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Preparation of a very stable immobilized *Solanum tuberosum* epoxide hydrolase

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Abstract—The covalent immobilization of the *Solanum tuberosum* epoxide hydrolase (*St*EH) was explored using highly activated Sepabeads-epoxy or Glyoxyl-agarose based supports. A Glyoxyl-agarose immobilizate, prepared under optimized experimental conditions, led to a material exhibiting excellent thermal and chemical stability. The key step of this immobilization process was the use of 164 kDa dextran as an additive during immobilization, which prevented the enzyme from inactivation at the high pH (pH 10) necessarily used for performing this immobilization. This afforded a Glyoxyl-agarose-*St*EH immobilizate with 80% initial enzymatic activity retention and a stabilization factor of at least 300 at 60 °C, as compared to the free enzyme. The high enantio- and regio-selectivity properties of this novel biocatalyst were shown to be nearly identical to those of the free enzyme.
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1. Introduction

Due to the recent release of new FDA marketing guidelines, biotechnological processes, which are generally recognized as being environmentally safe since they avoid production of potentially toxic waste in industrial processes, are becoming increasingly important. This in particular applies to the synthesis of enantiomerically pure pharmacological building blocks or end products.[1] We and others have previously amply demonstrated that the preparation of enantiomerically pure epoxides and/or of their corresponding vicinal diols, which are very attractive chirons, can be efficiently performed by running a hydrolytic kinetic resolution of a given racemic epoxide.[2] This implies the use of dedicated enzymes—that is epoxide hydrolases (EHs), such as the commercially available *Aspergillus niger* epoxide hydrolase (EC 3.3.2.3).[3] An even more stringent industrial demand, triggered by the necessity to set up as cost effective as possible processes, relates to the possibility to run so-called *enantioconvergent* strategies, which allow approaching the ideal '100% yield 100% ee' goal starting from a racemic mixture. In this context, some of us have previously described the surprising enantioconvergent behavior of the recombinant plant *Solanum tuberosum* epoxide hydrolase (*St*EH) on differently substituted styrene oxide derivatives[4,5] (Scheme 1).

Immobilization of the biocatalyst may constitute as another step further as far as process cost aspects are concerned. Hence, with enantioconvergent single enzyme processes still being very scarce, we pursued the exploration of the potential of this highly interesting EH and, in particular, of the possibility to efficiently immobilize it on various supports. To date, only one study has been reported regarding this question, which led to poor to moderate success (only 10% of recovered activity and a stabilization factor of about 7 at 52 °C).[6] Obviously, a more efficient immobilization protocol allowing us to obtain higher enzymatic activity recovery and improved stability still remains highly desirable. As previously documented,[7–11] the use of adequate supports and experimental conditions permitting the multipoint covalent attachment of an enzyme to a highly activated support via short spacer arms, has in some cases been reported to increase enzyme stability. This is essentially due to the fact that the residues involved in the immobilization process could preserve their relative

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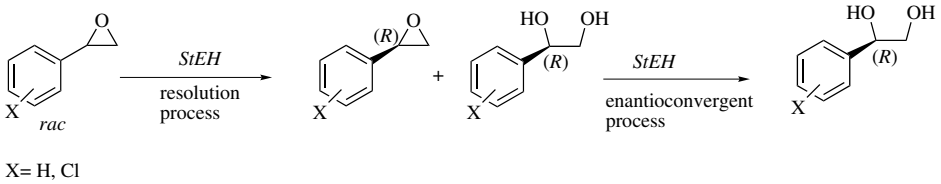

Scheme 1.

position nearly unaltered during any conformational change promoted by heat, solvents or any other distorting agent.[12,13] For instance Glyoxyl-agarose (GA), a support bearing aldehyde functions on its surface, was shown to direct immobilization of an enzyme to the areas of the protein bearing the highest primary amino group density and, as a consequence, to allow high stabilization.[14,15]

Herein, we explored the possibility to immobilize the *St*EH onto different such supports and describe our results related to the activity, stability, and selectivity of the thus obtained materials.

2. Results and discussion

2.1. Immobilization of the *St*EH onto different supports

Four types of supports were prepared starting either from commercially available Sepabeads-epoxy support (Sepabead-EP) or from an Agarose based support, and were used to conduct preliminary experiments aimed at determining the most promising material. Our results are summarized in Table 1. The amount of immobilization was estimated by measuring the enzymatic activity remaining in the aqueous solution after incubation of the enzyme solution with the support. The amount of enzymatic activity recovered on the support was measured using our previously described methodology (UV spectrometry test against styrene oxide, based on the oxidation of the formed diol to benzaldehyde using sodium periodate as chemical oxidant).[16] The results obtained clearly appeared to depend on the nature of the support.

Incubation of the *St*EH with (commercially available) hydrophobic Sepabeads-epoxy support (moderately hydrophobic polymethacrylate resin containing oxirane groups) in 1 M sodium phosphate at pH 7 led to nearly total recovery of the enzyme activity in the supernatant, indicating that no noticeable immobilization occurred. Conversely, incubation with Sepabeads-EP-amino supports (partial modification of the epoxy groups with ethylene diamine as previously described)[17] at pH 7 led to almost total activity disappearance in the supernatant. However, nearly no activity was recovered on the support either, suggesting that the enzyme was inactivated after reaction with the support. Better results were obtained using the Sepabeads-EP-Cu support (partial modification of the epoxy groups with iminodiacetic acid followed by incubation with $CuSO_4$)[17] at pH 7, where almost 70% of immobilized activity was recovered. In order to allow further reaction of the enzyme residues with the epoxide moieties born by this support, the already immobilized enzyme was re-incubated at pH 9 for an additional 24 h period. However, the activity then decreased to about 40%, thus suggesting some enzyme-support reaction.

Glyoxyl-agarose supports (GA) are very suitable for providing multipoint and non-distorting covalent immobilization of enzymes, but the immobilization protocol requires the reaction to be performed at about pH 10 in order to have enough unprotonated primary amino residues during the first immobilization process.[12,14] Incubation of *St*EH with this support at pH 10 led to a considerable loss of enzymatic activity, with only about 10% being recovered on the immobilizate. This can be explained by the fact that the *St*EH is deactivated in less than 1 h at this pH, at 25 °C. Even at 4 °C the activity rapidly decreased at this pH.

Very satisfactorily however the same immobilization procedure, conducted using a solution of enzyme containing an initial 20% (w/v) amount of 164 kDa dextran as additive,[18–21] led to nearly full recovery of enzymatic activity on the support after only 1 h incubation, at 4 °C and pH 10, indicating that dextran did efficiently protect the enzyme from any pH induced inactivation.

Table 1. Immobilization of *St*EH on different supports

Support	Immobilized enzyme (%)	Recovered activity (%)
Sepabead EP	0	0
Sepabead-EP-Cu	100	70
Sepabead-EP-NH_2	100	< 10
Glyoxyl-agarose (GA)[a]	100	>95

[a] Immobilization carried out in the presence of 20% of 164 kDa dextran.

2.2. Stability of the different immobilizates

An additional parameter to be determined when preparing such immobilizates is the thermal stability of the material obtained. The soluble *St*EH is a reasonably stable enzyme, its half-life at 60 °C and pH 7 being about 30 min. The stability of the above prepared immobilizates was therefore tested using identical experimental conditions (Fig. 1). The enzymatic activity of the Sepabeads-EP-Cu immobilizate appeared to be slightly more stable than that of the soluble enzyme and the stabilization factor was even higher when the immobilized enzyme preparation was re-incubated at pH 9 before the epoxy blocking step, suggesting an increase of the number of covalent enzyme-support bonds. The stability reached using these protocols was

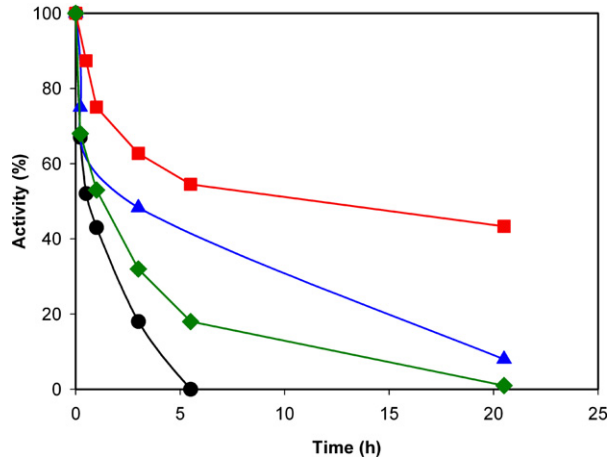

Figure 1. Thermal stability of the enzyme immobilized on different supports. The different preparations were incubated at pH 7 and 60 °C. Circles: soluble enzyme; rhombus: enzyme immobilized on Sepabeads-EP-Cu at pH 7 during 24 h; triangles: enzyme immobilized on Sepabeads-EP-Cu at pH 7 during 24 h and re-incubated during 24 h at pH 9; squares: enzyme immobilized on Glyoxyl-agarose support (GA).

about 10-fold as compared to the soluble enzyme. Even better results were obtained using the GA support for which a stabilization factor of about 20 was observed (half-life about 10 h).

2.3. Optimization of the GA immobilizate stability

Owing to this promising result, we decided to improve the experimental conditions for the preparation of an optimal GA immobilizate. Indeed, more finely tuned experimental conditions could allow us to increase the number of covalent bonds of the *St*EH to the support.[22,23] Some of us have recently showed a direct correlation between the number of enzyme-support linkages and the stability of an immobilized protein on Glyoxyl-agarose.[24] Our results indicated that the enzymatic stability of the thus obtained immobilizate was strongly dependent on the incubation conditions, as illustrated in Figure 2.

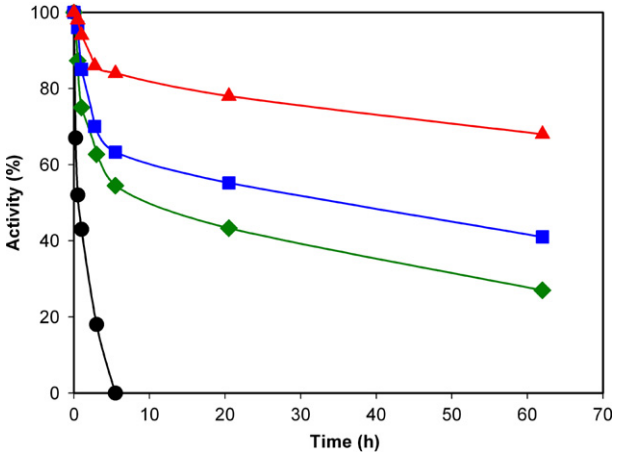

Figure 2. Stability optimization of the *St*EH immobilized on Glyoxyl-agarose supports (GA). The different preparations were incubated at pH 7 and 60 °C in presence of 20% dextran 164 kDa. Circles: soluble enzyme; rhombus: incubation during 20 min at 4 °C; squares: incubation during 3 h at 4 °C; triangles: incubation during 3 h at 4 °C and additional incubation at room temperature during 3 h.

Thus, in two separate experiments, the enzyme was first incubated at 4 °C for 20 or 180 min. An additional experiment was conducted by first incubating the enzyme at 4 °C for 180 min, and then for an additional 3 h period at 25 °C. This last experiment led to a very interesting result, since it allowed an activity recovery as high as 80% (i.e., about 23 U per gram of support) and to an excellent (thermal) stabilization factor of at least 300.

2.4. Enantio- and regio-selectivity of the GA immobilizate

Contrary to most enzymatic mechanisms, which are intrinsically unambiguous as far as their regioselectivity is concerned, the epoxide hydrolase catalyzed ring opening of an oxirane ring may imply different regioselectivities, due to the possible attack of either carbon atom of the oxirane ring by the nucleophylic enzyme residue. As a consequence, the stereochemical outcome of such a reaction will be strongly governed by the intimate mechanism of a given epoxide hydrolase, which partly implies the optimized positioning of the substrate into its active site. Some of us have previously proposed to quantify this behavior by using the so-called regioselectivity coefficients $\alpha(S)$ and $\alpha(R)$.[25] Interestingly enough, these coefficients may vary from one enantiomer to the other of a given racemic epoxide, which in fact is the fundamental reason of the enantioconvergency observed in the *St*EH catalyzed hydrolysis of various styrene oxide derivatives.[5] Therefore, creating covalent bonds between an enzyme and a given support could eventually, due to discrete deformation of the enzyme in its immobilized form, alter the positioning of each enantiomer in the active site and, as a consequence, modify the stereo- and regiochemical outcome of the reaction. Therefore, a comparative biohydrolysis between the free *St*EH enzyme and its GA immobilizate using *rac*-styrene oxide as substrate were performed. The results (Fig. 3) indicated that, in both experiments (a) the (*S*)-epoxide was preferentially hydrolyzed to the (*R*)-diol, (b) the (recovered) less reactive enantiomer was the (*R*)-epoxide, (c) the ee of this epoxide (ee_s) reached a value of nearly 100% after about 90 min, (d) the ee of the produced diol (ee_p) only slightly decreased from 93% to 89% after 2 h. Interestingly, overlapping ee curves were obtained (after fine tuning of the amount of immobilizate to be used) indicating that both the *E* value ($E = 30$) and the regioselectivity remained unaltered. To further confirm these observations, we also determined the regioselectivity of the immobilizate catalyzed reaction by separately hydrolyzing pure (*R*) and (*S*) styrene oxide enantiomers. The values thus obtained, that is, $\alpha(S) = 97\%$ and $\alpha(R) = 10\%$ are very similar to those we had determined previously for the free *St*EH [$\alpha(S) = 97\%$ and $\alpha(R) = 8\%$].[5] This result further confirmed that the immobilization of *St*EH on the GA support did not modify the global stereoselectivity of the enzyme.

Finally, we also have controlled that, using this material, it was possible—as we have previously described using the

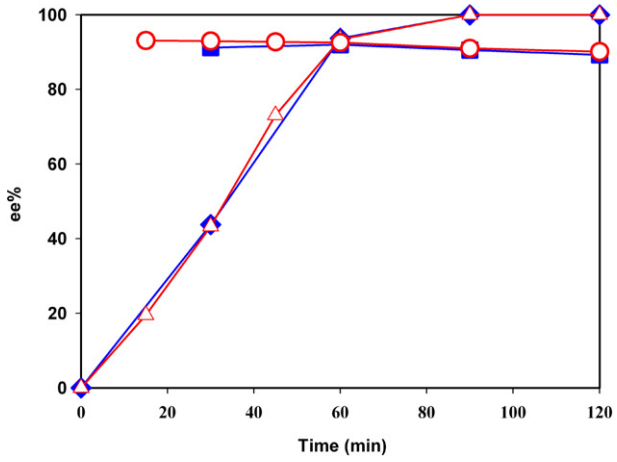

Figure 3. Biohydrolysis of *rac*-styrene oxide by the free and immobilized *St*EH. The enantiomeric excesses of the residual epoxide (ee_s) and the formed diol (ee_p) were monitored over time using a substrate concentration of 10 mM at 27 °C. Open triangles: ee_s (free *St*EH); filled rhombus: ee_s (immobilized *St*EH); open circles: ee_p (free *St*EH); filled squares: ee_p (immobilized *St*EH).

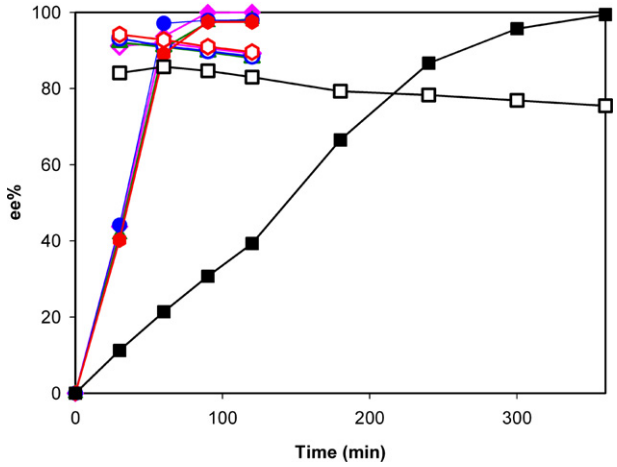

Figure 4. Repeated-batch reactor conducted with heating of the immobilizate between each resolution cycle. The ee of the residual epoxide (ee_s) and the formed diol (ee_p) were monitored over time using an initial styrene oxide concentration of 10 mM at 27 °C. First cycle: filled rhombus: ee_s, white rhombus: ee_p. Second cycle: filled triangle: ee_s, white triangle: ee_p. Third cycle: filled circle: ee_s, white circle: ee_p. Fourth cycle: filled hexagon: ee_s, white hexagon: ee_p. Fifth cycle: filled squares: ee_s, white squares: ee_p.

free enzyme—to perform the enantioconvergent biohydrolysis of *meta*-chlorostyrene. As expected, total (analytical scale) biohydrolysis of this epoxide exclusively afforded the corresponding (*R*)-diol, in a nearly quantitative yield and an excellent ee of 96%.[5] This (*R*)-diol is an important building block in the synthesis of various biologically active molecules.[26]

2.5. Confirmation of the GA–*St*EH immobilizate temperature resistance using repeated-batch reactors

During our stability studies, we showed that the GA–*St*EH immobilizate seemed to be a thermally very robust biocatalyst ($t_{1/2}$ >200 h at 60 °C). In order to confirm this result, we performed 'repeated-batch' experiments, using *rac*-styrene oxide as substrate. After each resolution process (performed at 27 °C in order to avoid chemical hydrolysis, which would prevent a good understanding of the results) the recovered biocatalyst was heated at 58 °C for several hours (20 then 44 then 68 h) before being re-used in the next experiment. As shown in Figure 4, the kinetic profile of the different experiments remained nearly identical after 120 min (the ee_s and ee_p stayed unaltered over the first four cycles), indicating that no loss of activity, enantio- or regio-selectivity occurred. After the fourth experiment, additional heating of the recovered immobilizate was performed, at 58 °C for 7 days (164 h). Surprisingly, 30% activity still was present and an ee_s higher than 98% still could be obtained, after 360 min reaction time. However, the ee_p value culminated at a value lower than in the previous experiments, suggesting a slight modification of the regio- and/or enantio-selectivity of the enzyme.

These results clearly confirm the very high thermal stability of the GA–*St*EH immobilizate, a very interesting property, which opens the way to very long-running repeat batch reactor experiments.

3. Conclusion

Immobilization of the recombinant *S. tuberosum* epoxide hydrolase was explored using different supports. The use of a Glyoxyl-agarose support allowed us to obtain an optimized immobilizate exhibiting excellent thermal and chemical stability. The key parameter of this successful immobilization process was the use of 164 kDa dextran as an additive, which prevented the enzyme from inactivation at the high pH (pH 10) necessarily used for performing this immobilization. The enantio- and regio-selectivity properties of the thus obtained GA–*St*EH immobilizate were shown to be very similar to those of the free *St*EH, indicating that the stereochemical properties of the enzyme were not noticeably altered. These results thus clearly pave the way to the efficient use of this novel biocatalyst for carrying out either the biocatalyzed hydrolytic kinetic resolution of an epoxide or the enantioconvergent preparation of the corresponding diol, thus obtained in nearly quantitative amount and highly enantiomerically enriched form, by running a packed bed downflow-column reactor. Work is currently in progress in our laboratories to further explore the preparative scale potentialities of this new biocatalyst.

4. Experimental

4.1. Materials

The plasmid containing the epoxide hydrolase gene from *S. tuberosum* (pGEFII-*St*EH) was kindly provided by Professor M. Arand (Institute of Pharmacology and Toxicology, Zürich, Switzerland). Agarose 10BCL was obtained from Iberagar S.A. and activated as described elsewhere.[12] Sepabeads epoxy-supports (Sepabead-EP) were kindly donated by Resindion S.R.L. and derivatized

as previously described.[17] Enantiomeric excesses were determined by chiral GC analysis carried out on a Shimadzu GC-20A equipped with FID and a fast Cyclosil-B column (Agilent, 13 m, 0.1 mm, 0.1 μm film, He) or a Chirasil-DEX CB (Varian, 25 m, 0.25 mm, 0.25 μm film, He).

4.2. Preparation of the enzymatic extract

Escherichia coli BL21(DE3) was used as the host for the cloning and functional expression of the EH gene from *S. tuberosum*. *E. coli* competent cells were transformed with the recombinant plasmid (pGEF-*St*EH). To produce native EH, cells grown to an initial OD_{600} of 1.5 in a 1 L fermentor were induced for 8 h with 0.4 mM IPTG (20 °C, LB broth, 200 μg/mL ampicillin). Cells were harvested by centrifugation, re-suspended in 20 mL of 20 mM carbonate ammonium buffer pH 7.6 and then broken by a single pass through a French press. After centrifugation at 20,000*g* for 30 min at 4 °C, the supernatant was freeze dried to produce about 0.5 g of crude enzymatic extract. This showed an activity of 1.4 U/mg and was used as such in all experiments.

4.3. Enzyme activity measurement

The enzymatic activity was measured against styrene oxide (at 25 °C and pH 7) as previously described by recording the absorbance increment at 290 nm promoted by the benzaldehyde formed.[16] Thus, 2 mL of water, 20 μL of a 200 mM sodium periodate solution, and various amounts of a styrene oxide solution (from a stock solution 50 mM in dimethyl formamide) were added to a spectroscopy cell. Different enzyme preparations were added to this solution. In parallel, a blank experiment was conducted using in the absence of substrate.

4.4. Immobilization on Sepabeads-EP supports

To 1 g of the different epoxy-supports (see Table 1) were added 10 mL of an enzyme solution (1 mg/mL) in the appropriate buffer (1 M sodium phosphate at pH 7 for the hydrophobic commercial supports and 10 mM sodium phosphate at pH 7 for the other supports). The suspension was kept under mild stirring for 24 h at 25 °C and the enzymatic activity of both the suspension and the (filtered) supernatant were determined. The suspension was filtered, dried under vacuum, and a sodium hydrogen carbonate (10 mL of 0.1 M, pH 9) solution was added. Finally, the residual epoxy groups were blocked by adding a glycine solution (15 mL of 3 M, pH 8.5).[27] The suspension was stirred during 24 h before washing and drying under vacuum.

4.5. Immobilization on the Glyoxyl-agarose support

4.5.1. Preparation of the Glyoxyl-agarose support (GA). Glyoxyl-agarose 10 BCL gels (agarose-O-CH_2-CHO) were prepared by etherification of agarose gels with glycidol (2,3-epoxypropanol) and further oxidation with sodium periodate.[12]

4.5.2. Standard procedure. GA support (2 g) was mixed with an enzyme solution (20 mL of 1 mg/mL water) and a sodium bicarbonate solution (2 mL, 0.2 M). The suspension was gently stirred for 1 h at 4 °C. After this period, sodium bicarbonate (6 mL of 0.2 M, pH 10) containing sodium borohydride (10 mg) was added and the suspension was stirred for 30 min. Finally, the preparation was washed with an excess of distilled water.

4.5.3. Optimized procedure. GA support (2 g) was mixed with an enzyme solution (20 mL of 1 mg/mL water), a sodium bicarbonate solution at pH 10 (2 mL, 0.2 M) and 20% (w/v) of 164 kDa dextran. The suspension was stirred gently for different time periods at 4 °C. In some cases, the preparation was incubated at 25 °C for different time periods. After this period, the enzyme preparation was reduced and washed as described above to furnish GA immobilizate.

4.6. Enzyme stability against temperature

The different immobilizates were incubated at the indicated temperature. At different time periods, samples were taken from the preparation and the activity remaining on the immobilizate measured. In each case, the initial activity was considered to be the 100% value.

4.7. Comparative selectivity study of the free *St*EH versus the GA immobilizate

The resolution process was started by the addition of *rac*-styrene oxide (20 μL of a 1 M solution in DMF) to a solution of free *St*EH (0.62 mg of enzymatic extract in 2 mL of phosphate buffer 10 mM, pH 7) or of immobilized *St*EH onto optimized Glyoxyl-agarose supports (150 mg in 2 mL of phosphate buffer 10 mM, pH 7). This mixture was maintained at 27 °C under gentle magnetic stirring. At time intervals, about 150 μL of the reaction mixture were withdrawn using a pipette tip equipped with a filter in order to avoid removal of some support and extracted with ethyl acetate (75 μL) under vortex agitation. The ee of the residual epoxide and of the formed phenyl ethanediol was determined by injection (3 μL) of the ethyl acetate phase on a fast chiral GC column (Cyclosil-B) using a temperature programming (90 °C, 8 min; 60 °C/min; 150 °C, 15 min). The following retention times were obtained: (*S*)-styrene oxide: 6.7 min and (*R*)-styrene oxide: 7.2 min; (*S*)-phenyl ethanediol: 16.7 min and (*R*)-phenyl ethanediol: 16.9 min.

4.8. Determination of the regioselectivity coefficients of *St*EH immobilized on the GA support

Biohydrolyses were started by the addition of pure (*R*)- or (*S*)-styrene oxide (2 μL) to a solution of immobilized *St*EH onto GA support (150 mg in 2 mL of phosphate buffer 10 mM, pH 7) maintained at 27 °C under gentle magnetic stirring. At three different time periods, the ee of the diols formed was analyzed as described above.

Complete biohydrolysis of *meta*-chlorostyrene oxide (10 mM) was carried out at 20 °C in 1 h using optimized Glyoxyl-agarose support (1 g in 2 mL of phosphate buffer 10 mM, pH 7). The ee of the formed diol was determined,

after derivatization into the corresponding acetonide, by injection on a chiral GC column (Chiralsil-DEX CB at 135 °C; (*R*)-diol: 12.9 min and (*S*)-diol: 14.9 min).

4.9. Study of the thermal stability of the GA–*St*EH immobilizate using a repeated-batch reactor

The thermal stability of the *St*EH immobilized on GA support was investigated using a repeated-batch reactor. A batch reactor similar to those described above was run at 27 °C until the enantiomeric excess of the residual styrene oxide reached 99%. The residual epoxide and the formed diol were then removed from the reactor by washing the support four times with water (2 mL). The support was either incubated in phosphate buffer (2 mL, 10 mM, pH 7) at 58 °C for several hours. The liquid phase was removed by filtration under vacuum and the enantioselectivity of the support was determined as described above before starting a new resolution cycle.

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