they are essentially used as soluble catalysts, which may be a practical problem for recovery and recycling. Therefore, efforts to circumvent these drawbacks are still of the utmost interest. Among the alternative approaches presently studied, the use of a biocatalytic strategy implying microbial enzymes called epoxide hydrolases (EH; EC 3.3.2.3)—an interesting group of 'easy to use' hydrolases that catalyse the addition of a water molecule to an epoxide—has recently proven to be one of the most efficient. Indeed, these enzymes have been shown to be ubiquitous in nature, do not require cofactors and are very effective biocatalysts for performing the hydrolytic kinetic resolution of various racemic epoxides (Scheme 1).[3]

This is particularly true for the *A. niger* epoxide hydrolase, which some of us have thoroughly studied and recently made commercially available.[4,5] A dimer of this (recombinant) enzyme has been crystallised and its 3D structure has been established, whereas the elution profile on Superose 12 suggested it is tetrameric in its native form.[6,7] Interestingly, this is the first (and still unique) fungal epoxide hydrolase structure



Scheme 1 General scheme of the hydrolytic kinetic resolution catalysed by an epoxide hydrolase.

described. In order to broaden the scope of potential applications of this enzyme, the preparation of an immobilized form, providing good stability and activity, was obviously a key prerequisite to its industrial implementation as a practical biocatalyst. Up to now, immobilization of epoxide hydrolases has only scarcely been studied, leading to a clearly unsatisfactory result as far as availability and/or increase of stability—and therefore re-usability of the biocatalyst—are concerned.[8,9,10]

Epoxy activated supports have been shown to often fulfil these requirements and allowed immobilization of several enzymes of industrial interest.[11] Recently, the development by some of us of a so-called 'second generation' modified epoxy support—*i.e.* implying a multi-point attachment strategy—allowed us to further extend the use of such supports to other enzymes, due to the fact that adsorption of the protein occurs *via* different areas of the protein. Therefore, we have explored the possibility of using this new approach for immobilization/stabilisation of the recombinant *A. niger* epoxide hydrolase. We describe in this work: (a) the set-up of such an optimised immobilization protocol and (b) the application of this new supported biocatalyst to a repeated batch process.

Results and discussion

The 'second generation' type of support is based on the multipoint covalent attachment of an enzyme which was first adsorbed to a support by ionic interactions.[11,12] This approach proceeds *via* a four step procedure as described in Scheme 2. It

† Part 54. For part 53 see ref. 21.

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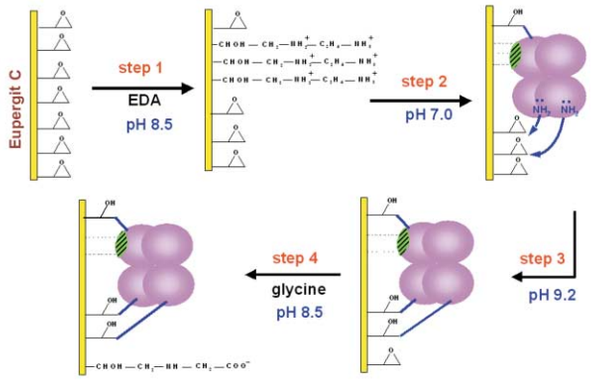


Scheme 2 Principle of 'second generation' supports allowing multipoint covalent attachment of an enzyme on epoxy-activated supports.

implies: 1) a chemical modification step of the (commercially available) support *via* reaction of some of its epoxide moieties with an appropriate nucleophilic linker, *i.e.* ethylene diamine (EDA) or iminodiacetic acid (IDA). This leads to a support bearing some (positively at pH 7) charged arms but also unreacted epoxide moieties; 2) adsorption of the enzyme at neutral pH on this 'partially modified support' *via* ionic interactions, thus triggering the formation of some covalent bonds between the enzyme and the support; 3) treatment of the thus obtained material at higher pH (*i.e.* pH 9–10), thus allowing additional covalent attachment of the protein molecules to the support *via* reaction of their nucleophilic groups (amine, thiol or hydroxyl) with other epoxy functions remaining on the support; 4) inactivation of the remaining unreacted epoxy groups using an additional (low molecular weight) nucleophile. This last step is aimed at preventing further (uncontrolled) reaction between the enzyme and/or the substrate with the support.[13]

Set up of an optimised immobilization protocol for the recombinant *A. niger* epoxide hydrolase

Selection of an efficient support. In an initial approach, we have conducted various experiments aimed at exploring the immobilization capacity of either commercially available solid supports (Eupergit C and Eupergit C 250L) or of different 'modified supports' we have prepared in our laboratory (*i.e.* Eupergit C/EDA, Eupergit C 250L/EDA, Eupergit C/IDA and Eupergit C/IDA/Cu). The results (Fig. 1) indicate that, in four cases out of five, nearly total adsorption of the epoxide hydrolase on the solid support (*i.e.* nearly complete loss of activity of the supernatant) was achieved within a few hours. Total adsorption on Eupergit C/EDA required a longer time of contact (*i.e.* about 24 h), whereas no consistent immobilization on the Eupergit C/IDA support occurred at all, even after 25 h.

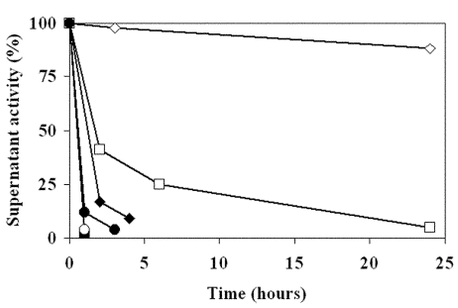


Fig. 1 Immobilization of *A. niger* epoxide hydrolase onto different supports. Experiments were performed as described in the Experimental section. The figure shows the decrease of enzymatic activity in the supernatant. (■) Eupergit C derivative; (●) Eupergit C/IDA/Cu derivative; (◆) Eupergit C 250L/EDA derivative; (○) Eupergit C 250L derivative; (□) Eupergit C/EDA derivative; (◇) Eupergit C/IDA derivative.

Table 1 Percentage of immobilized EH and activity recovered after immobilization onto different epoxy activated supports

	Immobilized EH (%)	Activity recovered (%)
Eupergit C	100	35
Eupergit C 250L	100	30
Eupergit C/EDA	100	>95
Eupergit C 250L/EDA	100	>95
Eupergit C/IDA	5–10	nd
Eupergit C/IDA/Cu	100	0

The enzymatic activity exhibited by the thus obtained biocatalyst was determined for each support. These values proved to be dependent on the nature of the support, as summarised in Table 1 and Fig. 1. Our results indicate that: (a) only about 30–35% of the initial enzyme activity was recovered (on the support) when commercial Eupergit C or Eupergit C 250L were used; (b) on the contrary, pre-treatment of these two supports with EDA (thus affording the 'modified supports' Eupergit C/EDA and Eupergit C 250L/EDA) led to nearly full recovery of enzymatic activity on the support; (c) in spite of the high proportion of enzyme immobilization on Eupergit C/IDA/Cu, no noticeable enzymatic activity was observed for the solid material, indicating that the enzyme was somehow totally inactivated. We have further checked, by submitting each preparation to desorbing conditions (see the Experimental section) that the observed immobilization was indeed due to covalent linkage.[11]

Thermal stability of the enzyme immobilized on the modified supports. Another interesting advantage of enzyme immobilization is the fact that, in certain cases, such procedures may allow the thermal stability of a given biocatalyst to increase noticeably. Fig. 2 shows the results obtained at 50 °C, using immobilized biocatalysts (prepared following step 2, using either Eupergit C, Eupergit C 250L/EDA or Eupergit C/EDA) as compared to the soluble enzyme. We thus observed that: (a) with commercial Eupergit C, loss of activity followed a biphasic figure rather than the expected linear decrease in activity. Very rapid loss of activity was first observed within a few hours, the remaining activity staying nearly unchanged for 15–20 h. This may suggest the formation of two different species of immobilized enzyme (one displaying a stability similar to the one of the soluble enzyme and the second one showing a higher stability; (b) similar results were obtained with Eupergit C 250L/EDA; (c) interestingly however, immobilization on Eupergit C/EDA increased the thermal stability, the half life being 12-fold higher than for the soluble enzyme.

Optimisation of step 3. Due to these results, we selected the Eupergit C/EDA enzyme-bearing support as the best candidate for additional optimisation. Further incubation of the

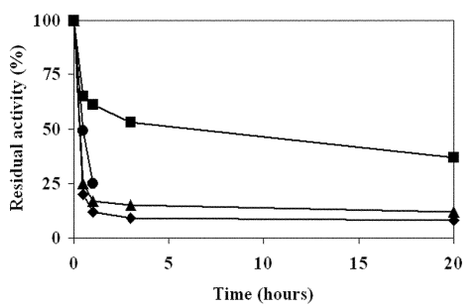


Fig. 2 Thermal inactivation of some enzymatic derivatives. Experiments were conducted at 50 °C in phosphate buffer (25 mM) at pH 7 as described in the Experimental section. In all cases the immobilization time was 24 h. After this period, the remaining epoxide groups were blocked by reaction with glycine (3 M solution at pH 8.5). (●) soluble enzyme; (▲) Eupergit C 250L/EDA derivative; (■) Eupergit C/EDA derivative; (◆) Eupergit C derivative.

biocatalyst (obtained after step 2) was performed for different time periods at pH 9.2. This was aimed at defining the optimum experimental conditions enabling formation of additional covalent linkages between the enzyme and the support. After inactivation of the remaining epoxide groups (step 4) the thermal stability of the thus obtained biocatalysts was evaluated. This indicated that the best thermal stabilisation was found after 72 h incubation (Fig. 3). Interestingly, this material (obtained after 'step 3 treatment') exhibited a 2.5-fold stabilisation as compared to the material obtained after the step 2 stage, which translates into an overall stability increase of about 30 times as compared to the soluble enzyme (Fig. 4). We have checked independently that no enzyme stabilisation was observed when using the commercial support which had been pre-treated with EDA (for 24 h) to fully destroy the epoxy groups, thus only allowing adsorption of the enzyme. This strongly suggests that the observed stabilisation was indeed essentially promoted by formation of covalent linkages between the protein and the support. However, as a consequence of this significant increment on enzyme stability, incubation at alkaline pH (step 3) led to a slight loss (about 26%) of total enzyme activity (Fig. 3).

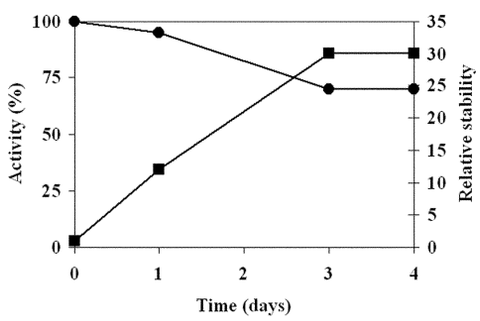


Fig. 3 Effect of the long-term incubation at alkaline pH of Eupergit C/EDA derivatives. At the indicated times, the remaining epoxide moieties were inactivated by reaction with glycine (3 M at pH 8.5). The residual activity was measured and the relative stability was determined by incubation at 53 °C as described in the Experimental section. (●) relative activity of the obtained material; (■) relative stability of the derivatives.

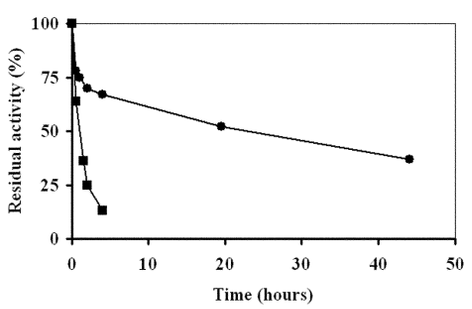


Fig. 4 Thermal stability of the optimal Eupergit C/EDA biocatalyst. Experiments were performed at 53° as described in the Experimental section. (■) soluble enzyme; (●) incubated solid support.

Optimum loading capacity of the support. In order to determine the optimum loading capacity of the selected Eupergit C/EDA support, different amounts of enzyme (exhibiting a 5.75 U mg^{-1} activity) were offered for immobilization. After step 2, the activity of the obtained (solid) biocatalyst was determined in order to detect possible diffusional problems. The results, summarised in Fig. 5, indicate that no such problems were observed in experiments using up to 20 mg of enzymatic powder per wet gram of support. Indeed, the thus obtained material exhibited nearly total immobilization of the available soluble activity. However, at 40 mg crude enzyme/g support (230 U g^{-1} support), although full immobilization yield was achieved, we only recovered about 175 U g^{-1} indicating a loss of about 26% of the initial activity. This can be attributed to the appearance of diffusion problems. After step 3 we interestingly observed that, after the slight decrease of enzyme activity, the apparent specific activity of the immobilized enzyme stayed constant (4.4 U mg^{-1} of enzymatic powder added) whatever the quantity of enzyme charged in the range of 2 to 40 mg g^{-1} of support. Therefore, these results indicate that the optimal load capacity of this support, using the crude enzyme preparation, is about 40 mg per gram of (wet) Eupergit/EDA. Higher loads would probably lead to diffusion problems.

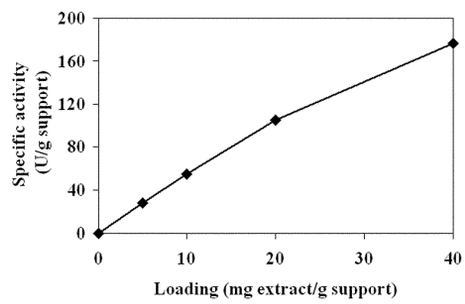
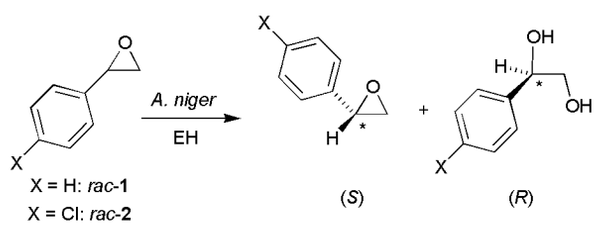


Fig. 5 Enzymatic activity of the solid supports with different loads of enzyme.

Interestingly, determination (using Bradford's method)[14] of the total protein content remaining in the aqueous solution after the immobilization process could be estimated to be about 50%, while all the available epoxide hydrolase activity was immobilized. This indicates that the enzyme was immobilized on this support preferentially to other proteins, leading in fact to purification of this enzyme by partially selective adsorption.

Applications of the Eupergit C/EDA immobilized epoxide hydrolase

Enantioselectivity enhancement. It has been shown previously by some of us that immobilization of multimeric enzymes could alter their enantioselectivity, due to possible slight spatial modification of the multimer upon formation of covalent linkages to the support.[15] Since the *A. niger* epoxide hydrolase used in this study was shown to be multimeric,[7] we examined the possible modification of enantioselectivity upon the above described immobilization methodology. Owing to the fact that the E value is the ratio of the V_{max}/K_M values for each one of the two enantiomers of a racemic mixture, a modification of enantioselectivity had obviously to reflect a modification of some of the kinetic constants implied. Therefore, these were determined for both the soluble and the (optimal) Eupergit C/EDA biocatalyst, using separately the two enantiomers of styrene oxide **1** as test substrate (Scheme 3). For the soluble enzyme, K_M values of 0.98 mM and 7.02 mM, together with V_{max} value of 6.7 and 1.91 U mg^{-1}, were determined for (*R*)-**1** and (*S*)-**1**, respectively. The E value calculated on the base of these values is 25.[16] For the Eupergit C/EDA immobilized enzyme, the V_{max} and K_M values of (*R*)-**1** and (*S*)-**1** were, respectively, 1.5 and 24 mM for K_M and 5.25 and 1.5 U mg^{-1} for V_{max}, leading to a calculated E value of 56. It therefore appears that the observed enhancement of enantioselectivity is essentially due to a noticeable (3-fold) increase of the K_M constant of



Scheme 3

the less reactive (*S*)-enantiomer. This enantioselectivity enhancement was also confirmed using another racemic epoxide, *p*-chlorostyrene oxide **2**. Thus, comparison of the *E* value of the soluble enzyme *versus* the one of the Eupergit C/EDA biocatalyst was performed, the *E* values being determined on the base of the ee of the unreacted epoxide and of the conversion ratio. Again, an *E* value enhancement from 40 to 100 was observed. This is a very interesting feature which makes this biocatalyst even more interesting for preparative scale applications.

Repeated-batch reactor. Obviously, one of the most attractive advantages of enzyme immobilization is the possibility of very easily separating the biocatalyst from the reaction medium, thus allowing one to perform so-called 'repeated-batch' experiments, which would noticeably lower the intrinsic cost of a potential biocatalytic process. This might be particularly attractive for large scale industrial application. Therefore, we checked the possibility of using our optimised epoxide hydrolase/Eupergit C/EDA biocatalyst for several reaction cycles. Thus, 1 g of biocatalyst was added to 80 mL of a 18 mM styrene oxide **1** solution. The ee of the remaining epoxide reached a value higher than 98% within about 75 min. As can be seen in Fig. 6, this value was reached within the same time period over the first five cycles, and began to very slightly decrease later on. After cycle 11, the ee obtained still reached a value of about 85%, indicating that nearly no loss of enzymatic activity occurred all over the process. Increasing the reaction time from 75 to 115 min for cycle 12 again allowed the ee value to reach higher than 98%. Due to the fact that small scale filtration of the support at each cycle necessarily led to a non-negligible loss of biocatalyst (around 1–2% per cycle), it can be extrapolated that, in fact, almost no loss of enzymatic activity was really occurring. This was further checked by measuring the enzymatic activity of the biocatalyst recovered after 5 cycles, which indicated that nearly all the initial enzymatic activity was indeed recovered at that stage.

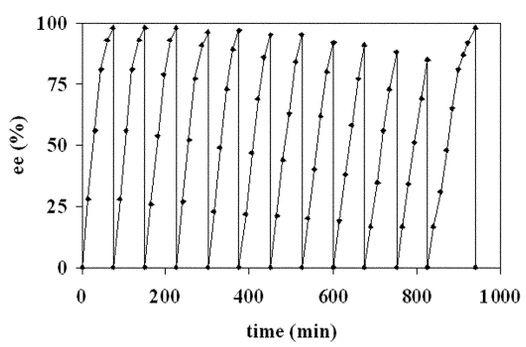


Fig. 6 Re-use of epoxide hydrolase/Eupergit C/EDA biocatalyst for several reaction cycles in a stirring batch reactor.

Conclusion

The aim of the present work was to set up an efficient and 'easy to use' protocol for immobilization of the *A. niger* epoxide hydrolase. This enzyme—which has been recently made commercially available[4]—is indeed a very promising new tool for achieving the hydrolytic kinetic resolution of various racemic epoxides. We have shown that direct use of commercially available Eupergit type supports are unsatisfactory, presumably due to the multimeric nature of this enzyme. However, by using a 'second generation' support—*i.e.* Eupergit C/EDA—we have set up an optimised experimental protocol, which involves a so-called 'multi-point attachment' strategy. This allows us to efficiently immobilize this enzyme without important loss of activity. Moreover, this novel supported biocatalyst showed a 30 times higher enzyme stability together with a noticeable improvement of enantioselectivity toward styrene oxide **1** as well as towards *p*-chlorostyrene oxide **2**. Thus, this material could be used, at room temperature and without noticeable loss of enzymatic activity, over a 12-cycle repeated-batch process (about 16 h overall time period). Obviously, this process could be continued for further cycles in order to optimise the total turnover number of such a reactor. This novel, high performance, biocatalyst therefore offers excellent potentialities for both lab-scale and industrial application, *i.e.* for the preparation of various interesting chiral building blocks and/or biologically active products in enantiopure form. Work is in progress in our laboratory in order to further explore the potential of this new supported biocatalyst.

Experimental

The crude powder of the recombinant *Aspergillus niger* epoxide used throughout this study showed an activity of about 5.7 U per mg against styrene oxide, as measured by our recently described spectrophotometric method.[16] The substrates were either commercial as styrene oxide **1** (Aldrich) or synthetized in our laboratory as *p*-chlorostyrene oxide **2**.[17] Eupergit supports were kindly provided by Degussa.[18] All the products used in the present paper were of analytical grade.

Enzymatic assay

The enzymatic activity was measured using either styrene oxide **1** or *p*-chlorostyrene oxide **2** as substrates following our new (sodium periodate based) spectrophotometric assay.[16]

Step 1: preparation of the modified supports[11,19]

Eupergit-C/EDA. 10 g of wet support was suspended in 100 mL of a 5% v/v ethylene diamine aqueous solution at pH 8.5. This suspension was put under gentle reciprocal stirring during 15 min at room temperature. The support was then washed (8 × 100 mL deionised water).

Eupergit-C/IDA. Similarly, 50 mL of a 1.8 M iminodiacetic acid solution were added to 10 g of wet support. The suspension was left under gentle stirring for 5 h, and then washed with deionised water.

Eupergit C/IDA/Cu. 50 mL of an aqueous solution of 2 mg mL^{-1} of copper sulfate were added to 10 g of Eupergit-C/IDA support. The suspension was stirred for 2 h, and the obtained support was washed with deionised water.

Step 2: immobilization of the *A. niger* epoxide hydrolase

5 g of the appropriate support were suspended in 10 mL of an aqueous (deionised water) enzyme solution at the desired concentration. Except in the indicated cases, the load was 2 mg of crude powder per gram of support using pH 7 phosphate buffer solutions at different concentrations (from 5 mM in the case of our modified supports to 1 M when using commercial Eupergit C or C 250L). Aliquots of the supernatant were withdrawn periodically and analysed for enzymatic activity. In order to check for ionic adsorption *versus* covalent linkage of the enzyme to these supports, aliquots of the suspension were incubated in the conditions where the enzyme was shown to be desorbed from supports bearing inactivated oxirane groups (0.5 M NaCl solution for the modified supports and 0.05 M phosphate buffer for the commercial ones).[11] In parallel, blanks of enzyme solution were incubated at the same pH, buffer and temperature conditions. In all cases total activity was recovered.

Step 3: promotion on additional covalent linkages

In order to promote the formation of additional protein-support linkages, 10 g of the covalently immobilized enzyme

obtained in step 2 were re-incubated at room temperature in 100 mL of disodium phosphate 0.1 M at pH 9.2 for different time periods.

Step 4: inactivation of the remaining epoxide moieties

At the end of step 3, 1 g of the immobilized support was treated for 24 h with 5 mL of 3 M glycine solution at pH 8.5 (room temperature) in order to inactivate the remaining epoxide moieties. The obtained derivative was then washed with an excess of distilled water to eliminate proteins non-covalently linked to the support.[12]

Optimised protocol

A typical experiment can be described as follows (all steps being conducted at room temperature): 1) 10 g of wet commercial Eupergit C were added to a solution of 5 mL of ethylene diamine dissolved in 100 mL of deionised water (final pH 8.5 adjusted by addition of HCl 20%). The solution was gently shaken for 15 min; 2) after filtration and washing with water, 5 g of the thus obtained material were added to 10 mL of an epoxide hydrolase solution containing 200 mg of enzymatic powder (5.7 U mg^{-1}) in phosphate buffer (pH 7, 5 mM) and gently shaken for 24 h; 3) the supernatant was sucked out of the flask and replaced by 100 mL of phosphate buffer (pH 9.2, 100 mM), then incubation was continued for 72 h; 4) the supernatant was removed and replaced by 50 mL of a 3 M glycine solution (pH 8.5) and further incubation was achieved for an additional 24 h period.

Thermal stability of the different derivatives

The supported biocatalyst obtained after enzyme immobilization was suspended in a 25 mM phosphate buffer solution at pH 7 and incubated at the indicated temperature. The enzymatic activity of the suspension was checked against time. In each case, the proportion of observed remaining activity was referred to the initial value. Stability is given as half-life and stabilisation as the ratio between the half-lives of the immobilized derivative and that of the soluble enzyme.

Enantiomeric excess and conversion ratio determination

Styrene oxide **1** or alternatively *p*-chlorostyrene oxide **2** were used as substrates. The enantiomeric excess of the unreacted epoxide was determined using chiral GC chromatography (Macherey Nagel, Lipodex-G column, 0.25 μm, 25 m, 0.25 mm column); (gas carrier He); 95 °C (styrene oxide) and 110 °C (*p*-chlorostyrene oxide). Reverse phase HPLC was used to quantify the formed *p*-chlorophenylethanediol. 30 μL of sample were injected into an Hypersil BDS-C18 column (220 × 4.6, 5 μm) and eluted using a mixture of acetonitrile–water (30 : 70) at 0.5 mL min^{-1} (UV detector at 220 nm). The E value of the resolution was calculated using the classical Sih's equation[20] using the ee of the unreacted epoxide and the conversion ratio.

Kinetic constants measurement

The activity of either the soluble enzyme or the obtained supported biocatalyst was measured using the commercially available enantiomers of styrene oxide separately, at concentrations varying from 0.1 to 18 mM.[16] The double inverse Lineweaver–Burk plots representation was used for K_M and V_{max} determination.

Repeated-batch experiment

In a 250 mL vessel, 1 g of supported biocatalyst (enzyme immobilized on Eupergit C/EDA), prepared using 20 mg of crude enzymatic powder per g support was added to 80 mL of an 18 mM (homogeneous) styrene oxide aqueous solution. The solution was put under gentle mechanical stirring at 20 °C for 75 min. Stirring was stopped and the solid material was decanted. The supernatant solution was sucked out of the flask under vacuum. For the next cycle, a new 80 mL portion of substrate solution was added to the enzymatic derivative. At time intervals, the ee of the remaining epoxide was determined by chiral GC after extraction from the aqueous solution with isooctane.

Acknowledgements

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