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Improvement of enantioselectivity by immobilized imprinting of epoxide hydrolase from *Rhodotorula glutinis*

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Abstract

The yeast *Rhodotorula glutinis* contains an enantioselective, membrane-associated epoxide hydrolase (EH). Partially purified EH was immobilized in a two-step procedure. In the first step, the proteins were derivatized with itaconic anhydride. In the second step, the derivatized proteins were co-polymerized with ethylene glycol dimethacrylate in water-free cyclohexane to form a bioplastic. Before co-polymerization, the derivatized enzyme had been imprinted by substrates or its analogues (called imprinters) in an aqueous phase. After removing the imprinters, an enzyme with rationally modified properties was obtained. This is the first time that the above-mentioned method was successfully performed with a membrane-associated enzyme of the α/β-hydrolase fold family to which EH belongs.

The enantioselective conversion of (±)-1,2-epoxyoctane was reversed from a preference for (*R*)-1,2-epoxyoctane to (*S*)-1,2-epoxyoctane when the enzyme had been imprinted with (*S*)-1,2-epoxyoctane prior to co-polymerization. The enzymatic reaction was performed in aqueous media.

Other benefits of immobilizing EH into a co-polymer were the ease of recycling of the biocatalyst and the separation of biocatalyst and its products. An unexpected benefit was the enhanced enzyme stability. The half-life of the immobilized and imprinted biocatalyst was enhanced at least 7-fold. Most remarkable was that washing the immobilized EH with HCl, followed by washing it with buffer, resulted in about 50% of the residual activity, while native EH completely lost its activity.
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1. Introduction

Enantiomerically pure epoxides are important building blocks in the industry for the production of pharmaceuticals or agrochemicals. To produce these epoxides, enzymes such as monooxygenases or epoxide hydrolases are used. These latter enzymes are omnipresent in nature, ranging from mammals and plants to microorganisms [1–5]. Epoxide hydrolases (EHs) are attractive tools in biocatalysis because

Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; BSA, bovine serum albumin; AOT, aerosol dioctyl sodiumsulfosuccinate; EGDMA, ethylene glycol dimethacrylate; AIBN, 2,2′-azobis-(2-methylpropionitril); TNBS, 2,4,6-trinitrobenzene sulfonic acid

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they selectively convert racemic epoxides by kinetic resolution without the use of any cofactors.

Besides a reasonable enantioselectivity, important aspects for enzymes to become a versatile tool in industry are high operational stability and recycling. The technique of immobilized imprinting of proteins [6] is able to achieve all these aspects at once. First, the substrate- or enantioselectivity of the enzyme is rationally modified by "bio-imprinting" [6–8] in aqueous solution. To achieve this, certain ligands are added to an enzyme solution and then the enzyme is precipitated or lyophilized before it is used in a non-aqueous environment. Due to the interactions with the ligands the "bio-imprinted" enzymes are believed to adopt a specific orientation in which they are more active, stable or enantioselective in organic solvents. The ligands used for bio-imprinting are usually called protein-imprinters or imprinting molecules. A variety of molecules such as substrates, products, inhibitors or their respective analogues can be chosen as imprinters. Imprinting of enzymes has been done with several hydrolases such as lipases and α-chymotrypsin [6–8] but most conversions took place in organic solvents only [9].

The clue in immobilized imprinting of proteins is that the manipulated protein conformation is fixed by polymerizing it in a water-free organic solvent [6]. Before the proteins can be radically polymerized, they have to be vinylated. Free amino-, hydroxyl- or sulfhydryl groups of amino acids are covalently coupled to itaconic anhydride (Fig. 1) [10]. The obtained derivatized proteins can then co-polymerize radically with ethylene glycol dimethacrylate as the monomer under UV irradiation. The enzyme of interest is thus immobilized in a kind of plastic framework [11]. In this way it is easy to recycle the enzyme.

Recently, the EH from *Rhodotorula glutinis* was purified [12], and this enzyme is now further investigated. At present, this native membrane-associated EH has a preference in hydrolyzing the (*R*)-enantiomer of aliphatic 1,2-oxides, whereas the (*S*)-enantiomer is much less affected.

In this paper we describe the process of immobilized imprinting of the EH resulting in a so-called cross-linked imprinted epoxide hydrolase (CLIP-EH). Substrates or their analogues did serve as imprinting molecules because these molecules were believed to interact with the active site of the enzyme. In this way, we tried to adjust the reaction towards one specific enantiomer and separate the enzymes and products easily. In contrast to genetic engineering techniques such as site directed or random mutagenesis [13,14], this method is directly applicable to the protein on the protein level and it is a very rapid and easy method.

2. Material and methods

2.1. Chemicals

DTT and *n*-octylglucoside were purchased from Roche. (±)-1,2-epoxyhexane, (±)-1,2-epoxyoctane

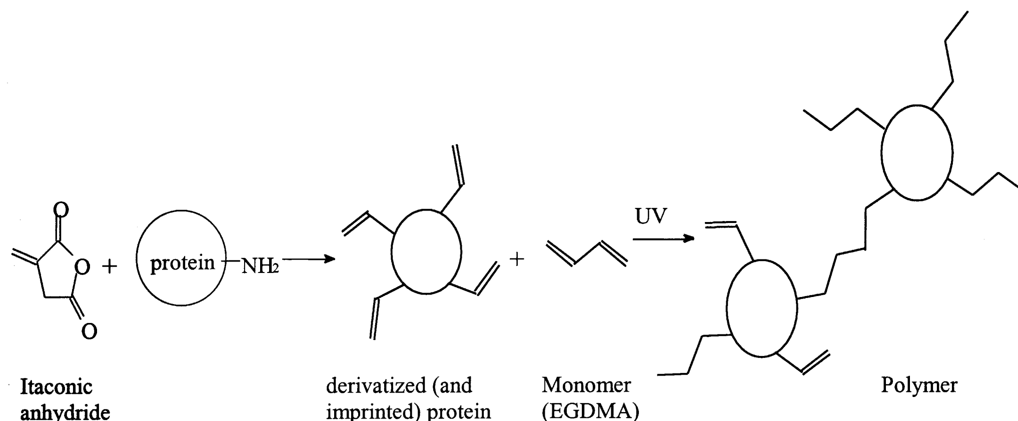

Fig. 1. Derivatization and polymerization of epoxide hydrolase (EH), Before polymerization, the derivatized enzyme can be imprinted to obtain a more active or enantioselective biocatalyst (for details see text).

and EGDMA were products of Aldrich Chemical Co. (*R*)- and (*S*)-1,2-epoxyoctane, water-free cyclohexane, isopropanol, AIBN and TNBS were obtained from Fluka. *N,N'*-methylene diacrylamide, styrene oxide and (±)-1,2-epoxybutane were purchased from Merck. EDTA was a product of Acros. BSA, itaconic anhydride and AOT were purchased from Sigma. (*R*)- and (*S*)-phenylethanediol are products of Janssen Chimica. All chemicals were of the highest available purity.

2.2. Preparation of enzyme samples

The yeast *R. glutinis* strain CIMW 147 (ATCC 201718) was cultivated and harvested as described before [12]. A partially purified enzyme extract was prepared as described earlier [15]. The working buffer was 50 mM potassium phosphate, pH 7.0, supplemented with 1 mM EDTA and 1 mM DTT.

A cell free extract was centrifuged (1 h, 100000 g, 4 °C) and the membrane fraction was treated with 30 mM *n*-octylglucoside for 5 min at 37 °C and again centrifuged. The supernatant was dialyzed overnight against two changes of working buffer (10 l) to remove the detergent.

2.3. Epoxide hydrolase acylation with itaconic anhydride

This method is derived from Peissker and Fischer [6]. The 0.5 ml enzyme solution (5 mg/ml) was diluted to 10 ml with working buffer and 11 mg itaconic anhydride per mg of protein was slowly added under constant magnetic stirring. The pH was monitored and maintained at 7.0 using 3 M NaOH. The mixture was stirred for 60 min (on ice). Non-reacted itaconic anhydride and other low-molecular mass compounds were removed by gel filtration (using four PD10 columns, Pharmacia) and the eluates of the columns were combined.

2.4. Imprinting of the derivatized protein

Imprinting molecules (5 mM final concentration) were added to the combined eluates (after PD10 columns). As imprinters were used the racemic substrate, the (*R*)-enantiomer of the substrate, or the (*S*)-enantiomer of the substrate. The substrates were 1,2-epoxybutane (only racemic), 1,2-epoxyhexane (only racemic), 1,2-epoxyoctane, or styrene oxide. The diols were (*R*)- and (*S*)-phenylethanediol. The samples were mixed and kept at room temperature for 20 min and then put on ice for 5 min. The imprinted proteins were precipitated with 30% (ice-cold) isopropanol that was supplemented with 10 mM (final concentration) of the imprinted molecule used. After mixing for 10 mm, the samples were put on ice for 60 min. After centrifugation (15 mm, 10000 g), the precipitates were washed once with ice-cold isopropanol and after a second centrifugation step the precipitates were lyophilized.

2.5. Determination of free amino groups in protein (TNBS assay)

The relative quantitative determination of amino groups of the native and covalently modified EH sample (after elution of the PD10 columns, before imprinting) was done according to Habeeb [16] and Hall et al. with TNBS [17]. The absorbance obtained with the native protein corresponded to 100% and the extent of modification was calculated according to Shetty and Kinsella (formula 1) [18]:

$$\text{modification (\%)} = \left[1 - \frac{A_{\text{mod}}}{A_{\text{nat}}}\right] \times 100 \qquad (1)$$

where A_{mod} and A_{nat} are the absorbance values obtained with modified or native protein solution, respectively.

To 0.3 ml protein solution (0.1–1 g/l) (native or modified), 0.3 ml $NaHCO_3$ (4%) and 0.3 ml TNBS (0.1%) were added. The samples were placed in a thermomixer at 37 °C (1000 rpm). After 60 min 0.47 ml 1 M HCl was added and the absorption was measured at 335 nm against a blank treated as above but with 0.3 ml demiwater instead of the protein solution.

2.6. Co-polymerization of the derivatized protein with ethylene glycol dimethacrylate

To 0.5 ml cyclohexane (water-free) 4 mg AIBN was added and mixed. The 0.2 ml EGDMA (water-free) was added to this mixture and mixed. The 30 mg derivatized or imprinted protein (lyophilized) was added and suspended by ultrasonification (waterbath, 30 min). The suspension was irradiated at 366 nm at

4 °C for 16 h. The resulting white pellet was washed twice with 4 ml isopropanol. The cross-linked enzyme was collected and finally lyophilized.

2.7. Enzyme activity measurements

To determine enzyme activities, the polymer was added to 1 ml of working buffer. Amounts (ranging from 10 to 30 mg polymer) were chosen so that the activities of the samples were comparable. Therefore, the protein amounts varied: 0.3 mg for 1,2-epoxyhexane, 0.25 mg for 1,2-epoxyoctane and 3.1 mg for styrene oxide. After pre-incubation of 10 min, 1–10 mM racemic substrate was added. The reaction was followed in time by taking headspace samples (100 μl) and analyzed by chiral GC. All samples were measured in at least duplicates and the standard errors were about ±4.5%. As substrates were used: (±)-1,2-epoxybutane, (±)-1,2-epoxyhexane, (±)-1,2-epoxyoctane and (±)-styrene oxide. The specific activities were calculated from the initial reaction rates, determined from the epoxide disappearance, and the protein concentration.

2.8. Calculation of the enantiomeric ratio (E-value)

The Eq. (2) used to calculate the enantiomeric ratio (E) was described by Morisseau et al. [19], where R_0 and S_0 are the substrate concentrations of the R- and S-enantiomer at $t = 0$ and, R and S are the substrate concentrations of the R- and S-enantiomers at $t = t_x$ (at least duplicate measurements).

$$E = \frac{[\ln(R_0/R)]}{[\ln(S_0/S)]} \quad (2)$$

This equation is valid if the R-enantiomer is degraded faster than the S-enantiomer, otherwise the equation is vice versa. By plotting $\ln(R_0/R)$ versus $\ln(S_0/S)$ (a change of concentration during time), the slope of this curve represents the E-value [15,19].

2.9. Stability experiments

To test the operational stability, one batch of EH-polymers (16 mg polymer, suspended in 1 ml buffer) was used repetitively. After each incubation (150 min) with 10 mM racemic substrate, the co-polymer was incubated with 100 μl 6 M HCl, to chemically hydrolyze remaining epoxides. After 10 min, the EH-polymers were thoroughly washed with working buffer and the preparation was ready for another incubation-cycle. The storage stability was tested by putting several samples (16 mg each) of the same polymer batch at 4 °C. Each new incubation was started with a batch from the fridge.

2.10. Analytical methods

The protein concentration was determined by the method of Bradford, with BSA as standard [20]. Gas chromatography was performed on a Chrompack CP9000 gas chromatograph as described before [12]. Analysis was done at oven temperatures of 47 °C for (±)-1,2-epoxyhexane, 97 °C for styrene oxide and 60 °C for (±)-1,2-epoxyoctane on a β-DEX 120 column (Supelco Inc.). (±)-1,2-epoxybutane was analyzed on a β-DEX 225 (Supelco Inc.) column at an oven temperature of 50 °C.

3. Results and discussion

3.1. Influence of acylation and co-polymerization on the catalytical properties of epoxide hydrolase (EH)

In order to cross-link the epoxide hydrolase (EH) via radical polymerization in organic solvents, the enzyme had to be vinylated first (Fig. 1). Itaconic anhydride which reacts with free amino-, hydroxyl- or sulfhydryl groups of proteins [10], was chosen as acylating agent. Initial experiments had shown that the optimum ratio (mg/mg) of itaconic anhydride to protein was 11.8:1. Typically, the resulting degree of protein derivatization was about 70% according to the TNBS assay (see Section 2). Higher levels of derivatization were possible with more itaconic anhydride per mg protein, but then the specific activity and the enantioselectivity were found to decrease (data not shown).

After derivatization, the enzyme was lyophilized and co-polymerized in cyclohexane. To determine possible effects of the derivatization and cross-linking procedures on the specific activity and enantioselectivity, enzyme activities and enantioselectivities towards the three substrates (±)-1,2-epoxyhexane, (±)-1,2-epoxyoctane and (±)-styrene oxide were

Table 1
The influence of protein derivatization and cross-linking on relative total initial activity (%) and enantioselectivity (E-ratio) of epoxide hydrolase (EH)[a]

Substrate	Free		Derivatized		Derivatized and cross-linked	
	Relative total initial activity (%)	E-ratio	Relative total initial activity (%)	E-ratio	Relative total initial activity (%)	E-ratio
1,2-Epoxyhexane	100[b]	3.7(*R*)	73	3.4(*R*)	62	3.1(*R*)
1,2-Epoxyoctane	100[c]	3.0(*R*)	85	3.0(*R*)	27	2.1(*R*)
Styrene oxide	100[d]	1.2(*R*)	100[d]	1.0	53	1.0

[a] The initial activities are given for the hydrolysis of the (*R*)-enantiomer.
[b] 100% activity is 129 nmol/min.
[c] 100% activity is 131 nmol/min.
[d] 100% activity is 135 nmol/min.

estimated for native EH, derivatized EH and cross-linked EH (Table 1). Derivatization did hardly influence the activity and the enantioselectivity of EH.

However, cross-linking of derivatized EH resulted in a decrease in activity of up to 73% in the case of 1,2-epoxyoctane as the substrate. The enantioselectivity (E-value) was slightly affected: a maximum decrease of 30% was found for the hydrolysis of 1,2-epoxyoctane.

3.2. Immobilized protein imprinting of EH

Attempting to influence the enantioselectivity of the immobilized EH, the derivatized EH was imprinted with different molecules (imprinters) prior to cross-linking. The idea is that these imprinters will interact with the active site of the enzyme and by this manipulating its conformation in a rational way when precipitation of the derivatized EH, by adding *n*-propanol into the enzyme/imprinter solution, takes place. The precipitated protein was dried and subsequently polymerized with EGDMA in water-free cyclohexane. The manipulated EH conformation obtained by protein imprinting ought to be covalently immobilized as it was successfully demonstrated in the case of serine proteases [6] This procedure may be called “immobilized protein imprinting”. Imprinters that should be able to manipulate the conformation of the EH at the active site are substrates, products, analogues of these two or may be detergents. Recently published experiments had shown that detergents can affect the specific activity and enantioselectivity of the native EH, although these effects are thought to be caused by changes in enzyme environment (forming of micelles) [15].

It should be mentioned here that cross-linking of the imprinted EH was also attempted in aqueous media with the detergent *n*-octylglucoside or the pure enantiomers of styrene oxide as imprinters, respectively. However, this method led to negative results as might be expected: either no activity of EH was recovered (*n*-octylglucoside as imprinter) or, the enantioselectivity of EH remained unchanged (data not shown). Since addition of detergents resulted in an unacceptable loss of activity in general they were omitted in further experiments.

In all further experiments the cross-linking was carried out in water-free cyclohexane. Firstly, the pure enantiomers of 1,2-epoxyoctane were chosen as imprinters because the specific activity of the EH with this substrate is high (compared to styrene oxide) and the enantioselectivity for this epoxide is poor. Thus, imprinting could have a distinct positive influence on the enantioselective performance of EH. In Fig. 2, the time course studies of the hydrolysis of (±)-1,2-epoxyoctane by cross-linked, imprinted EH (CLIP-EH) and by just cross-linked EH (blank) is shown. The activity of the blank (B) is nearly the same for both enantiomers, whereas in comparison, the activity for the *R*-enantiomer of the EH-polymer imprinted with (*R*)-1,2-epoxyoctane (R) was increased and, the activity for the *S*-enantiomer decreased. Using the EH-polymer imprinted with (*S*)-1,2-epoxyoctane as biocatalyst (S), the activity towards the (*R*)-enantiomer was decreased and, the activity for the (*S*)-enantiomer was increased, resulting

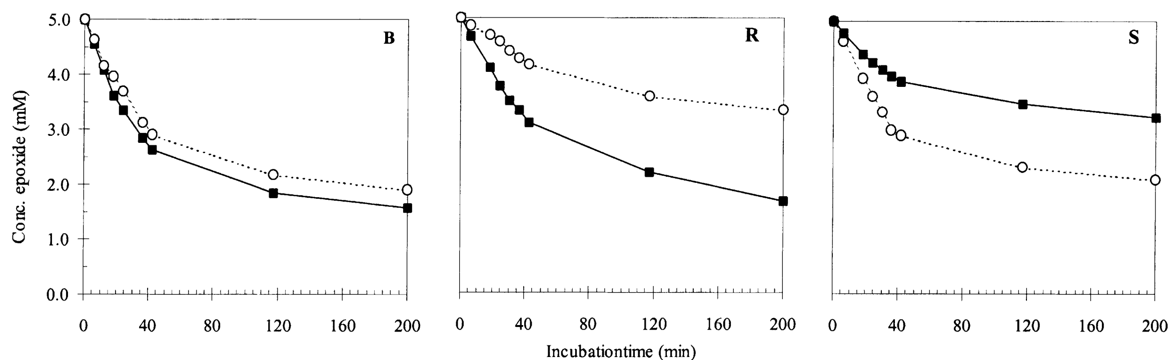

Fig. 2. Time course studies of the hydrolysis of (±)-1,2-epoxyoctane using non-imprinted and imprinted EH-polymers. B: blank, without imprinting; R: imprinted with (*R*)-1,2-epoxyoctane; S: imprinted with (*S*)-1,2-epoxyoctane. ■: (*R*)-enantiomer; ○: (*S*)-enantiomer.

in inverted enantioselectivity when compared to B and R.

Table 2 summarizes the results of these experiments and additionally, data of the conversion of 1,2-epoxyhexane by CLIP-EH imprinted with (*R*)-epoxyoctane are shown. In the case of 1,2-epoxyhexane as the substrate, the initial specific activities were 2-fold enhanced when imprinted with (*R*)-1,2-epoxyoctane, however the *E*-ratio remained more or less unchanged (*E*-ratios 1.7 or 1.8, respectively). When 1,2-epoxyoctane was used as the substrate, there were changes towards initial activity and enantioselectivity. With (*R*)-1,2-epoxyoctane as imprinter, the expected raise in initial activity towards the (*R*)-enantiomer did not occur. The resulting activity was only 66% compared to the non-imprinted EH-polymer. However, the initial activity towards the (*S*)-enantiomer decreased more dramatically. A consequence of this effect was that the *E*-ratio of (*R*)-imprinted EH-polymer was positively changed towards the (*R*)-enantiomer (*E*-ratio of 5.3 compared to 1.3 with the blank). An analogous effect was observed when the enzyme was imprinted with (*S*)-1,2-epoxyoctane: the initial activity towards the (*S*)-enantiomer was slightly decreased (93%) when compared with the blank, but the initial activity towards the (*R*)-enantiomer decreased much more (43%). As mentioned earlier (see Fig. 2) the enantioselectivity of EH was inverted; the *E*-ratio changed from 1.3 (*R*) to 1.8 (*S*). To our knowledge this is the first time that a membrane-associated enzyme, in this case an EH from *R. glutinis*, was successfully imprinted in aqueous phase and subsequently co-polymerized in organic phase in order to rationally manipulate its enantioselectivity in racemic resolution carried out in a buffer solution. It was possible to reverse the enantioselectivity of the EH by protein imprinting (Fig. 2 and Table 2).

Polymerizing the derivatized EH as such did show only minor effects on the enantioselectivity (Table 1), clearly leading to the conclusion that the strong effects

Table 2
Relative total initial activities (%) and *E*-ratios of non-imprinted and imprinted EH-polymers hydrolysing racemic 1,2-epoxyhexane or 1,2-epoxyoctane, respectively

Substrate	Imprinter	Relative total initial activity (%)		*E*-ratio
		R	*S*	
1,2-Epoxyhexane	None	100	100	1.7(*R*)
	(*R*)-epoxyoctane	200	200	1.8(*R*)
1,2-Epoxyoctane	None	100	100	1.3(*R*)
	(*R*)-epoxyoctane	66	29	5.3(*R*)
	(*S*)-epoxyoctane	43	93	1.8(*S*)

on the enantioselectivity observed were completely due to the imprinting procedure. However, the change in $\Delta G^{\#}$ necessary for the switch of the enantiopreference of EH is relatively small.

3.3. Effect of different imprinters on the catalytical properties of EH-polymers

As shown in Table 2, the enantioselectivity of EH could be inverted by imprinting with the appropriate enantiomer of 1,2-epoxyoctane. To establish this principle, several other imprinters were tested for their abilities to manipulate the catalytic properties of EH-polymers (Table 3).

As in the case with (*S*)-1,2-epoxyoctane imprinted EH-polymers, imprinting with (*S*)-styrene oxide also inverted the enantioselectivity of EH-polymers from *R* to *S* (no imprinter: *E*-ratio 1.0; *S* imprinter: *E*-ratio 1.4 (*S*)). Racemic substrate imprinters of 1,2-epoxyoctane and styrene oxide, respectively, also manipulated the initial activities and enantioselectivities of EH-polymers compared to the non-imprinted ones: the initial activities decreased and the enantioselectivity (*E*-ratio) versus the "natural" enantiomer increased (2-fold in case of styrene oxide). The pure enantiomers of 1,2-epoxyhexane were not available and the racemic substrate imprinter had nearly no effect on the activity and enantioselectivity of the hydrolysis of 1,2-epoxyhexane.

Table 3
Influence of various imprinters on the total initial activity (%) and enantioselectivity (*E*-ratio) of non-imprinted and imprinted EH-polymers[a]

Substrate	Imprinter	Relative total initial activity (%)		*E*-ratio
		R	*S*	
1,2-Epoxyhexane[b]	None	100	100	3.1(*R*)
	R/*S*	106	89	3.3(*R*)
1,2-Epoxyoctane[b]	None	100	100	1.3(*R*)
	R	71	36	5.3(*R*)
	S	30	101	1.8(*S*)
	R/*S*	34	36	1.9(*R*)
Styrene oxide	None	100	100	1.0
	R	55	33	2.0(*R*)
	S	52	100	1.4(*S*)
	R/*S*	52	48	2.0(*R*)

[a] The protein amounts per sample were different (0.3 mg for 1,2-epoxyhexane, 0.25 mg for 1,2-epoxyoctane and 3.1 mg for styrene oxide). The activity of the blank polymer (not imprinted) was set at 100%. The absolute activity values were 80 nmol/min for 1,2-epoxyhexane, 84 nmol/min for 1,2-epoxyoctane and 72 nmol/min for styrene oxide.

[b] Data presented were independently obtained from data in Table 2 (newly prepared EH-polymers).

Another possibility to manipulate the enantioselective properties of EH-polymers could be imprinting with the enantiopure products. For these experiments, we used the enantiopure products of hydrolyzed styrene oxide: (*R*)-, (*S*)- and also (±)-1,2-phenylethanediol. However, these imprinter molecules did not show comparable good results as with the enantiopure substrates. Only imprinting with (*R*)-1,2-phenylethanediol seemed to have a "positive" effect: the *E*-value (*R*) obtained in hydrolysing styrene oxide was 2.1 times better when compared with the blank (2.3 versus 1.1).

3.4. Stability of the immobilized EH-preparation

Two types of stability were considered: the operational and the storage stability. To test the operational stability, the same batch of a polymer (non-imprinted or imprinted with the *R*- or *S*-enantiomer of 1,2-epoxyoctane) was subjected to four repetitive incubations. After each conversion experiment the polymers were washed with HCl in order to completely hydrolyse remaining epoxide chemically. The initial specific activities of the polymers in the first and the fourth batch versus the *R*- or *S*-enantiomer of racemic 1,2-epoxyoctane are shown in Table 4. Although the washing procedure is very harsh, the activity of the EH-polymers decreases not more than about 55% (*S*-activity of *R*-imprinted polymer). The enantio-

Table 4
Operational stability of EH-polymers (substrate 1,2 epoxyoctane)

Number of incubations	Imprinter	Initial specific activity (nmol/min mg)		*E*-ratio
		R	*S*	
1	None	76.4	59.9	1.3(*R*)
	R	95.5	44.9	5.3(*R*)
	S	54.2	77.3	1.8(*S*)
4	None	49.7	47.3	1.2(*R*)
	R	59.6	20.4	1.3(*R*)
	S	31.3	47.3	1.2(*S*)

Table 5
Half-lives of native EH and EH-polymers (substrate 1,2-epoxy-octane)

EH preparation	Half-life (days)
Free enzyme	2
Blank EH-polymer	30
R imprinted EH-polymer	14
S imprinted EH-polymer	21

selectivities decreased as well but still, the *S*-imprinted polymer demonstrated the above-mentioned inverted preference for the *S*-configurated substrate (*E*-ratio of 1.2). In comparison, free native EH did not survive one incubation-cycle with HCl and lost all its activity immediately. Thus, the enhanced stability of EH due to immobilized imprinting is striking and it reflects a major finding of our studies as well.

The storage stability was tested in the time course of 1 week. In this case, the free native EH and the EH-polymers (non-imprinted and imprinted with *R*- or *S*-1,2-epoxyoctane) were kept at 4 °C. After 1 week the remaining activities of the polymers were about 76–90%. The free enzyme almost completely lost its activity after 4 days when stored under the same conditions (without glycerol). The corresponding calculated half-lives of the various EH preparations are presented in Table 5. Storage of wet EH-polymers at −20 °C resulted in high loss of activity, probably due to damage of the polymer network by crystallization of the water molecules.

Another option to improve enantioselectivity or stability of enzymes is by directed evolution (random mutagenesis), error prone PCR or DNA shuffling. In the case of an esterase from *Pseudomonas fluorescens*, this has already been successfully [13]. In the case of a hydantoinase for the production of L-methionin in *E. coli*, an inversion of enantioselectivity was established with random and saturation mutagenesis [14]. However, the method of immobilized protein imprinting seems easier, since one can change the enantioselectivity of the enzyme directly on the protein level without any screening efforts or the requirement of a special screening assay. In addition, immobilized protein imprinting is also fast: we resulted in an improved enzyme in 3 days (from whole cells to EH-polymer). No false positives were determined using this method, unlike the method of directed evolution [13,14].

4. Conclusions

Immobilized protein imprinting was able to rationally modify the enantioselectivity of an EH. Most remarkably, by this one could reverse the enantioselectivity of an enzyme from the same source, exactly the same strain, in favor of the other enantiomer. Usually, it is known that opposite enantiopreferences towards a substrate were only obtained by using EHs from different bacteria [21]. The method of immobilized protein imprinting includes stabilization of the biocatalyst and offers the possibility of its re-use. In the case of imprinted and polymerized EH from *R. glutinis* the EH-polymer survived harsh washing with hydrochloric acid, whereas the free enzyme was completely irreversibly inactivated. However, more work has to be performed in order to optimize this procedure.

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