# Properties of epoxide hydrolase from the yeast *Rhodotorula glutinis*



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**Nicole A.E. Ariës-Kronenburg**

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   *Leontievsky et al., Fems Microbiol. Lett. (1997),* ***156****, 210*

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Wageningen, 2002.

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# CHAPTER 1

## GENERAL INTRODUCTION

This thesis describes an investigation with respect to the enantioselective hydrolysis of a racemic epoxide by epoxide hydrolases (EH) as formulated in Fig. 1.1. The investigation has been carried out within the framework of the Innovation Oriented Research Program on Catalysis.

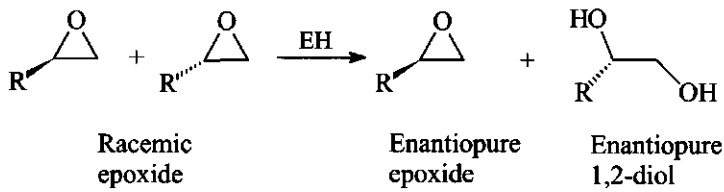
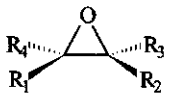


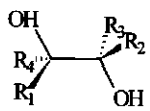

**FIG. 1.1. ENANTIOSELECTIVE ENZYMATIC HYDROLYSIS OF A RACEMIC EPOXIDE TO ITS VICINAL DIOL AND ONE REMAINING EPOXIDE ENANTIOMER**

## 1.1. EPOXIDES

Enantiopure epoxides (oxiranes) (**1**) as well as their corresponding 1,2-diols (**2**) are valuable and versatile intermediates for the synthesis of a broad range of fine chemicals.




**1**




**2**

Potentially, there are three principal routes to prepare **1**, **2** and mixtures thereof in an enantiopure form:

1) Enantioselective epoxidation of alkenes with the aid of a chiral catalyst or a microorganism giving **1**.
2) Enantioselective dihydroxylation of alkenes with a chiral catalyst or microorganism giving **2**.
3) Enantioselective hydrolysis of a racemic epoxide with an enzyme giving a mixture of **1** and **2**.

### 1.1.1. CHEMISTRY OF EPOXIDES

Epoxides are highly strained cyclic ethers that react smoothly with nucleophiles. The ring opening of epoxides occurs in much the same way as in other ethers, but under much milder conditions. Instead of a strong acid, the epoxide is hydrolyzed with dilute aqueous acid at room temperature into 1,2-diols, also known as glycols. Unlike other ethers, epoxides can also be cleaved by base, because of the strain of the three-membered ring.

The direction of the ring opening does not only depend on the substituents present, but also on the reaction conditions. Under basic conditions, the nucleophile attacks via a typical $S_N2$ reaction: attack takes place at the less hindered carbon atom. Acid-catalyzed ring opening occurs via a different reaction course: the nucleophilic attack takes place at the *more* highly substituted carbon atom [79].

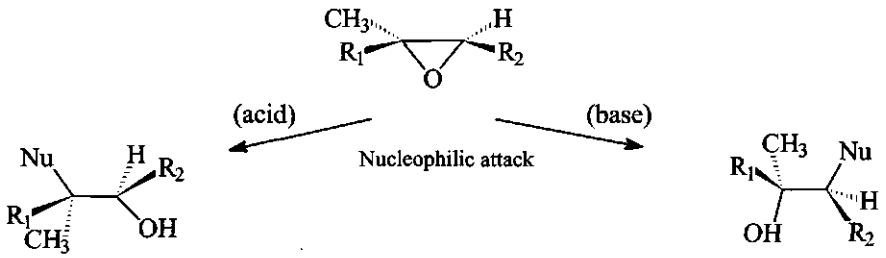



**FIG. 1.2. NUCLEOPHILIC RING-OPENING OF EPOXIDES ARE STEREOCONTROLLED**

The enzymatic hydrolysis of monoalkyl substituted epoxides to produce the vicinal diols can occur regioselectively in two ways (see Fig. 1.3.). Nucleophilic ring-opening at the unsubstituted, less hindered oxirane carbon atom proceeds with retention of configuration at the substituted oxirane carbon atom. Hydrolysis at the alkylsubstituted ring carbon atom proceeds with inversion of configuration at this carbon atom [146]. Such regioselective hydrolysis also takes place with di- and trialkylsubstituted oxiranes. The enzymatic nucleophilic water attack occurs preferentially at the (*S*)-configurated ring carbon atom [147].

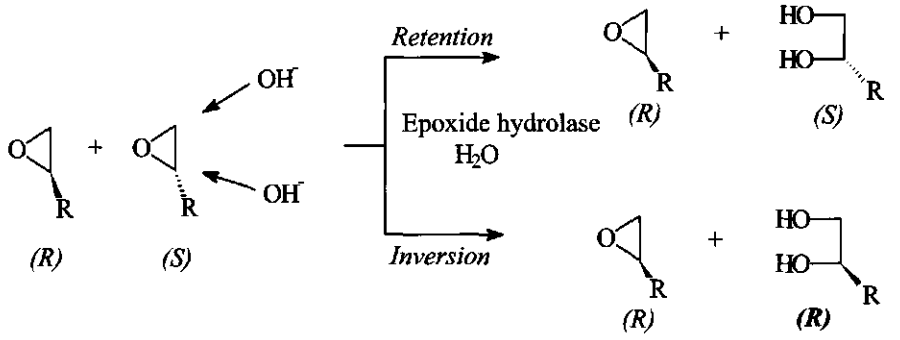



**FIG. 1.3. RETENTION OR INVERSION OF CONFIGURATION DURING ENZYMATIC EPOXIDE HYDROLYSIS**

### 1.1.2. NOMENCLATURE IN STEREOCHEMISTRY

Compounds with the same molecular formula and their atoms connected in the same order, but with different geometry are called stereoisomers. If the difference is only the fact that they are their mirror images, they are called enantiomers. An enantiomer, *i.e.* an enantiopure compound, is also often called an optically active compound, reflecting that it is not superimposable on its mirror image and is also able to rotate the plane of polarized light. This rotation can take place to the left (signed –) or to the right (signed +) and it is expressed by the specific rotation $[\alpha]_D$ [79].

An optically active compound is also called a chiral compound. Molecules that do contain a plane of symmetry are called achiral or nonchiral. A chiral center is the central carbon atom with 4 different groups attached. Another type of stereoisomers are the meso-compounds. These compounds also contain one or more chiral centers, but they also have a symmetry plane and therefore no net optical rotation.

To discriminate between the enantiomers, they are given an (*R*) or (*S*) notation that describes the stereochemical configuration around the chiral atom. (*R*) means that the configuration is to the right (R from *rectus* (Latin for right)) and *S* means to the left (S stands for *sinister* (Latin for left)) according to the Cahn-Ingold-Prelog rule [19].

The stereochemical configuration has nothing to do with the way the polarized light is rotated. An optical rotation to the left and a stereochemical configuration to the right are therefore possible, as in (S)-(+)-lactic acid and (R)-(-)-lactic acid.

The last term is racemic mixture, also called racemate. This is a 50:50 mixture of enantiomers, sometimes denoted (±) or (*R/S*). This mixture shows zero optical rotation because the rotations of both enantiomers extinguish each other. A racemic mixture has different properties than those of each of its enantiomers separately, *e.g.* a different melting point, density and solubility [79].

### 1.1.3. Enantiomeric ratio and enantiomeric excess

If a reaction occurs enantioselectively only one of the two enantiomers is formed or converted. If one enantiomer of a racemic mixture is transformed faster into a product than the other, it is called Kinetic Resolution (KR). If the reaction takes place with water and a biocatalyst, it is called Biocatalyzed Hydrolytic Kinetic Resolution (BHKR). At the end of the reaction, one enantiomer is available in excess. A drawback of this type of reaction is the theoretical maximum yield of 50%.

The enantiomeric composition of a racemate during the course of the reaction is represented by the enantiomeric excess, denoted ee (or as %ee). It can be calculated according to the following equation, in case the incubation mixture is enriched with the *R*-enantiomer (otherwise it is vice versa) :

$$\%\ ee = \frac{[R]_t - [S]_t}{[R]_t + [S]_t} \times 100\% \qquad \text{Eq. 1.1}$$

In this equation $[R]_t$ and $[S]_t$ are the concentrations of the enantiomers *R* and *S* at time = t. The %ee can be calculated for the enantiomeric composition of the starting material as well as for the product (mixture). Pure kinetic resolution will give of course ee = 100% for the product.

However, the ee gives insufficient information about the efficiency of the reaction as it is dependent on the conversion. For an industrially interesting process, kinetic resolution should not only take place with a high enantioselectivity but also at a high degree of conversion. This efficiency is usually given as the (biochemical) stereoselectivity factor E or enantiomeric ratio *E* (or *E*-ratio or *E*-value) (equations 1.2 and 1.4) [20, 109, 123], which is independent of the conversion. Equation 1.2a is used for the description of the reaction of the substrate and 1.2b is used for the formation of the product. ($ee_s$ is the enantiomeric excess of the substrate and $ee_p$ is the enantiomeric excess of the product).

$$E = \frac{\ln\{(1-c)(1-ee_s)\}}{\ln\{(1-c)(1+ee_s)\}} \qquad \text{Eq. 1.2a}$$

$$E = \frac{\ln \{ (1 - c)(1 - ee_p) \}}{\ln \{ (1 - c)(1 + ee_p) \}} \qquad \text{Eq. 1.2b}$$

In this equation the conversion of the reaction (c) is represented by equation 1.3, in which [*R*] and [*S*] are the concentrations of the enantiomers at time = t and [$R_0$] and [$S_0$] are the initial concentrations of the individual enantiomers.

$$c = 1 - \frac{[R] + [S]}{[R_0] + [S_0]} \qquad \text{Eq. 1.3}$$

Another representation of the E-value is given in equation 1.4. Plotting ln {[$R_0$] / [*R*]} against ln {[$S_0$] / [*S*]} gives s straight line with a slope representing the E-value.

$$E = \frac{\ln \{ [R_0] / [R] \}}{\ln \{ [S_0] / [S] \}} \qquad \text{Eq. 1.4}$$

A kinetic resolution is quite effective when the *E*-value exceeds 100, *i.e.* almost 50% conversion is possible with an enantiomeric excess of 100%. For industrial applications an E-value over 20 is already satisfactory [121], although at lower *E*-values higher conversions will be needed to obtain the remaining enantiomer 100% pure, resulting in a lower yield.

### 1.1.4. Production of racemic and enantiopure epoxides

Ethylene oxide is the smallest possible epoxide. It is an intermediate in the manufacture of both ethylene glycol (antifreeze in cars) and polyester polymers. More than 1 million tons of ethylene oxide are produced industrially each year (1988) by air oxidation of ethylene over a silver oxide catalyst at 300°C. This catalytic procedure, however, is not applicable to the epoxidation of higher alkenes.

Racemic epoxides are produced by treatment of an alkene with a peroxy acid ($RCO_3H$). Many different peroxy acids can be used, but *m*-chloroperoxybenzoic acid is preferred on a laboratory scale, because this peroxy acid is stable, crystalline and an easily handled material, in contrast to other peroxy acids (they are highly reactive and they readily decompose). Another (laboratory) process is the use of halohydrins, prepared by electrophilic addition of HOCl or HOBr to alkenes. When chloro- or bromohydrins are treated with a base, HCl or HBr is eliminated and an epoxide is produced. This is an intramolecular Williamson ether synthesis, with the nucleophilic O-atom and the electrophilic C-atom in the same molecule [79].

Chemical synthesis of enantiopure epoxides is possible by the Sharpless epoxidation [52] and by using the Jacobsen catalyst [50]. The enantiopure Jacobsen/Katsuki catalyst contains a metal-ion (manganese) [53, 54]. This catalyst is highly effective but also very expensive. A reaction with hypochlorite is the Jacobsen epoxidation: epoxidation with sodium hypochlorite used in the synthesis of Diltiazem (a drug) and in the first step of the production of L-735,524 (from indene to indene oxide) [121]. Disadvantages of these methods are the use of very acidic or basic conditions, the use of heavy metals like in the Sharpless epoxidation or Jacobsen catalyst [50, 52], and the relatively high costs for the required product isolation. The Jacobsen catalyst is also restricted to the resolution of terminal epoxides and the enantioselective ring opening of meso-epoxides [49, 132].

Other methods have a biochemical approach. The use of whole cells, purified enzymes (like lipase catalyzed reactions) or microorganisms in industrial approaches is very common [12]. Examples related to epoxides and glycols are: asymmetric reduction of haloketones, enantioselective dehalogenation of haloketones, production of optical pure halohydrins from prochiral substrates, direct epoxidation of alkenes with monooxygenases [142] or enantioselective hydrolysis of epoxides.

Biocatalytic methods to produce these epoxides include the use of epoxide hydrolases in the enantioselective hydrolysis of racemic mixtures. These enzymes use water as a co-substrate and are cofactor independent. By such kinetic resolution, one may obtain the remaining enantiomer as the enantiomeric pure epoxide together with the enantiopure vicinal diol, both in 50% yield. A disadvantage however, is the coupled production of two different intermediates.

## 1.2. INDUSTRIAL ENANTIOPURE EPOXIDES AND PRODUCTS THEREOF

Several sources mention that epoxides and their vicinal diols are relevant building blocks for the pharmaceutical and agrochemical industries. For pharmaceutical products, the biologically active compound has to fit into the chiral receptor at the target site. Most of the times, only one of the enantiomers of the active compound fits in this target site while the other enantiomer has no pharmaceutical impact or an opposite (negative) impact. In this section several of these important epoxide and diols are described. What are they and for which products are they building blocks?

The pharmaceuticals can be divided into groups according to their function, but this division is not exclusive. Examples of groups of pharmaceuticals are painkillers, anti-inflammatory drugs, β-blockers, inhibitors or pheromones (sex-attractant hormones). Sometimes one product is a pheromone but it can also act as an antagonist (blocker).

In Fig. 1.4. an example of a blocker (Diltiazem) and two other examples of pharmaceuticals (CL316.243 and LEDOL) are given. A highly successful group of antihypertensive drugs is comprised of calcium antagonists. Diltiazem is one of these

drugs and it belonged to the Chiral Top Ten in 1994: 300 tons per year were produced (worth 1500 million dollar) [57, 121].

CL316.243 is an anti-obesity drug which is under development by American Cyanamid. Its precursor is (*R*)-*meta*-chlorostyrene oxide. LEDOL is an anti-tussive (anti-coughing), produced by Dompe, prepared from glycidol.

Another example is 2-pyridyloxirane, a building block for phencyclidine (PCP). Derivatives of this PCP are etoxadrol and dioxadrol (mixture of dexoxadrol and levoxadrol). These compounds can cause hyperthermia (dexoxadrol) or hypothermia (levoxadrol) in rats [131]. Applications in human pharmaceuticals are not known yet, although the Merck index called etoxadrol an anaestheticum and dioxadrol besides an anaestheticum also a relaxant, anti-depressant, stimulant or even a painkiller.

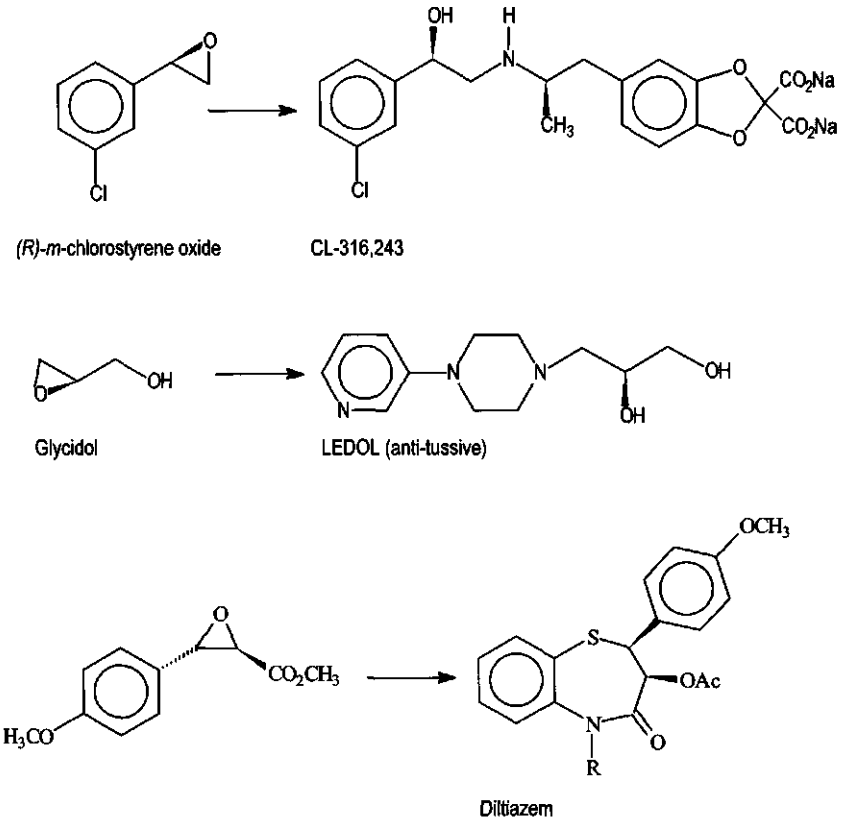



**FIG. 1.4. SOME INDUSTRIALLY IMPORTANT CHIRAL EPOXIDES AND THE FINAL PRODUCTS THAT CAN BE SYNTHESIZED THEREOF**

An important group of pharmaceuticals are painkillers and anti-inflammatory drugs. Most of these compounds are made from α-arylpropionic acid derivatives. α-Methylstyrene oxide is a building block for a variety of these derivatives. The following compounds belong to a major class of nonsteroidal anti-inflammatory drugs and household painkillers: (*S*)-Ibuprofen (2-[4-isobutyl-phenyl]-propanoic acid), one of the top-ten drugs sold worldwide (8000 ton per year (1994)), and (*S*)-Naproxen (2-(4-methoxynaphtyl)propanoic acid) (1400 ton per year (1994)) [23]. The *S*-enantiomer is responsible for the desired effect, while it has been demonstrated that the *R*-enantiomer of Ibuprofen accumulates in fatty tissue as a glycerol ester. The long-term effect of this accumulation is not known yet [144, 145]. (*S*)-Ibuprofen can be made from racemic 4-isobutyl-α-methylstyrene oxide in four steps. The epoxide hydrolase from *Aspergillus niger* catalyzes the first step. The other steps are chemical reactions [23].

In Fig. 1.5. an overview is given of a wide variety of industrial interesting products that can be produced from epoxides. All of these compounds are chiral compounds. Some compounds are further described.

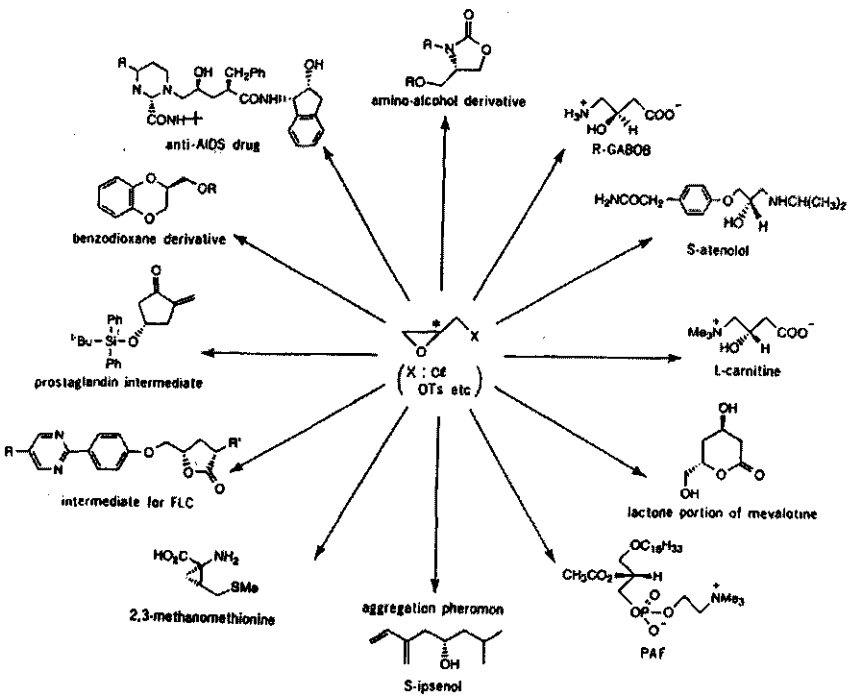



**FIG. 1.5. OVERVIEW OF INDUSTRIALLY INTERESTING PRODUCTS FROM EPOXIDES [141]**

The anti-AIDS drugs and channel blockers belong to the best-known inhibitors and blockers, respectively. Indene oxide is an important epoxide nowadays because it is used for the preparation of (*R,S*)-aminoindanol. This is a key intermediate for L735.524, or called Indinavir [72], a HIV protease inhibitor (developed by Merck) (top-left in Fig. 1.5.) [141].

Propanolol derivatives resemble the anti-AIDS drug Indinavir, but they are made from the precursor GLYTO (GLYcidol TOsylate). They find application as β-blockers and various companies produce these compounds.

Some other industrially interesting compounds are the platelet aggregation factor (PAF), some hormones (prostaglandin derivatives) and for example Eliprodil. This is a highly promising neuroprotective agent (as a glutamate antagonist) [78], produced from *para*-chlorostyrene oxide.

Epoxides are highly reactive and most of them are instrumental in causing cancer in mammals, but there are also epoxides that are important for the survival of a species. An example is Disparlure, the pheromone (sex-attractant) of the gypsy moth [10]. The pheromone is produced in the abdomen by the female moth. The moth is a harmful insect as its larvae can denude a tree in a few weeks. The insect is therefore a serious threat to hardwood forests and orchards. The active isomer of Disparlure is (7*R*,8*S*)-(+)-7,8-epoxy-2-methyloctadecane, the other configurations are not biologically active. Industrially produced enantiopure Disparlure can be used to lure and trap or kill the male insects and by doing this preventing the multiplication of this insect.

Another application is the use of (*S*)-(–)-Frontalin. This compound is an aggregation pheromone of pine beetles of the *Dendroctonus*-family. It is used in the field in pheromone traps. Kroutil et al. reported a chemoenzymatic synthesis route for this enantiopure compound [64]. Another aggregation pheromone, calles *S*-ipsenol, belong also to the industrially interesting compounds.

Also other families of insects make use of epoxides in their pheromone components [81], in which the enantiomer of the pheromone component sometimes acts as an antagonist.

## 1.3. EPOXIDE HYDROLASES

### 1.3.1. EPOXIDE HYDROLASES IN GENERAL

Epoxide hydrolases (EH) (EC 3.3.2.3) are hydrolyzing enzymes that catalyze the addition of water to epoxides, resulting in the formation of vicinal diols. Most EH belong to a very large family of hydrolases that includes esterases, proteases, dehalogenases and lipases [6]. This family is the α/β-fold hydrolase family, so called because of the 6 α-helices and 8 β-sheets, present in these enzymes (Fig. 1.6.). The parallel β-strands 3 to 8 are connected with α-helices A to E which are located at both sides of the β-sheets. The last α-helix (F) is situated at the C-terminus of the enzyme.

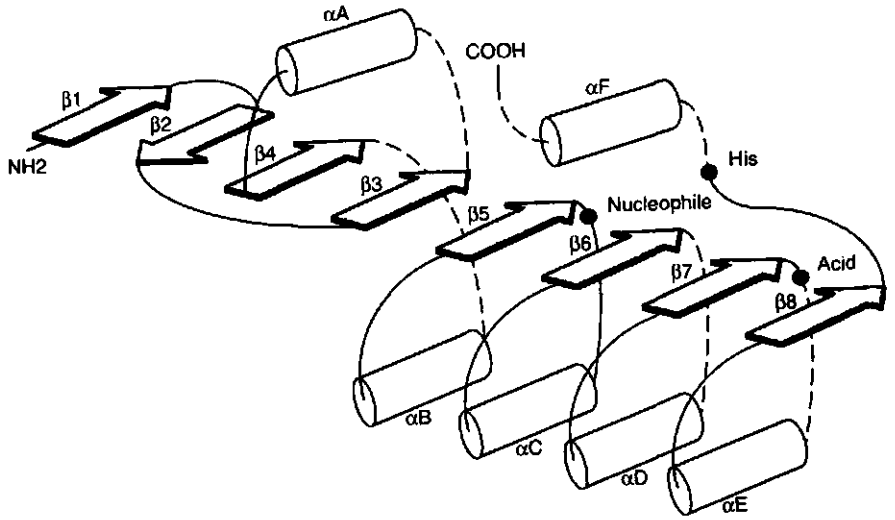



**FIG. 1.6. SCHEMATIC REPRESENTATION OF THE α/β HDROLASE FOLD**

β1-β8 are β-sheets. αA-αF are α-helices. The positions of the members of the catalytic triad are indicated (Nucleophile, Acid and Histidine) [97].

Epoxide hydrolases are ubiquitous and especially mammalian enzymes have been extensively studied over the years [4, 18, 93]. In mammals 5 different EH are found: the microsomal epoxide hydrolases (mEH), the cytosolic or soluble epoxide hydrolases (sEH), the cholesterol 5,6-oxide hydrolase, hepoxilin $A_3$ EH and leukotriene $A_4$ EH [31]. This last EH was recently cloned from the yeast *Saccharomyces cerevisiae*, characterized and improved [67, 68]. Each enzyme class has its own chemical and immunological properties. The cholesterol EH and mEH are located in microsomes while the other three EH are located in the cytosol, or, as in the case of sEH, in the peroxisomes. They have their own substrate specificity and specific inhibitors [31].

EH are also found in plants [11, 56], insects [147], bacteria [66, 77, 82] and fungi [87]. Recently, EH have been purified from yeast [14, 63] and these enzymes are useful in the enantioselective hydrolysis of epoxides [15, 139, 143].

As said earlier, the EH belongs to the α/β-hydrolase fold family. However, only mEH and sEH belong to this group of enzymes, although there is only a low level of similarity between both amino acid sequences [2, 69]. Their mechanisms and catalytic triads are fairly similar [69]. The α/β-hydrolase family hydrolyses its substrate by a catalytic triad. As the first step, they form a covalent enzyme-substrate ester [89].

The cholesterol 5,6-oxide hydrolase, however, does not seem to act in this way, and probably is not structurally related to mEH and sEH [89]. The catalytic mechanism for hepoxilin $A_3$ hydrolase is largely unknown and the enzyme can therefore not be categorized yet. Based on sequence alignment studies and other characteristics, the

leukotriene $A_4$-hydrolase is not likely to be related with the other EH [6, 40]. For example, the enzyme contains a $Zn^{2+}$ and it exhibits also aminopeptidase activity [39].

Because the EH from yeast is thought to belong to the large $\alpha/\beta$-hydrolase fold family, the specific features of sEH and mEH enzymes are summarized below. More detailed information about the different epoxide hydrolases and the sources is well summarized by H. Visser in Chapter 2 of his thesis [135].

### 1.3.2. Soluble epoxide hydrolases (sEH)

The mammalian sEH are xenobiotic enzymes that also participate in the metabolism of endogeneously derived fatty acid epoxides [80]. They belong to an extensively studied enzyme class: they have been purified and characterized, their genes and/or cDNAs are cloned and characterized [7, 35, 116-118, 129]. They are expressed in nearly all tissues [119]. Because the sEH possess an imperfect peroxisomal targeting sequence at the C-terminus, sEH activity is also found in peroxisomes. In the early days, the latter EH was thought to be a different EH [3, 7, 129].

Rodent sEH could be induced if the rodents were treated with peroxisomal proliferation agents such as 2,4-dichlorophenoxy acetic acid, Wyeth-14.643, or hypolipidemic drugs such as clofibrate. Other possible inducers were: nafenopin, di-(2-ethylhexyl)phthalate, mono-(2-ethylhexyl)phthalate, ICI-55.897, S-8527, 2,4,5-trichlorophenoxy acetic acid and 2-ethylhexanoic acid. These compounds have very different structures but are all known to induce proliferation of peroxisomes [75] by an as yet unknown mechanism. Induction in humans has not been reported yet.

A typical substrate for sEH is *trans*-stilbene oxide, because this epoxide is not hydrolyzed by mEH [95]. Other specific sEH substrates, like epoxy esters or carbonates have been synthesized. An example is 4-nitrophenyl (2*S*,3*S*)-2,3-epoxy-3-phenylpropyl carbonate [26]. Endogenous substrates are EETs (epoxyeicosatrienoic acids) and leukotoxin. Besides typical sEH substrates, there are also typical inhibitors of sEH which do not inhibit mEH. Examples are chalcone oxide and *trans*-3-phenylglycidols [27, 28]. Other inhibitors are compounds based on urea and carbamates but also the heavy metals $Cd^{2+}$ and $Cu^{2+}$ [29, 86].

### 1.3.3. Microsomal epoxide hydrolases (mEH)

The major action of mEH is directed towards the metabolism of xenobiotic compounds, like sEH. Typical substrates include therefore toxic and procarcinogenic compounds. The most common compounds that are metabolized by mEH are epoxide derivatives of polycyclic aromatic compounds like *cis*-stilbene oxide, butadiene monoxide and benzo[$\alpha$]pyrene-4,5-oxide. The enzyme is also well documented: it was purified from a large number of organisms, the proteins characterized and the genes and cDNA's have been cloned and characterized [1, 6, 17, 42, 43, 99, 124].

For the mEH, several inhibitors have been reported. 1,1,1-Trichloropropene-2,3-oxide was the first inhibitor studied. Later studies revealed that also cyclopropyl

oxiranes were competitive reversible inhibitors [108]. Recently discovered inhibitors are divalent metal ions, with the most potent inhibitors being $Zn^{2+}$ and $Hg^{2+}$ [29].

Although *trans*-stilbene oxide is not a substrate for mEH, it induces the enzyme, like nearly all classical inducers of xenobiotic metabolizing systems: Phenobarbital, 3-methylcholanthrene and 2-acetylaminofluorene [75].

## 1.4. *RHODOTORULA GLUTINIS* AND ITS DERIVED EPOXIDE HYDROLASE(S)

*Rhodotorula glutinis* is a red-pigmented yeast, isolated from (over)ripe fruits like apples. The red color of *Rhodotorula glutinis* originates mainly from red carotenoids, which contain 12 to 13 double bonds and methoxy- and oxo- groups [120]. Carotenoids or carotenoid-like pigments are of interest as vitamin precursors and effective antioxidants. In *Rhodotorula glutinis* CBS 20, β-carotene, β-zeacarotene, torulene and torularhodin are the four most abundant carotenoid pigments. They may protect the organism against visible and ultraviolet light [104]. The yeast grows very well on several media. It can grow on different carbon sources, and it is very comfortable on growing on just glucose as carbon-source. The yeast best grows under aerobic conditions. Under anaerobic conditions, the growth rate is much lower and the distinctive red-orange color disappears which makes the yeast turn colorless. This, however, has no influence on the activity or production of the epoxide hydrolase.

*Rhodotorula* strains belong to the group of asporogenous yeast. They can be considered having a limited life cycle as they multiply almost completely by budding (asexual reproduction), with few mycelial networks. Because of this characteristic, *Rhodotorula glutinis* is also called an imperfect yeast, or the anamorphous phase. The perfect state (or telemorphous phase), having sexual reproduction, of *Rhodotorula glutinis* is better known as *Rhodosporidium toruloides*.

Yeasts are, like mammals and other higher organisms, eukaryotic organisms, having a defined nucleus in which the genetic material is enclosed. This was a reason to assume that the properties of the EHs from *R. glutinis* would resemble the mammalian EHs and not the bacterial EHs. In dematiaceous fungi, epoxide hydrolase is constitutively expressed. Its expression is coincident with secondary metabolite pigment production in stationary phase or in idiophase [36]. It is interesting to know if the pigment production in *Rhodotorula glutinis* can be induced and coincidentally the EH production be enhanced.

## 1.5. INDUSTRIAL JUSTIFICATION AND GOAL OF THIS THESIS

As stated by the overall objective of the Innovative Oriented Program on Catalysis, new industrial processes must meet both economic and environmental requirements. These include a clean production method (*i.e.* less reagents, no difficult purification processes), high production rate (fast reaction, few reaction steps), and waste prevention (no heavy metals, strong acids or bases, no pathogenic organisms).

The use of EH from the yeast *Rhodotorula glutinis* could satisfy these requirements. To produce this yeast in high quantities only water, glucose and some salts are necessary. The yeast grows fast and contains an enantioselective EH, which is capable of hydrolyzing a wide range of substrates [140], whereby the aliphatic 1,2-epoxides are converted at a very high rate [143] with only water as a cosubstrate. By this enzymatic kinetic resolution, the remaining epoxide may be obtained in high yield together with the vicinal diols formed.

The main goal of this investigation was to isolate and purify the epoxide hydrolase from the yeast *Rhodotorula glutinis*. Subsequently, we would characterize the purified enzyme and study the substrate specificity for aliphatic epoxides in comparison with other known epoxide hydrolases. Finally, we would try to develop a suitable method to separate the epoxides from the diols.

This investigation was related to a similar study at the University of Groningen on the applicability of bacterial EHs for the kinetic resolution of aromatic epoxides. Their role was to test available and newly isolated biocatalysts with a broad range of epoxides. The most promising enzymes would be further characterized and the mechanism elucidated. Finally, they would try to optimize cultivation and production of the appropriate biocatalysts and the production and isolation of enantiomerically pure epoxides and diols.

The Groningen study was focussed on the soluble EH from *Agrobacterium radiobacter* AD1. The enzyme was purified and its primary structure, X-ray structure and the catalytic mechanism was elucidated [48, 90, 91, 110, 111, 113]. By site-directed mutagenesis, an EH with improved enantioselectivity has been developed [112]. With this recombinant enzyme several reactions and transformations has been investigated [33, 76, 77]. A novel item was the tandem reaction of an EH combined with a halohydrin dehalogenase to produce glycerol from epichlorohydrin [76].

## 1.6. OUTLINE OF THIS THESIS

Chapter 2 gives a description of the purification of the epoxide hydrolase from *Rhodotorula glutinis*. Some difficulties had to be overcome, since this EH appeared to be a membrane-associated enzyme. A partial purified sample was separated by preparative electrophoresis. Peptide analysis of the obtained fragments clearly showed homology with other $\alpha/\beta$ fold hydrolases.

Chapter 3 shows that the activity and enantioselectivity of this membrane-associated enzyme were remarkably affected by detergents used during the purification procedures.

Chapter 4 describes the influence of the presence of other hydrophobic environments such as a second phase of substrate added to the reaction mixture. This second phase was found to have a stabilizing effect on the hydrophobic enzyme.

Chapter 5 describes the immobilization of the EH in polymers. As the enzyme seems to be sensitive towards its environment, "freezing" its conformation into a polymer maintains its properties better. When the enzyme is simultaneously incubated with a particular substrate, the active site is "frozen" in its active form. In this way, it is possible to alter the active site in a variable way and enhance the stability of the enzyme by at least 7 times. The chosen substrate determines the preference of the active site for one of the epoxide enantiomers.

Finally, in Chapter 6, the scope and limitations of the epoxide hydrolase from *Rhodotorula glutinis* is discussed: what are the characteristics of this EH compared to other EHs, what experiments could be done to gain more knowledge about this EH in order to improve the enzyme. In this respect, the present as well as the potential industrial use of EH is discussed.

At the end of this thesis a list of used abbreviations together with summaries in English and Dutch are given.

# CHAPTER 2

## PURIFICATION OF AN EPOXIDE HYDROLASE FROM *RHODOTORULA GLUTINIS.*

**ABSTRACT**

The epoxide hydrolase from *Rhodotorula glutinis* was isolated and initially characterized. The enzyme was membrane associated and could be solubilized by Triton X-100. Purification yielded an enzyme with a specific activity of 66 μmol 1,2-epoxyhexane hydrolyzed $min^{-1}$ $mg^{-1}$ protein. The enzyme was not completely purified to homogeneity but, nevertheless, a major protein was isolated by SDS-PAGE for subsequential amino acid determination of peptide fragments. From sequence alignments to related enzymes, a high homology towards the active site sequences of other microsomal epoxide hydrolases was found. Molecular mass determinations indicated that the native enzyme exists as a homodimer, with a subunit molecular mass of about 45 kDa. Based upon these, this epoxide hydrolase is structurally related to other microsomal epoxide hydrolases.

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## 2.1. INTRODUCTION

Enantiopure epoxides are important building blocks in the pharmaceutical industry. Chemical production methods of these epoxides are often only specific for a narrow range of substrates and environmentally hazardous compounds, *i.e.* heavy metals, have to be used as catalysts. Therefore, research has been directed to the development of biocatalytic methods to produce these chiral compounds. One of these methods involves the use of hydrolyzing enzymes like epoxide hydrolases (EHs). Advantages of these enzymes are that they are cofactor independent and they use water as substrate. By hydrolytic kinetic resolution of epoxides, the degraded enantiomer of the epoxide can be produced as an optically pure diol.

Epoxide hydrolases are ubiquitous and most of them belong to the α/β-hydrolase fold family. The folding of these enzymes is common to enzymes of widely differing phylogenetic origin and catalytic function. The arrangement of the catalytic residues is conserved. In the primary sequence, the order is always: nucleophile, acid, histidine [97]. The catalytic mechanism involves the formation of a covalent intermediate between the substrate and the nucleophilic amino acid residue (e.g. Asp) of the enzyme [89].

Since epoxide hydrolases are omnipresent, it is possible that they are suitable for use in biotechnological applications. As source of biocatalysts, microorganisms are more suitable than higher organisms like mammals, because it is easier to cultivate large amounts of biomass to obtain large quantities of enzymes. The first EH from yeast has been described by Weijers in the strain *Rhodotorula glutinis* [140]. Recently, other closely related yeast genera were found which are also able to enantioselectively hydrolyze epoxides [15]. EH from *R. glutinis* can convert a broad range of substrates: aryl, alicyclic and aliphatic epoxides were hydrolyzed [140].

For biocatalytic purposes, the yeast EH should be overexpressed in a suitable host. Therefore, the enzyme now has been partly purified, peptides were obtained and their amino acid sequences analyzed. With these peptides, it is possible to make primers and probes to pick up the gene encoding for EH in *R. glutinis*. The results from the purification and peptide analysis are described in this paper.

## 2.2. METHODS

### 2.2.1. GROWTH CONDITIONS FOR *RHODOTORULA GLUTINIS*

*Rhodotorula glutinis* strain CIMW 147 (ATCC 201718) was obtained from our own laboratory culture collection. It was grown on mineral salts medium supplemented with 0.2% (w/v) yeast extract and 1% (w/v) glucose with 600 mL medium in 5 L flasks [140]. After 2 days shaking at 30°C, the cells were harvested by centrifugation at 10,000 *g* for 10 min, washed with 50 mM potassium phosphate buffer pH 7.0, concentrated by centrifugation and resuspension, and stored at -20°C.

### 2.2.2. Preparation of cell free extract

Cell free extracts (CFE) were prepared as follows: 5 mL cell suspension (approx. density 1.1 g cells $mL^{-1}$) was diluted with 2 mL 50mM potassium phosphate buffer, pH 7.0, with 1 mM EDTA and 1 mM DTT (working buffer). The cells were broken by a beadmill (Retsch MM 2000) for 15 min at 100% amplitude with glass beads of 1-1.5 mm in diameter (50% of the total volume were beads). Unbroken cells and debris were removed by centrifugation (10,000 *g*, 8 min, 4°C) and the remaining supernatant was used as cell free extract.

### 2.2.3. Purification of an epoxide hydrolase activity

CFE was centrifuged at 100,000 *g* for 1 h at 4°C. The supernatant was discarded, the pellet was mixed with 2% (v/v) Triton X-100 for 5 min at 37°C, recentrifuged at 100,000 *g*, 1h 4°C and the supernatant contained solubilized membrane proteins. Glycerol was added to give a final concentration of 20% (v/v). Following steps were performed at 5-7°C. If necessary, pooled fractions were concentrated by ultrafiltration with an Amicon ultrafiltration unit, using a Filtron 10K membrane. The purification procedure is a modification of the method described by Papadopoulous *et al.* [99].

The 100,000 *g* supernatants were applied onto a DEAE-cellulose 52 column (Whatman) (2.5×32 cm), equilibrated and eluted with buffer A (working buffer supplemented with 0.1% (v/v) Triton X-100 and 20% (v/v) glycerol) at a flow rate of 1 mL $min^{-1}$. Fractions (20 mL) with EH activity were pooled.

Concentrated DEAE fractions were diluted with buffer B (20 mM TrisHCl, pH 8.2, with 1 mM EDTA, 1 mM DTT, 0.1% (v/v) Triton X-100 and 20% (v/v) glycerol) and applied to a Q-Sepharose fast flow (Pharmacia) column (5×10 cm). The column was equilibrated with buffer B and eluted with an increasing linear gradient of NaCl from 0 to 0.5 M in the same buffer at a flow rate of 3 mL $min^{-1}$. Fractions (20 mL) with EH activity were pooled.

The Q-Sepharose pools were applied onto a Sephacryl S300 (Pharmacia) column (2.5×98 cm) for further purification. The column was equilibrated with buffer B with 0.15M NaCl at a flow rate of 0.7 mL $min^{-1}$ and the fraction size was 5 mL.

A part of the Q-Sepharose pool was applied onto a Superose 6 column to determine the molecular weight. The column was equilibrated with buffer B with 0.15 M NaCl. (Aldolase (158,000), bovine serum albumin (67,000), ovalbumin (43,000), and chymotrypsin A (25,000) were used as reference proteins).

### 2.2.4. Enzyme assay

Samples (0.5 mL) of enzyme were added in buffer to test tubes and closed with rubber caps. The reaction mixture was pre-incubated at 35°C for 10 min in a shaking waterbath. The reaction was started by addition of 10 mM (*R,S*)-1,2-epoxyhexane. Every 2 min headspace samples (100 µL) were taken, followed by analysis on chiral GC. Initial reaction rates were determined from the epoxide disappearance and

correlated to the protein content. The enantiomeric ratio E was calculated using the equation described by Morisseau *et al.* [85].

### 2.2.5. ISO-ELECTRIC FOCUSSING

For iso-electric focussing, samples were concentrated by ultrafiltration with an Amicon ultrafiltration unit, using a Filtron 10K membrane and subsequently desalted using a PD-10 column (Pharmacia). Electrophoresis was carried out with a Phast System (Pharmacia). The pI was deduced from a pH 3-9 iso-electric focussing gel using the standards from the broad pI calibration kit (Pharmacia). The gels were stained with Coomassie Brilliant Blue G.

### 2.2.6. ANALYTICAL METHODS

Peptide analysis was performed by the Protein Analysis Center (PAC) of the Karolinski Institutet, Stockholm, Sweden. Proteins from the SDS-PAGE gel were sequenced via in-gel digestion, resulting in tryptic peptides. These were extracted and separated into fragments using a micro-column reverse phase HPLC and fractionated onto a PVDF strip (Microblotter, PE-Applied Biosystems) for subsequent high-sensitivity Edman degradation (Procise cLC from PE-Applied Biosystems).

Gas chromatography (GC) was performed as described by Weijers [140]. Analysis was done at an oven temperature of 45°C for 1,2-epoxyhexane.

## 2.3. RESULTS AND DISCUSSION

### 2.3.1. PURIFICATION OF EH FROM *RHODOTORULA GLUTINIS*

Epoxide hydrolase in whole cells of *Rhodotorula glutinis* converts 1,2-epoxyhexane at over 100 nmol per min per mg protein with an enantiomeric ratio (E-ratio) of 84 [143]. Of various methods used to prepare CFE, a beadmill (see Methods) gave the most reproducible results. Recovery of EH activity from whole cells in CFE was approx. 92%. Specific activities in whole cells and in CFE were comparable at approx. 120 nmol (*R*)-1,2-epoxyhexane degraded per mg protein per min. When the CFE was centrifuged at 100,000 *g*, 77% of EH activity was recovered in the pellet indicating it to be a membrane-associated enzyme. For further purification, EH was solubilized from the membrane fraction using Triton X-100 at 2% (v/v), which gave satisfactory results. The membrane fraction was recentrifuged at 100,000 *g*. Curiously, the total EH activity recovered in this supernatant was higher than in the starting material (pellet). Storage of the supernatant at -20°C for 8 weeks did not result in an appreciable loss of activity.

To avoid denaturation of the EH, Triton X-100 was present in all the following column steps. The supernatant was applied to DEAE cellulose, to which the bulk of proteins bound. EH activity was collected just after the void volume. In this way, 96% of the proteins present in the supernatant were removed, but 48% of the total activity was lost. The active fractions were pooled, concentrated and then applied to a Q-Sepharose column. EH eluted at 65 mM NaCl. In this case, 97% of the proteins from

the DEAE-fractions were removed and loss of activity was 48%. The pooled, active fractions obtained from this fractionation were applied to a Sephacryl S-300 column. In this way, further protein separation was obtained and salts were removed. A high percentage (90%) of the applied proteins was removed, but also a similarly high percentage of the activity (87%) was lost. Consequently, no significant increase in specific activity was obtained in this step. The results of the purification are presented in Table 2.1. In conclusion, the EH was purified 565 fold with a yield of 3%.

**TABLE 2.1. PURIFICATION SCHEME OF EPOXIDE HYDROLASE FROM *RHODOTORULA GLUTINIS***

| Steps | Total protein (mg) | Total act. (μmol $min^{-1}$) | Spec. act. (nmol $min^{-1}$ $mg^{-1}$) | Yield (%) | Purification fold |
|---|---|---|---|---|---|
| CFE | 10200 | 1200 | 118 | 100 | 1 |
| Pellet | 5250 | 900 | 170 | 75 | 1 |
| Supernatant | 4900 | 1100 | 225 | 92 | 2 |
| DEAE-cellulose | 225 | 575 | 2550 | 48 | 22 |
| Q-Sepharose | 5.8 | 300 | 51725 | 25 | 438 |
| Sephacryl S300 | 0.6 | 40 | 66650 | 3 | 565 |

Activities were determined using (*R,S*)-1,2-epoxyhexane as substrate and the specific activities given represent the degradation rate of the *R*-enantiomer.

Microsomal epoxide hydrolases from different mammals have been purified, mainly from liver microsomes. Mammalian sources were human [37, 94], horse [18], rat [9, 74], dog [4] and rabbit [44]. All these enzymes belong to the α/β hydrolase fold family. Apparently, no specific problems were encountered during purification of these enzymes and they all could be purified to homogeneity. Also, yields were quite reasonable during purification schemes and were often over 10%. In our case, the procedure was more cumbersome. No completely purified preparation was obtained (Fig. 2.1) and our yield of 3% was relatively low. The problems encountered during the purification procedure may indicate that the interaction of the enzyme with the membranes differs for the mammalian enzymes and this yeast enzyme, respectively. A further indication for differences between the mammalian enzymes and the yeast enzyme was obtained by determining the pI value. The yeast enzyme has a pI value close to 7, which is higher than the pI of mammalian mEHs, which are between 4.8 and 6.0 [98].

### 2.3.2. DETERMINATION OF THE MOLECULAR WEIGHT

A Superose 6 column was used to determine the molecular weight of the enzyme under non-denaturing conditions. The EH eluted at approx. 80 kDa. All mammalian microsomal EH are monomers and have a molecular weight in the order of 47-54 kDa [4, 74, 99]. On the basis of this gel filtration, the yeast EH seemed to be

larger than the mammalian microsomal enzymes. Subsequently, 12.5% SDS-PAGE was used not only to verify the purity of the various fractions but also to determine the molecular weight under denaturing conditions. The most pure fraction (59) obtained from the Sephacryl S300 column still revealed at least 3 distinct bands (Fig. 2.1).

The molecular weights of these bands were in the range of 43-49 kDa. The band marked EH has a molecular weight of approx. 45 kDa and had become more intense during purification. The molecular weight on SDS-PAGE is not in agreement with the molecular weight on Superose 6.

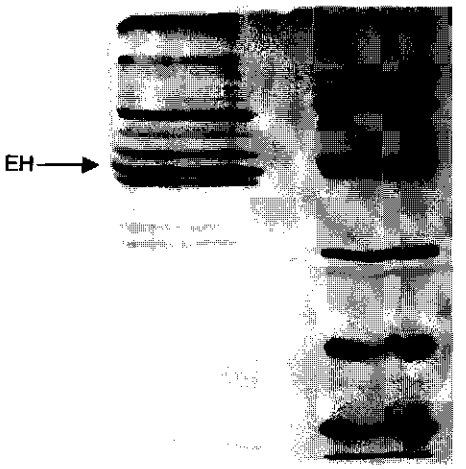


**FIG. 2.1. SDS-PAGE OF SEPHACRYL S300 FRACTION 59**
SDS-PAGE was performed according to the method of Laemmli [71] using a 12.5% gel, stained with Coomassie Brilliant Blue G. Lane 1 is the concentrated sample (Microsep microconcentrator) of fraction 59 of the Sephacryl S300 column. Lane 2 are the markers: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) (Pharmacia low-molecular-weight calibration kit). Band EH was cut out and peptides were made by in-gel digestion and analyzed for their amino acid sequences.

However, since Triton X-100 was present in the samples during Superose 6 chromatography, it is very well possible that aggregates have been formed resulting in a non-monomeric molecular weight of about 80 kDa. The value of 80 kDa may indicate that EH is present in these samples as a homodimer. Similar results have been obtained with other membrane-associated enzymes. It is known that non-denaturing detergents may not fully dissociate protein complexes or aggregates and as a result detergent-protein complexes may exhibit twice the apparent molecular weight expected for the monomeric protein [9, 74, 130].

The molecular weight of EH from *R. glutinis* under denaturing conditions is in the same range as the molecular weights of mammalian microsomal EHs.

### 2.3.3. HYDROLYSIS OF 1,2-EPOXYHEXANE

The hydrolysis rate and enantioselectivity in the hydrolysis of 1,2-epoxyhexane was determined to check whether the purified enzyme showed the same characteristics as in whole cells. The reaction rate of the purified protein obviously is much higher than in whole cells (66 μmol $min^{-1}$ $mg^{-1}$ protein versus 120 nmol $min^{-1}mg^{-1}$ protein), but the enantioselectivity had changed.

From the results given in Fig. 2.2, an enantiomeric ratio of E=17 can be calculated. In whole cells, an enantiomeric ratio of E=84 was observed [143]. The loss of enantioselectivity can be due to the fact that the enzyme is no longer in its natural environment of the membranes. Apparently, Triton X-100 is not able to completely stabilize the conformation of the enzyme.

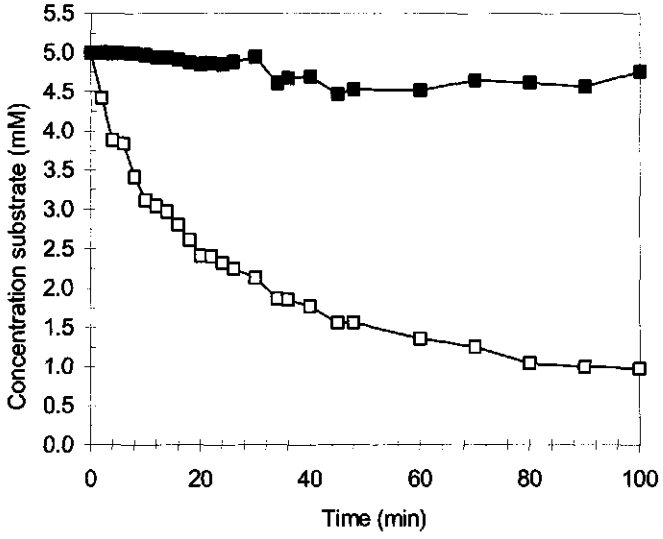


**FIG. 2.2. ENANTIOSELECTIVE DEGRADATION OF 1,2-EPOXYHEXANE BY PURIFIED EPOXIDE HYDROLASE**
Material of fraction 59 of Sephacryl S300 was used to follow the enantioselective degradation of (*R,S*)-1,2-epoxyhexane in time. The incubation mixture contained 0.5 mL of concentrated fraction 59 (12 µg protein) and 0.5 mL buffer (50 mM $KP_i$, pH 7.0 + 1 mM EDTA, 1 mM DTT, 20% (v/v) glycerol and 0.1% (v/v) Triton X100). The open squares (❑) represent the (*R*)-enantiomer and the closed squares (■) represent the (*S*)-enantiomer.

### 2.3.4. PEPTIDE ANALYSIS

The purity of the protein did not allow to directly perform Edman degradation and a further purification step by preparative SDS-PAGE was necessary. The most distinct band (marked EH) in Fig. 2.1 had a molecular weight indicative of an epoxide hydrolase (45 kDa). This band was cut and tryptic peptides were made via in-gel digestion. The amino acid sequences of five peptides, designated P16, P19, P27, P29 and P30, were analyzed and the respective sequences were: P16 (15 aa): GPAYGVMQQLTP(E)(D)F; P19 (13 aa): DAPEGGHFFAALK; P27 (12 aa): YHLHNFASK(S)(G)(R); P29 (15 aa): WPATEIVPEDYTD(D or E)(P or I or A) and P30 (15 aa): (S or T or Y)IGTSFLPVSLNPHF. The amino acids between brackets were difficult to assign. Peptides 16, 27, 29 and 30 showed no clear homology with any known partial EH sequence. However, the sequence of P19 clearly showed homology

with the sequence of other EHs: microsomal EHs from rabbit (RBmEH) [45] and rat (RTmEH) [107] and a microsomal juvenile hormone EH (JHEH) from an insect (*M. sexta*) [148]. This peptide showed homology to the catalytic histidine region of these 3 mEH as shown in Fig. 2.3.

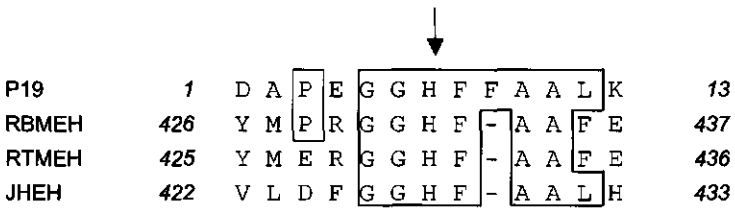


**FIG. 2.3. COMPARISON OF THE P19 PEPTIDE SEQUENCE WITH EH AMINO ACID SEQUENCES.**
The P19 sequence is aligned to the corresponding putative catalytic histidine regions of rabbit mEH (RBMEH) [45], rat EH (RTMEH) [107] and juvenile hormone EH (JHEH) [148]. The arrow indicates the catalytic histidine residue. Amino acids identical to the P19 sequence are framed. Numbers in italics designate the position of the amino acid in the respective sequences. Dashes indicate spaces inserted in the amino acid sequence to maximize homology.

From these results, it is concluded that EH indeed is an epoxide hydrolase that belongs to the large family of α/β-hydrolase fold enzymes. Peptide 19 showed homology with the highly conserved sequence of the catalytic histidine region in α/β fold hydrolases although an extra amino acid (phenylalanine) was present. In the near future, the enzyme will be studied in more detail by overexpressing it in a suitable host.

# CHAPTER 3

## EFFECTS OF DETERGENTS ON SPECIFIC ACTIVITY AND ENANTIOSELECTIVITY OF THE EPOXIDE HYDROLASE FROM *RHODOTORULA GLUTINIS*

**ABSTRACT**

The yeast *Rhodotorula glutinis* contains an enantioselective epoxide hydrolase. Previous work showed that the enzyme is a membrane-associated enzyme that can be solubilized from the membranes by a detergent treatment. Now, the effect of detergents on reaction rate and on enantioselectivity in particular was investigated. Three types of detergents were tested: non-ionic, anionic and zwitterionic. Non-ionic detergents stimulated the specific activity of the enzyme. Enantioselectivity of the enzyme was strongly affected by several detergents. Thesit and sucrosemonolaurate had the most pronounced effects and enantiomeric ratios were strongly enhanced. The effects are most likely due to the ability of detergents to stabilize membrane-proteins by forming micelles and thus mimicking the membrane structure.

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## 3.1. INTRODUCTION

Enantiopure epoxides are important building blocks in the pharmaceutical industry. Biocatalytic methods to produce these compounds include the use of epoxide hydrolases in the enantioselective hydrolysis of racemic mixtures. These enzymes use water as a cosubstrate and are cofactor independent. By kinetic resolution, it is possible to obtain the less reactive enantiomer as the enantiomeric pure epoxide.

Epoxide hydrolases (EH) are ubiquitous and especially mammalian enzymes have been extensively studied over the last few years [4, 18, 93]. EHs are also found in plants [11, 56], insects [148], bacteria [65, 66, 77, 82] and fungi [87]. Recently, EHs have been purified from yeasts [14, 63] and these enzymes are useful in the stereoselective hydrolysis of epoxides [15, 139, 143]. Both the reaction rate and the enantioselectivity of EHs are strongly dependent on the substrate. Interestingly, we noticed that detergents have an effect on these parameters [63] and this initial observation has now been studied in more detail.

Detergents are commonly used to solubilize membrane-bound enzymes. Above their critical micellar concentration, they form micelles that can serve as artificial membrane structures [25]. In this way, the micelles are also capable of stabilizing enzymes [66]. Another effect of detergents is their ability to influence the reaction rate of hydrolytic enzymes as has been observed for lipases [149].

Here we describe the study of the influence of detergents on the membrane-associated EH from *R. glutinis* in partially purified forms. Three classes of detergents were tested: non-ionic, anionic and zwitterionic. As representative substrates we tested both styrene oxide and 1,2-epoxyhexane.

## 3.2. MATERIALS AND METHODS

### 3.2.1. CHEMICALS

The detergents Gal 12Ac, MGal 12OH and MGal 12Ac were a kind gift from P. Bogaerts of ATO-DLO (Wageningen, The Netherlands). AOT and bovine serum albumin were purchased from Sigma (Zwijndrecht, The Netherlands). All other detergents and DTT were purchased from Roche (Almere, The Netherlands). (*R,S*)-1,2-epoxyhexane and decane were products of Aldrich (Zwijndrecht, The Netherlands). Ethylacetate and (*R,S*)-styrene oxide were purchased from Merck (Darmstadt, Germany). EDTA was a product of Acros ('s-Hertogenbosch, The Netherlands). All other reagents and chemicals used were of analytical grade.

### 3.2.2. GROWTH CONDITIONS FOR *RHODOTORULA GLUTINIS*

The yeast *Rhodotorula glutinis* strain CIMW 147 (ATCC 201718) was cultivated and harvested as described in Chapter 2 (p. 22).

### 3.2.3. PREPARATION OF CRUDE EXTRACT

Cell free extract (CFE) was prepared as described previously (Chapter 2, p. 23) and centrifuged at 100,000 *g* for 1 h at 4°C. In total, a 40 mL cell suspension was obtained from 1-liter cell-culture. The supernatant (*S*1), containing the cytosolic fraction of the cells, was stored at -20°C. The pellet (P1) was resuspended in a volume equal to the first supernatant in Buffer A (50 mM potassium phosphate buffer pH 7.0, supplemented with 1 mM DTT and 1 mM EDTA). A small aliquot of this suspension (0.5 mL) was stored for activity measurements. Solid n-octylglucoside was added to the suspension to give a final concentration of 33 mM. The suspension was incubated for 5 min at 37°C, followed by centrifugation (100,000 *g*, 1h, 4°C) and the supernatant (S2) now contained solubilized membrane proteins. Supernatant S2 was dialyzed overnight against 2 changes of buffer A (10 L). The dialyzed material contained 2.9 mg protein per mL and aliquots of 1 mL were stored at -20° C until further use. After 8 weeks of storage under these conditions, only 10% of the activity was lost.

### 3.2.4. ENZYME ASSAY

Crude enzyme (1 mL) was diluted with 1 mL of buffer A and divided between 2 vials (4 mL, Supelco) and closed with mininert valves (Supelco). To the samples a detergent was added whereas the blank did not receive an addition. Detergent concentrations were chosen within the range recommended by the supplier [Roche 1999]. The reaction mixture was pre-incubated at 35°C for 10 min in a shaking water bath. The reaction was started by the addition of 10 mM (*R,S*)-1,2-epoxyhexane or 5 mM (*R,S*)-styrene oxide. Every 2 min headspace samples (100 µL) were taken, followed by analysis on chiral GC.

To examine the distribution of the substrate over the vapor and liquid phase, also liquid samples were taken. To a reactionvessel of 30 mL, 5 mL enzyme material in buffer A (protein content of 1.5 mg $mL^{-1}$) was added. At appropriate time-intervals, samples of 0.5 mL liquid phase were taken, saturated with 0.3 g NaCl and extracted for epoxides with 0.8 mL ethylacetate. The epoxide/ethylacetate samples were stored in glass vials at –20°C until further analysis.

The specific activities were calculated from the initial reaction rates, determined from the epoxide disappearance, and the protein concentration. Two different equations were used to calculate the enantiomeric ratio (E). Equation 3.1 was described by Morisseau *et al.* [85], where $R_0$ and $S_0$ are the concentrations of the *R*- and *S*-enantiomer at t = 0 and *R* and *S* are the concentrations of the *R*- and *S*-enantiomers at t = t.

$$E = [\ln (R_0/R)] / [\ln (S_0/S)] \qquad \text{Eq. 3.1}$$

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. The second equation used was:

$$E = [\ln ((1 - c)(1 - ee))] / [\ln ((1 - c)(1 + ee))] \quad \text{Eq. 3.2}$$

In this equation, c is the conversion and ee the enantiomeric excess of the substrate. But this equation is less accurate, since the E-ratio is obtained by single-point observations. Equation 3.2 is useful if just one enantiomer is degraded, and the other enantiomer is not hydrolyzed at all. When the first enantiomer is totally degraded (ee = 100%), the conversion is 50% and the E-ratio is at its maximum value. Since the second enantiomer will not be degraded, the E-ratio remains at this value. However, in this paper both enantiomers of the substrates used are degraded, although at different rates, and therefore only Eq. 3.1 will be used to calculate the E-ratio.

#### 3.2.5. Determination of the effect of the detergent concentration

Enzyme material (1 mL) was diluted with 1 mL of buffer A and divided between 2 vials. Detergents in a solid form were added to obtain different endconcentrations. The concentration ranged from below the critical micellar concentration (CMC) to far above. Thesit and sucrosemonolaurate were chosen as detergents (CMC 0.09 mM and 0.2 mM, respectively). After pre-incubation for 10 min, 10 mM (*R,S*)-1,2-epoxyhexane was added and the course of the reaction was followed by headspace sampling.

#### 3.2.6. Analytical methods

Protein concentrations were determined by the method of Bradford, with bovine serum albumin as standard [16]. Gas chromatography (GC) was performed on a Chrompack CP9000 gas chromatograph according to Kronenburg *et al.* [63]. Analysis was done at oven temperatures of 47°C for (*R,S*)-1,2-epoxyhexane and 95°C for (*R,S*)-styrene oxide.

All fractions and samples were tested for enzyme activity with 10 mM (*R,S*)-1,2-epoxyhexane or 5 mM (*R,S*)-styrene oxide. Headspace analysis (vapor phase) was done by injecting 100 µL headspace. Liquid samples were analyzed by injecting 1 µL of the epoxide/ethylacetate samples.

## 3.3. Results

#### 3.3.1. Effect of various detergents on specific activity and on enantiomeric ratio

Cell free extracts (CFE) of *R. glutinis* contained EH activity and when racemic 1,2-epoxyhexane was used as a substrate, it was converted enantioselectively with an enantiomeric ration E = 40. Upon centrifugation at high speed (100,000 *g*, 1 h, 4°C), 76% of the epoxide hydrolase activity was recovered in the pellet (P1) with an enantiomeric ratio of E = 13. Subsequently, this pellet was treated with the detergent n-octylglucoside to release membrane-associated EH and this suspension was centrifuged at 100,000 *g*. Most of the activity from the CFE was now recovered in the supernatant (S2) in which E was 61 (Table 3.1). We have optimized this system by

varying detergent-concentration and incubation times (results not shown). This resulted in an n-octylglucoside concentration of 33 mM and a pre-incubation time of 5 min at 37°C.

**TABLE 3.1. EPOXIDE HYDROLASE ACTIVITY IN CRUDE EXTRACTS.**

| Fraction | Total activity (nmol $min^{-1}$) | Yield % | Specific activity (nmol $min^{-1}$ $mg^{-1}$) | E-ratio |
|---|---|---|---|---|
| CFE | 3800 | 100 | 109 | 40 |
| Pellet 1 | 2900 | 76 | 102 | 13 |
| Supernatant 1 | 875 | 23 | 9.1 | 5 |
| Pellet 2 | 250 | 7 | 7.8 | 5 |
| Supernatant 2 | 3425 | 90 | 196 | 61 |

The intriguing effect of the detergent n-octylglucoside on specific activity and E-ratio was further investigated by testing 15 other detergents (Table 3.2). Epoxide hydrolase was solubilized from the membranes (fraction P1) using n-octylglucoside and after ultracentrifugation the supernatant S2 was dialyzed in order to remove the detergent. The specific activities and enantiomeric ratios of the enzyme towards the substrates (*R,S*)-1,2-epoxyhexane and (*R,S*)-styrene oxide (Fig. 3.1) were determined in either the presence or absence of detergents (Table 3.2). The concentrations of all tested detergents exceeded their critical micellar concentration (CMC) [114]. Each detergent has a different optimum work concentration [114]; the concentrations used and the CMCs are indicated in Table 3.2.

Three classes of detergents were tested: anionic, zwitterionic and non-ionic (Table 3.2). The addition of anionic detergents had a negative effect on enzyme activities and enantioselectivities of the reaction. The specific activity for both substrates was reduced by at least 30%. The E-ratio of the (*R,S*)-epoxyhexane conversion was reduced, whereas the E-ratio of styrene oxide degradation was not affected. The zwitterionic detergents had a slightly positive effect on both reaction rates and enantiomeric ratios. The non-ionic detergents form a heterogeneous group of compounds and most of them did not have a strong effect on either specific activity or enantioselectivity for both substrates tested. But considerable effects were obtained when Thesit, sucrosemonolaurate or n-dodecylmaltoside were employed in case 1,2-epoxyhexane was used as a substrate. The detergents were very effective in increasing both the reaction rates (especially the *R*-enantiomer) and the enantiomeric ratios. The most spectacular increase in enantiomeric ratio was in the presence of sucrosemonolaurate with an E-ratio that was approx. 11 times higher than in the blank. The specific activity was only twice as high, but the specific activity for the *S*-enantiomer was decreased. Thesit increased both reaction rate and enantiomeric ratio

nearly three times. The effects with styrene oxide were similar: Thesit, Triton X100 and n-dodecylmaltoside were very effective in increasing the reaction rate, while the addition of Thesit, n-dodecylmaltoside and n-octylglucoside increased the enantiomeric ratio. The largest increase in reaction rate was 8 times, while the increase in enantiomeric ratio is maximum 4.8 times (both Thesit).

**TABLE 3.2. DETERGENT EFFECTS ON EPOXIDE HYDROLASE ACTIVITY AND ENANTIOMERIC RATIO**

| | | | 1,2-Epoxyhexane | | | Styrene oxide | | |
|---|---|---|---|---|---|---|---|---|
| Detergent | Conc. | CMC[a] | Spec. act.[b] | | E- | Spec. act.[b] | | E- |
| | (mM) | (mM) | *R* | *S* | Ratio | *R* | *S* | Ratio |
| No addition | - | - | 90 | 29 | 3.7 | 4.8 | 4.4 | 1.2 |
| *Anionic* | | | | | | | | |
| AOT | 49 | n.k[c] | 63 | 15 | 1.1 | n.d | n.d | n.d |
| Deoxycholic acid | 23 | 1-4 | 18 | 14 | 1.0 | 2.4 | 2.8 | 1.0 |
| SDS | 66 | 8.3 | 18 | 6 | 1.0 | 3.4 | 4.5 | 1.0 |
| *Zwitterionic* | | | | | | | | |
| CHAPS | 10 | 4.0 | 198 | 37 | 4.2 | 10 | 6.6 | 2.0 |
| Zwittergent 3-12 | 25 | 3.6 | 225 | 63 | 5.9 | 23 | 6.6 | 3.0 |
| *Non-ionic* | | | | | | | | |
| Octyl-glucoside | 30 | 15 | 180 | 29 | 13 | 15 | 10 | 2.5 |
| Dodecyl-maltoside | 15 | 0.2 | 252 | 39 | 17 | 16 | 14 | 2.6 |
| Non-idet $P_{40}$ | 1 | 0.3 | 108 | 31 | 2.8 | 14 | 11 | 1.5 |
| Sucrosemonolaurate | 48 | 0.2 | 198 | 19 | 40 | 15 | 9.6 | 2.3 |
| Digitonin | 8 | 0.6 | 117 | 15 | 7.5 | 7.2 | 3.1 | 1.7 |
| Thesit | 48 | 0.1 | 261 | 33 | 10 | 39 | 11 | 5.8 |
| Tween 20 | 1 | 0.1 | 99 | 27 | 5.3 | 15 | 9.3 | 2.0 |
| Triton X-100 | 1 | 0.2 | 198 | 31 | 4.0 | 28 | 19 | 1.5 |
| Gal 12 Ac | 20 | n.k[c] | 81 | 24 | 3.3 | 13 | 11 | 1.3 |
| Mgal 12 OH | 26 | n.k[c] | 99 | 18 | 6.1 | 29 | 27 | 1.1 |
| Mgal 12 Ac | 36 | n.k[c] | 99 | 26 | 4.2 | 15 | 12 | 1.3 |

[a] CMC = critical micelle concentration. Values were obtained from [114]

[b] The specific activity is represented in nmol $min^{-1}$ per mg protein

[c] n.k. = not known

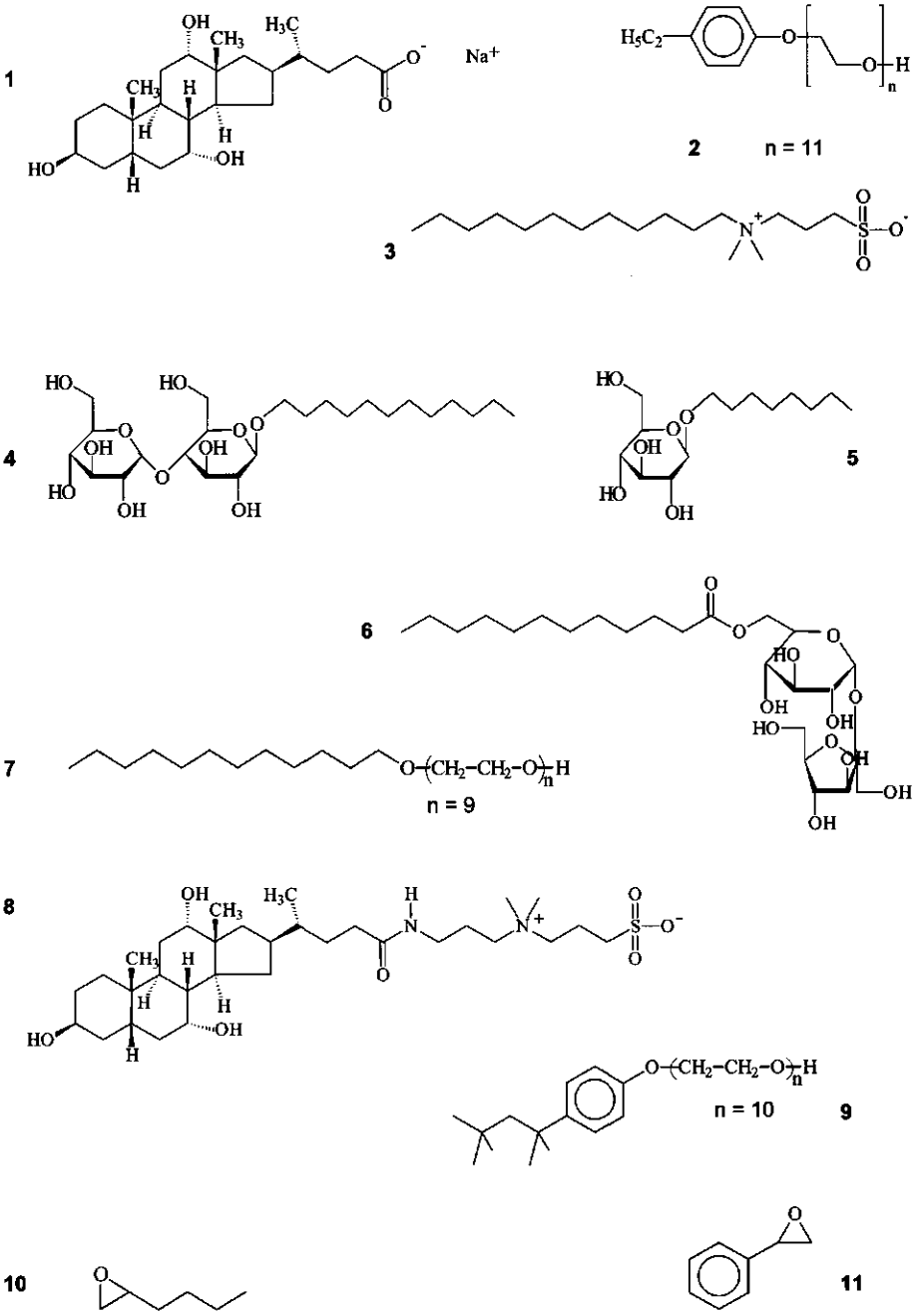


**FIG. 3.1. STRUCTURES OF SOME OF THE TESTED DETERGENTS AND THE TWO SUBSTRATES.**

1: Deoxycholate sodium; 2: Non-Idet P40; 3: Zwittergent 3-12; 4: n-Dodecylmaltoside; 5: n-Octylglucoside; 6: Sucrosemonolaurate; 7: Thesit; 8: CHAPS; 9: Triton X-100; 10: 1,2-epoxyhexane; 11: Styrene oxide

As an illustration, the reactioncurves for the hydrolysis of racemic 1,2-epoxyhexane with dialyzed enzyme material in the absence (Fig. 3.2A) and presence (Fig. 3.2B) of the detergent octylglucoside are shown. From these graphs it is clear that the *R*-enantiomer of the substrate is hydrolyzed faster in the presence of the detergent. Because the hydrolysis of the *R*-enantiomer proceeds at a much higher rate than the hydrolysis of the *S*-enantiomer, the E-ratio is positively influenced. These curves were more or less the same for the other detergents. In Fig. 3.2C, the effect of the addition of detergent on the substrate-concentration in the gas phase is shown. Heat inactivated enzyme and 10 mM racemic 1,2-epoxyhexane were added to the reaction buffer and incubated with and without the detergents octylglucoside, Thesit or sucrosemonolaurate. There is no difference between both enantiomers in the vapor phase, whether a detergent is added or not. Also, there is no difference in peak area between the vapor and liquid phase, indicating no reduction of the partial vapor pressure of the substrate (curve not shown). Similar effects were found for the three detergents tested (results not shown).

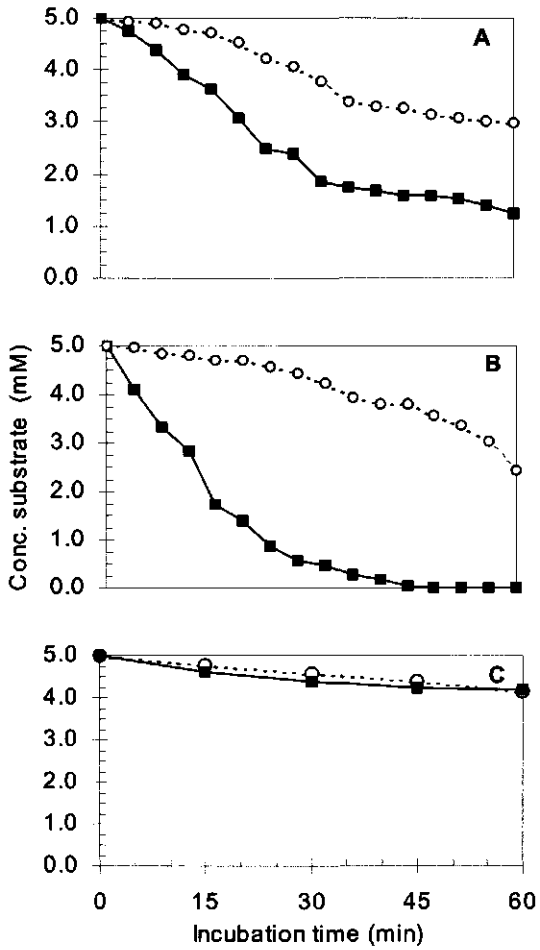


**FIG. 3.2. REACTIONCURVES OF HYDROLYSIS OF 10 MM RACEMIC 1,2-EPOXYHEXANE**

A: Blank (dialyzed enzyme);
B: Detergent (dialyzed enzyme with addition of 30 mM octylglucoside);
C: control (heat inactivated enzyme, no detergent).

—■— : concentration of (*R*)-1,2-epoxyhexane;
---○--- : concentration of (*S*)-1,2-epoxyhexane.

### 3.3.2. EFFECT OF DETERGENT CONCENTRATION ON THE REACTION RATE AND ENANTIOSELECTIVITY

Different amounts of detergents were added to the dialyzed material in order to test whether the increase of the reaction rate and enantiomeric ratio was concentration-dependent. Thesit and sucrosemonolaurate were chosen because these detergents had the largest effect on reaction rate and/or enantioselectivity. (*R,S*)-1,2-epoxyhexane was used as a substrate because the reaction rate was high in comparison with styrene oxide. In Fig. 3.3A and 3.3B it is shown that the hydrolysis rate is high when the concentration of the detergents is significant higher than the critical micellar concentration (CMC, indicated with arrow in Fig. 3.3). Also the enantiomeric ratio has its optimum above the CMC, but it is not at the same concentrations as the specific activity. The maximum specific activity of the enzyme was in the presence of 50 mM Thesit (196 nmol $min^{-1}$ $mg^{-1}$ protein), while the maximum enantiomeric ratio was at 100 mM Thesit (E = 22). The results with sucrosemonolaurate were similar: the maximum specific activity is at 50 mM sucrosemonolaurate (160 nmol $min^{-1}$ $mg^{-1}$ protein) and the maximum enantiomeric ratio is at 125 mM (E = 43). The first maximum in E-ratio is at the same concentration as the maximum in specific activity. However, there is an unexplainable drop in the E-ratio between 50 and 125 mM. This drop results in the second maximum in E-ratio at 125 mM.

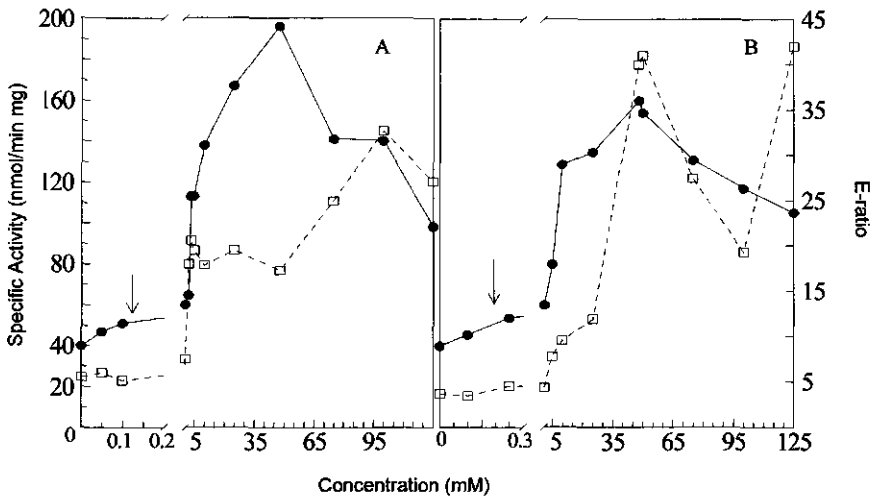


**FIG. 3.3. EFFECT OF THE CONCENTRATION OF THESIT (A) OR SUCROSEMONOLAURATE (B) ON THE SPECIFIC ACTIVITY (●) AND ENANTIOMERIC RATIO (E) (□) OF EPOXIDE HYDROLASE.**
All activities were determined from at least 2 independent duplo measurements from the decrease of (*R*)-1,2-epoxyhexane. The arrow indicates the CMC (critical micelle concentration): 0.1 and 0.2 mM resp.

## 3.4. DISCUSSION

Recently, the purification of the epoxide hydrolase from *R. glutinis* was described [63]. This enzyme is a membrane-associated enzyme that could be solubilized from the membranes by a detergent. This solubilization had both positive effects on the specific activity and the enantiomeric ratio (E-ratio). In this report, we describe the change in specific activities and enantioselectivity of the EH in crude extracts by adding of a range of detergents.

### 3.4.1. ENZYME PREPARATION

The CFE and the fractions of the crude preparation (S1, S2, P1 and P2) after cell breakage have lower values for the E-ratio compared with whole cells because the EH was freed from the membranes. Lack of a protective environment causes lower activity and enantioselectivity, but addition of detergents can help to stabilize the enzyme by serving as artificial membrane structures [25, 66]. Oesch & Daly [92] and Bentley & Oesch [9] already observed the increased activity, but the increase of the E-ratio of EH by using a detergent is new. It is, however, not new, as for i.e. lipases also the E-ratio was affected by detergents [149]. However, these authors had used anionic detergents in combination with organic solvents. The detergents are not capable of completely mimicking the membranes since the E-ratio in the fraction S2 is lower than in whole cells (E = 84, Weijers *et al.* [143]).

### 3.4.2. EFFECT OF VARIOUS DETERGENTS ON SPECIFIC ACTIVITY AND ENANTIOMERIC RATIO

Fifteen other detergents were added to the crude preparation to further investigate the effects mentioned above. Activities were measured by the decrease of 1,2-epoxyhexane and styrene oxide. Two different substrates were chosen to investigate whether the change in the E-ratio and/or specific activity by the detergents is dependent on the structure of the substrates, as has been found for lipases [149]. Removing the detergent by dialyzing fraction S2 showed a decrease in the specific activity and E-ratio. These decreases were less when 10% glycerol was added to both sample and dialysis buffer, indicating a stabilizing effect. Re-introducing detergents to the samples resulted in an increase in specific activity. Bentley & Oesch [9] found an apparent activation of 30-40% when styrene oxide was used as a substrate. We found activations between 50% (digitonin) and 800% (Thesit), and the detergent thus clearly influences the conversion rate of a substrate. Most non-ionic and zwitterionic detergents gave positive results, except Non-idet P40, Tween 20 and Gal 12Ac. They did not affect the conversion rates either positively or negatively. The anionic detergents decreased the specific activity for both substrates, probably caused by the negative charge of these detergents.

According to Zou *et al.* [150] a part of the EH from *A. niger* (amino acids 250-283) forms a lid above the active site of the enzyme (formed by mostly C-terminal residues). The catalytic His is located near the lid in the enzyme. Based on amino acid

sequence homology between the epoxide hydrolase from *A. niger* and *R. glutinis* [137], it is concluded that they are quite closely related. If the charges of the detergent are large enough, they can affect this histidine in the active site. The histidine is essential, because it activates the water-molecule that will be added to the epoxide to form the diol. When an anionic detergent attracts this histidine, the His can move away from the active site and a conformation change of the enzyme will occur. Water can still be activated, but not close to the active site and thus not specific, resulting in non-selective hydrolysis [150]. The zwitterionic detergents, although charged but with the net charge zero, have in this way little effect on the conformation of the enzyme.

The results suggest that the specific activity of only the *R*-enantiomer and the E-ratio are not interdependent: a rise in specific activity of the *R*-enantiomer does not result in a comparable rise in E-ratio. And removal of the detergent decreased the E-ratio while the specific activity of the *R*-enantiomer remained the same. However, when the activity of the *S*-enantiomer is considered, the results can be better explained. Because the specific activity of the *S*-enantiomer is mostly not affected by the addition of detergents, in some cases there is also an increase in activity, leading to a lesser increase in E-ratio (e.g. for the zwitterionic detergents). In the case of sucrosemonolaurate, the very high E-ratio can be explained by the decrease in specific activity of the *S*-enantiomer.

Whether the increase in reaction rate and E-ratio by Thesit, n-dodecylmaltoside and sucrosemonolaurate is caused by the fact that these detergents are themselves optically active, is not known. It is possible that the effects observed are related structurally. Structures of detergents that had a considerable effect on both activity and enantioselectivity are presented in Fig. 3.1. Also, there is a substrate-dependent effect. Detergents did affect the E-ratio if 1,2-epoxyhexane was the substrate while with styrene oxide these effects were less pronounced. Because both substrates are themselves hydrophobic (log $P_{o/w}$= 1.9 for 1,2-epoxyhexane and 1.6 for styrene oxide), it is likely that they are incorporated in the micelles and this can influence the hydrolysis rate or the enantioselectivity. Substrate concentrations were measured in the vapor phase and the liquid phase. The concentrations were determined by peakareas of the GC-analysis. During time, the peakareas in vapor and liquid phases were comparable and remained the same, although there was a slight overall decrease (Fig. 3.2C). When substrates are incorporated into micelles, one might expect a difference in peakarea between vapor and liquid phase. Since we found no significant difference, incorporation of substrates in micelles is not likely to occur. However, it is possible by our extracting method, that we also extracted epoxides from the micelles, indicating an easy removal from the micelles. In this way, the reaction rate or selectivity is not highly influenced.

### 3.4.2. Effect of detergent concentration on the reaction rate and enantioselectivity

The concentration of the detergent influences both the specific activity and enantioselectivity (Fig. 3). When the concentration is above the CMC, the specific

activity and E-ratio increase. The formation of micelles has a positive effect on these parameters. Above the CMC, there is a distinct rise in specific activity and E-ratio. For Thesit, the specific activity still increases, but the E-ratio remains constant until the specific activity begins to decrease. This effect might be explained that the detergent influences both the enantiomers of the substrate at different concentrations. At higher detergent concentrations, the specific activity towards the *S*-enantiomer might be more decreased than the specific activity towards the *R*-enantiomer, resulting in this higher E-ratio.

### 3.4.3. In conclusion

Detergents can cause positive effects on both specific activity and enantioselectivity of the epoxide hydrolase from *R. glutinis*. There seems to be a correlation between the effects of the detergents and the substrates used. Effects with an aliphatic substrate and a certain detergent are sometimes opposite to the effects accomplished with an aryl substrate. Choosing a specific combination detergent/substrate can create an optimal system.

# CHAPTER 4

## 1,2-EPOXYHEXANE APPLIED AS A SECOND PHASE AND ITS EFFECTS ON ACTIVITY AND ENANTIOSELECTIVITY OF EPOXIDE HYDROLASE FROM *R. GLUTINIS*

**ABSTRACT**

The membrane-associated epoxide hydrolase (EH), as cell-free extract as well as whole cells from yeast *Rhodotorula glutinis*, was investigated for the enantioselective hydrolysis of 1,2-epoxyhexane. In particular, the effect of increasing epoxide amounts (up to 10 mmol per 10 mL of water, leading to phase separations) on both the activity and enantioselectivity has been studied, including the effect of detergents on such two-phase enzymatic conversions. It appeared that cell-free extracts without detergents gave the highest activity at 10 mmol epoxide per 10 mL of water added, without loss of enantioselectivity as compared to 1 mmol epoxide per 10 mL of water emulsions ($E \approx 3$). Homogeneous epoxide solutions (0.1 mmol per 10 mL of water), however, are preferred for high enantioselectivities ($E \approx 30$) of the (R)-enantiomer.

## 4.1. INTRODUCTION

Enantiomerically pure epoxides are important building blocks in the industry for the production of pharmaceuticals or agrochemicals [23, 78]. There are different ways to produce these epoxides and nowadays most industries are searching for environmentally friendly and cheap processes ('green chemistry') to produce these intermediates. [23, 78]. Epoxide hydrolases (EH), omnipresent in nature [11, 48, 93, 140] are attractive tools for the biocatalyzed hydrolytic kinetic resolution because they enantioselectively convert racemic epoxides in only water as solvent and reactant, without the use of any cofactors.

Both the reaction rate and the enantioselectivity of EH are strongly dependent on both the epoxide substrate and the presence of detergents (Chapter 3). Another observation was the ability of the EH to convert efficiently amounts of 1 mmol epoxide per 10 mL of water [21, 22]. However, at industrially relevant substrate concentrations (up to 10 mmol epoxide per 10 mL of water) product inhibition of the EH seemed to occur.

In this chapter, the effects of the 1,2-epoxyhexane amount and of detergents were studied with enzyme preparations in *R. glutinis* cells as well as in recombinant *Escherichia coli*. Visser et al. have recently cloned and over-expressed the EH from *R. glutinis* in *E. coli* [136]. The specific activity of this recombinant *E. coli* EH was at least 200 times higher than the activity in *R. glutinis*.

Three different 1,2-epoxyhexane amounts per 10 mL of water were investigated: 0.1 mmol (epoxide completely dissolved); 1 mmol (above the solubility of the epoxide, resulting in an emulsion) and 10 mmol (epoxide is present as a clearly visible second phase).

## 4.2. MATERIAL AND METHODS

### 4.2.1. CHEMICALS

DTT (Dithiotreitol), Thesit, sucrose monolaurate (SML) and n-octyl β-D-glucoside (OG) were purchased from Roche. (±)-1,2-epoxyhexane and (±)-1,2-hexanediol were products of Aldrich Chemical Co. Glycerol (87%) was obtained from Fluka. EDTA was a product of Acros. BSA was purchased from Sigma. IPTG was obtained from Diagnostic Chemicals Limited, UK.

### 4.2.2. PREPARATION OF ENZYME SAMPLES FROM *R. GLUTINIS*

The yeast *R. glutinis* strain ATCC 201718 (previously described as strain CIMW 147) was cultivated and harvested as described in Chapter 2. It was grown on mineral salts medium supplemented with 0.2% (w/v) yeast extract and 1% (w/v) glucose with 600 mL medium in 5 L flasks [140]. After 2 days shaking at 30°C, the cells were harvested by centrifugation at 10,000 *g* for 10 min, washed with 50 mM potassium phosphate buffer pH 7.0 (Buffer A), concentrated and stored at –20°C. A partially purified enzyme extract was prepared as described in Chapter 3. The buffer used

throughout the experiments was Buffer B (= Buffer A supplemented with 1 mM EDTA, 1 mM DTT). 10% $^{v}/_{v}$ Glycerol was added for stabilization during the storage at –20°C.

Cell free extracts (CFE) were prepared as follows: 5 mL cell suspension (approx. density 1.1 g cells $mL^{-1}$) was diluted with 2 mL Buffer B. The cells were broken by a beadmill (Retsch MM 2000) for 15 min at 100% amplitude with glass beads of 1-1.5 mm in diameter (50% of the total volume were beads). Unbroken cells and debris were removed by centrifugation (10,000 *g*, 8 min, 4°C) and the remaining supernatant was used as CFE.

The CFE was centrifuged at 20,000 *g* for 1 h at 4°C. In previous work, the CFE was centrifuged at 100.000 *g*. Due to technical problems, the CFE had to be centrifuged at a lower speed (20.000 *g*), which was still sufficient to separate the, apparently, large membrane sections with EH-activity from the cytosolic fraction. The obtained supernatant (S1), containing the cytosolic fraction of the cells, was discarded. The pellet (P1) was resuspended in a volume equal to the first supernatant in Buffer B. Solid n-octyl β-D-glucoside (OG) was added to the suspension to give a final concentration of 30 mM. The suspension was incubated for 5 min at 37°C, followed by centrifugation (20,000 *g*, 1h, 4°C) and the resulting supernatant (S2) now contained solubilized membrane proteins. Supernatant S2 was dialyzed overnight against 2 changes of Buffer B (10 L). The dialyzed material (S3) contained 3.2 mg protein per mL and aliquots of 10 mL were stored at –20° C until further use.

### 4.2.3. Preparation of recombinant *E. coli* cells

Recombinant cells of *Escherichia coli* BL21(DE3)*Rg*Eph1 [136] were taken from an LB agar plate supplemented with kanamycin (50 μg $mL^{-1}$), and used to inoculate 50 mL LB medium in a 250 mL flask (37°C, 200 rpm). Ten mL of this pre-culture was used to inoculate a 200 mL culture. After growing for 3 hours at 37°C, (until the $OD_{600}$ reached 0.5-1), the *EPH1* gene expression was induced by adding 1 mM IPTG. After an additional 4 hours of incubation at 21°C, the cells were harvested by centrifugation at 10.000 *g* for 10 min and washed with Buffer A. The cells were resuspended in 7 mL of Buffer B. This concentrated cell suspension was stored at –20°C.

The higher growth temperature is for obtaining a large amount of cells in a relatively short time. The induction took place at a lower temperature to avoid the formation of inclusion bodies [134].

### 4.2.4. Enzyme activity measurements

To determine enzyme activities, S3 (the dialyzed second supernatant) was diluted twice with Buffer B and amounts were added to glass bottles of 60 mL to give a final volume of 10 mL (after addition of substrates). The bottles were closed with caps with rubber septa. After 10 minutes preincubating at 35°C (in a water bath, shaking at 200 rpm), (±)-1,2-epoxyhexane was added to the incubation mixture. in amounts of 12 μL (0.1 mmol), 120 μL (1 mmol) or 1.2 mL (10 mmol). No concentrations are given because 1 and 10 mmol epoxide per 10 mL of water is above the maximum solubility of the epoxide in the aqueous phase (approx. 0.6 mmol in 10 mL).

The reaction was followed in time by taking liquid samples (0.5 mL) that were saturated with 0.3 g NaCl and extracted with 0.8 mL ethyl acetate. After 10 s. of centrifugation (phase separation), the ethyl acetate phase was transferred to vials and analyzed by chiral GC. The samples with high epoxide concentrations (10 mmol added) were diluted ten times with ethyl acetate to avoid bad peak resolution on the GC-column). All samples were measured in at least duplicates and the standard errors were about ± 5%.

The specific enzyme activities were calculated from the initial reaction rates, determined from the epoxide disappearance. The diol formation could not be determined quantitatively since only partial extraction from the aqueous into the ethyl acetate phase takes place. As no by-products are formed in the epoxide hydrolysis reaction, 1,2-diol formation is set equal to epoxide disappearance.

To determine the effect of detergents OG (33 mM), Thesit or SML (both 45 mM) were added to the reaction buffer and preincubated for 10 min. These concentrations were chosen on the basis of previous experiments (see Chapter 3).

Besides the above mentioned enzyme preparations, the incubations were also performed with whole cells of *R. glutinis* and the recombinant *E. coli* strain. Cells of *R. glutinis* were diluted to give final protein concentrations of 10 mg $mL^{-1}$. Cells of recombinant *E. coli* were diluted to give a final protein concentration of 0.4 mg $mL^{-1}$.

### 4.2.5. Calculation of the enantiomeric ratio (E-value) and %ee

Equation (4.1) [85] is used to calculate the enantiomeric ratio (E), 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 $t = t_x$ (from duplicate measurements).

$$E = [\ln (R_0/R)] / [\ln (S_0/S)] \qquad \text{Eq. 4.1}$$

Plotting ln $(R_0/R)$ versus ln $(S_0/S)$ gives a straight line with a slope that represents the E-value [61, 85].

The enantiomeric excess (%ee) for the formed diols were calculated according to the following formula:

$$\%ee = \{ ([Rdiol]_t - [Sdiol]_t) / ([Rdiol]_t + [Sdiol]_t) \} \times 100 \qquad \text{Eq. 4.2}$$

In this formula, $[Rdiol]_t$ and $[Sdiol]_t$ are the diol concentrations at time = t. Although the absolute diol concentrations could not be determined (see above), the relative *R*/*S* concentrations of the diol fraction extracted are still valid.

### 4.2.6. ANALYTICAL METHODS

The protein concentration was determined by the Bio-Rad *DC* Protein Assay (Bio-Rad Laboratories BV, Veenendaal, The Netherlands), with BSA as standard. Gas chromatography (GC) was performed on a HP6890 gaschromatograph equipped with a FID detector with an autosampler using $N_2$ as carrier gas. Determination of the enantiomeric excesses was performed by chiral GC using a fused silica cyclodextrin capillary β-DEX 120 column (30m length, 0.25 mm ID and 0.25 mm film thickness, Supelco Inc., Zwijndrecht, The Netherlands). This analysis was done at oven temperatures of 47°C for (±)-1,2-epoxyhexane and 125°C for (±)-1,2-hexanediol.

To determine the absolute epoxide concentrations, calibration curves of 1,2-epoxyhexane and 1,2-hexanediol were made with heat-inactivated enzyme samples in buffer with or without detergents.

The high epoxide amounts (1 and 10 mmol epoxide per 10 mL of water) formed an emulsion in the buffer with the yeast cells. Before taking a sample, the bottles were put upside down to demulsify the sample. Samples were taken through the septum of the cap and by putting the bottles upside down, which prevented taking pure epoxide instead of the dissolved epoxide in the reaction mixture.

## 4.3. RESULTS AND DISCUSSION

The results of the enzymatic hydrolysis of 1,2-epoxyhexane are summarized in Table 4.1. The data are based on the corresponding time to conversion measurements (A-F) as presented in Figure 4.1.

The first column of Table 4.1 gives the different enzyme samples used, i.e. whole cells of *R. glutinis*, the dialyzed second supernatant (twice diluted, denoted S3), both without and with detergents, and the cells of the recombinant *E. coli*. The second column shows the added amount of epoxide in mmol per 10 mL of water instead of concentrations, because 1 and 10 mmol added to 10 mL buffer-incubation mixture exceeds the maximum solubility of the epoxide. The third column shows the specific activities of the hydrolysis of (*R*)-1,2-epoxyhexane together with the E-ratio of this reaction. As a practical measure the % ee of the formed (*R*)-1,2-hexanediol are given at an epoxide conversion of 40%. This conversion is chosen because the epoxide conversion must be kept below 50% to obtain a high enantioselectivity together with a reasonable yield of (*R*)-1,2-hexanediol.

**TABLE 4.1: EFFECT OF 1,2-EPOXYHEXANE AMOUNTS AND DETERGENTS ON THE ENZYMATIC HYDROLYSIS RATE OF EPOXIDE HYDROLASE**

| Time course graph of Figure 4.1 | Enzyme Sample | Amount of epoxide (mmol per 10 mL of water) | (*R*)-epoxide hydrolysis | | % ee (*R*-diol) (at 40% conversion) |
|---|---|---|---|---|---|
| | | | Spec. Act. (μmol/min, mg) | E-ratio | |
| A 1 | *R. glutinis* | 0.1 | 0.11 | 30 | 92 |
| A 2 | | 1 | 0.30 | 6.1 | 69 |
| A 3 | | 10 | 0.34 | 4.4 | 52 |
| B 1 | S3[a] | 0.1 | 0.09 | 3.7 | 46 |
| B 2 | | 1 | 0.83 | 2.4 | 35[b] |
| B 3 | | 10 | 2.44 | 2.9 | 39 |
| C 1 | S3[a] + OG | 0.1 | 0.20 | 13 | 81 |
| C 2 | | 1 | 1.00 | 3.2 | 45 |
| C 3 | | 10 | 0.82 | 2.9 | 44 |
| D 1 | S3[a] + Thesit | 0.1 | 0.26 | 10 | 62 |
| D 2 | | 1 | 1.09 | 4.9 | 65 |
| D 3 | | 10 | 1.24 | 4.3 | 58 |
| E 1 | S3[a] + SML | 0.1 | 0.20 | 40 | 71 |
| E 2 | | 1 | 0.75 | 3.9 | 56 |
| F 1 | *E. coli* RgEH1 | 0.1 | 3.30 | 24 | 95 |
| F 2 | | 1 | 9.02 | 17 | 92 |

[a]S3 is the twice diluted second supernatant after overnight dialysis (no detergent present). The additions mentioned are 33 mM n-octyl β-D-glucoside (OG), 45 mM Thesit (Thesit) and 45 mM sucrose monolaurate (SML).

[b] 40% conversion was not yet reached at the end of the incubation. The % ee of the diol is therefore estimated by extrapolation.

The data show that there is a significant increase of the specific hydrolysis rates upon increasing the epoxide amounts from 0.1 to 1 mmol per 10 mL of water. However, further increase of the epoxide amount to 10 mmol, in which there is a distinct epoxide layer present, does not show a further increase of reaction rate in the case of whole cells or when detergents are added to the supernatant. Remarkably, S3 (dialyzed, diluted second supernatant) without any detergent gave the highest conversion rate at an epoxide amount of 10 mmol per 10 mL of water, i.e. at the industrially interesting two-phase 10% w/w epoxide/water system. Clearly, a distinct secondary epoxide phase mimicks the cell-membrane's apolar phase without serious diffusion limitations. The much higher rate of hydrolysis of 1,2-epoxyhexane (30 times) with whole cells of recombinant *E. coli* is in accordance with the approximately 25 times higher protein content as compared with whole cell and supernatant samples of *R. glutinis* . So, no principal difference is observed, except the better enantioselectivity of the *E. coli* species at 1 mmol per 10 mL of water epoxide amounts.

Conversion graphs of the 1,2-epoxyhexane hydrolyses (Figure 4.1) did not show serious product inhibition effects as found by Choi et al. at high product (diol) concentrations [22]. They showed that in the presence of (extra added) 50 mM diol, the diol formation rate (and thus the epoxide hydrolysis rate) decreased. As a possible explanation for the lower diol production rate, Choi et al. stated that diols at high concentrations are toxic to the yeast cells. The hydrophobic diols enters the hydrophobic cell membrane, thus destroying the structure of the membrane and possibly affecting the EH-activity since the EH is a membrane-associated enzyme [22].

Although the results of Chapter 3 showed that the addition of detergents (in concentrations where micelles are formed) gave an improvement in both specific activity and enantioselectivity, the combination of detergents and a second epoxide phase had no additional positive effects (Figure 4.1, graphs D, E and F). Both hydrolysis rate and enantioselectivity were worse when higher (> 1 mmol per 10 mL of water) amounts of epoxides were added to the incubation mixture, compared to the situation when the epoxide is completely dissolved in the mixture (0.1 mmol per 10 mL of water).

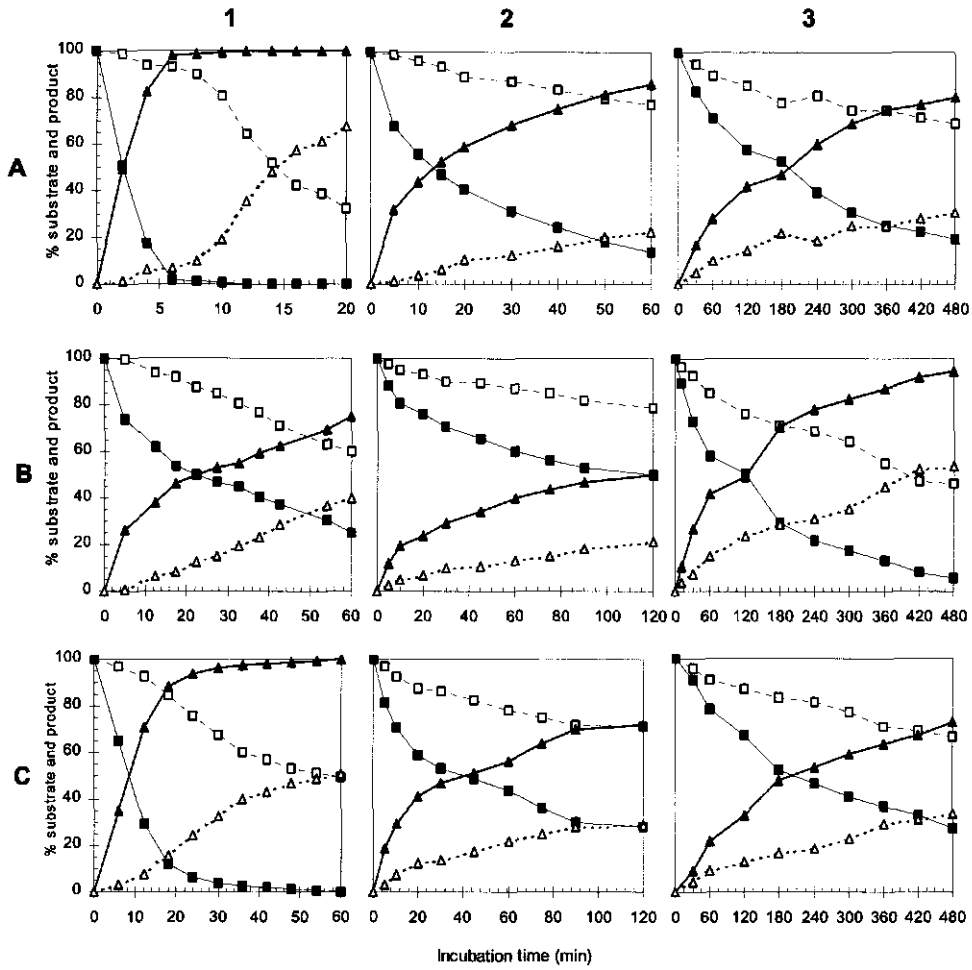


**FIG. 4.1. TIME COURSE OF ENZYMATIC 1,2-EPOXYHEXANE HYDROLYSIS AND 1,2-HEXANEDIOL PRODUCTION**

**A:** Whole cells of *Rhodotorula glutinis*; **B**: *Rhodotorula glutinis* dialyzed second supernatant (twice diluted) (S3) without detergent; **C**: Dialyzed second supernatant (twice diluted) (S3) with 33 mM n-octyl β-D-glucoside.

**1, 2, 3**: 0.1, 1 and 10 mmol of 1,2-epoxyhexane respectively.

The closed squares (■) represent the *R*-epoxide, the open squares (□) the *S*-epoxide. The closed triangles (▲) represent the *R*-diol and the open triangles (Δ) the *S*-diol.

On the y-axis, the percentage hydrolyzed epoxide and formed diol (calculated from the epoxide disappearance as described in 4.2.4) are shown. On the x-axis, the total incubation time (in min) is shown.

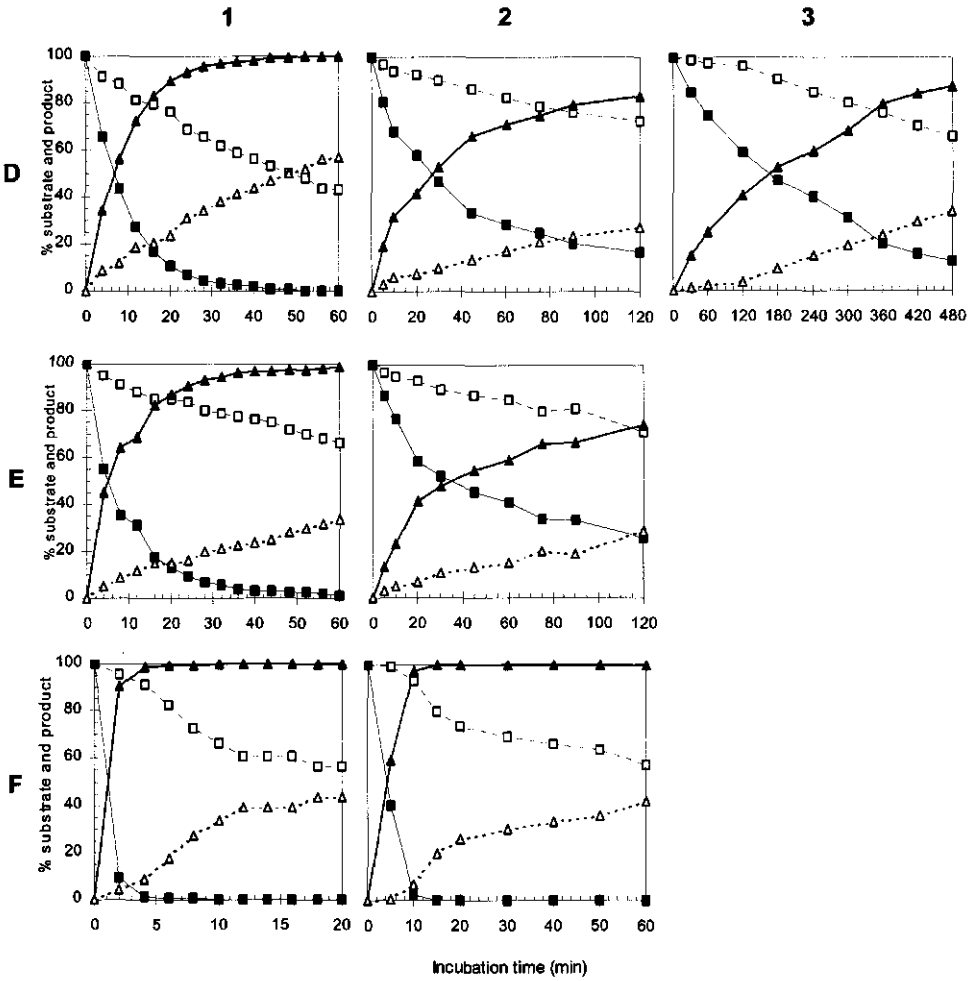


**FIG. 4.1 (CONTINUED). TIME COURSE OF ENZYMATIC 1,2-EPOXYHEXANE HYDROLYSIS AND 1,2-HEXANEDIOL PRODUCTION**

**D**: S3 with 45 mM Thesit; **E**: S3 with 45 mM sucrose monolaurate (SML); **F**: Whole cells of recombinant *E. coli*.

**1, 2, 3**: Different amounts of epoxide added: 0.1, 1 and 10 mmol respectively.

The closed squares (■) represent the *R*-epoxide, the open squares (□) the *S*-epoxide. The closed triangles (▲) represent the *R*-diol and the open triangles (△) the *S*-diol.

On the y-axis, the percentage hydrolyzed epoxide and formed diol (calculated from the epoxide disappearance as described in 4.2.4) are shown. On the x-axis, the total incubation time (in min) is shown.

## 4.4. CONCLUSIONS

Lab-scale synthesis of enantiopure (*R*)-1,2-epoxyhexane in amounts of 0.1 mmol (per 10 mL incubation volume) can be satisfactorily carried out using whole *R. glutinis* cells or the supernatant thereof together with a detergent. These enzymatic conversions showed moderate E-ratio's, which allow the preparation of both (*R*)-1,2-epoxyhexane and (*R*)-1,2-hexanediol. Amounts that are industrially interesting (10 mmol per 10 mL incubation volume, *i.e.* a 10% w/w epoxide/water two-phase system), should be prepared with the supernatant of *R. glutinis* cells without any detergent. The epoxide in a second phase seems to stabilize the enzyme. Former positive effects of detergents are not present anymore when a second phase is available. In addition, the absence of a detergent is a big advantage for easy phase separation and isolation of the reaction products.

Higher (30 times) enzyme activities per weight of total protein are achieved by recombinant *E. coli* cells and are the best choice for industrial scale conversions using the above-mentioned two-phase epoxide-water system. The decrease in E-ratio (enantioselectivity) is less than that for whole cells of *R. glutinis.*

# CHAPTER 5

# IMPROVEMENT OF ENANTIOSELECTIVITY BY IMMOBILIZED IMPRINTING OF EPOXIDE HYDROLASE FROM *RHODOTORULA GLUTINIS*

**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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## 5.1. 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 [11, 66, 84, 94, 139]. Epoxide hydrolases (EHs) are attractive tools in biocatalysis because 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 [103] is able to achieve all these aspects at once. First, the substrateselectivity or enantioselectivity of the enzyme is rationally modified by "bio-imprinting" [34, 103, 127] 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 [34, 103, 127] but most conversions took place in organic solvents only [55].

The clue in immobilized imprinting of proteins is that the manipulated protein conformation is fixed by polymerizing it in a water-free organic solvent [103]. 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. 5.1) [30]. 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 [138]. In this way it is easy to recycle the enzyme.

Recently, the EH from *R. glutinis* was purified [63], 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 chapter, we describe the process of immobilized imprinting of the EH resulting in a so-called CLIP-EH (**C**ross**L**inked **I**m**P**rinted epoxide hydrolase). 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, 106], this method is directly applicable to the protein on the protein level and it is a very rapid and easy method.

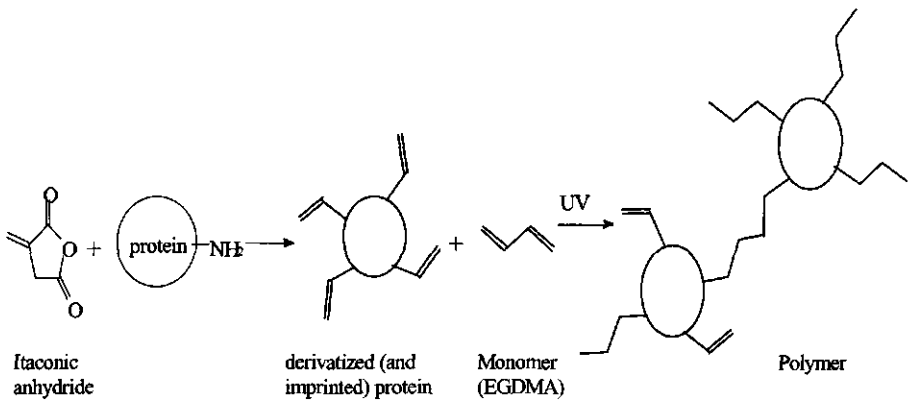


**FIG. 5.1. DERIVATIZATION AND POLYMERIZATION OF EPOXIDE HYDROLASE (EH)**
Before polymerization, the derivatized enzyme can be imprinted to obtain a more active or enantioselective biocatalyst (for details, see material and methods).

## 5.2. MATERIAL AND METHODS

### 5.2.1. CHEMICALS

DTT and n-octylglucoside were purchased from Roche. (±)-1,2-epoxyhexane, (±)-1,2-epoxyoctane and EGDMA were products of Aldrich Chemical Co. (*R*)- and (*S*)-1,2-epoxyoctane, waterfree cyclohexane, iso-propanol, 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.

### 5.2.2. PREPARATION OF ENZYME SAMPLES

The yeast *R. glutinis* strain CIMW 147 (ATCC 201718) was cultivated and harvested as described before [63]. A partially purified enzyme extract was prepared as described earlier [61]. 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 (1h, 100.000*g*, 4°C) and the membrane fraction was treated with 30 mM n-octylglucoside for 5 minutes at 37°C and again centrifuged. The supernatant was dialyzed overnight against two changes of working buffer (10 L) to remove the detergent.

### 5.2.3. Epoxide hydrolase acylation with itaconic anhydride

This method is derived from Fischer and Peißker [30]. 0.5 mL enzyme solution (5 mg $mL^{-1}$) 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 minutes (on ice). Non-reacted itaconic anhydride and other low-molecular-mass compounds were removed by gel filtration (using 4 PD-10 columns, Pharmacia) and the eluates of the columns were combined.

### 5.2.4. Imprinting of the derivatized protein

Imprinting molecules (5 mM final concentration) were added to the combined eluates (after PD-10 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 min, the samples were put on ice for 60 minutes. After centrifugation (15 min, 10.000 *g*), the precipitates were washed once with ice-cold isopropanol and after a second centrifugation step the precipitates were lyophilized.

### 5.2.5. 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 [38] and Hall *et al.* with TNBS [41]. The absorbance obtained with the native protein corresponded to 100% and the extent of modification was calculated according to Shetty and Kinsella (equation 5.1) [122]:

$$\text{Modification (\%)} = [1 - (A_{mod}/A_{nat})] \times 100\% \qquad \text{Eq. 5.1}$$

$A_{mod}$ 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 minutes 0.47 mL 1M 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.

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

To 0.5 mL cyclohexane (waterfree) 4 mg AIBN was added and mixed. 0.2 mL EGDMA (waterfree) was added to this mixture and mixed. 30 mg derivatized or imprinted protein (lyophilized) was added and suspended by ultrasonification

(waterbath, 30 minutes). 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 crosslinked enzyme was collected and finally lyophilized.

### 5.2.7. Enzyme activity measurements

To determine enzyme activities, the polymer was added to 1 mL of working buffer. Amounts (ranging from 10-30 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 minutes, 1-10 mM racemic substrate was added. The reaction was followed in time by taking headspace samples (100 µL) and analyzed by chiral GC. 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.

### 5.2.8. Calculation of the enantiomeric ratio (E-value)

Equation (5.2), used to calculate the enantiomeric ratio (E), was described by Morisseau *et al.* [1997]. $R_0$ 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).

$$E = [\ln (R_0/R)] / [\ln (S_0/S)] \qquad \text{Eq. 5.2}$$

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 [61, 85].

### 5.2.9. 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 minutes, 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 polymerbatch at 4°C. Each new incubation was started with a batch from the fridge.

### 5.2.10. Analytical methods

The protein concentration was determined by the method of Bradford, with BSA as standard [16]. Gas chromatography was performed on a Chrompack CP9000 gas chromatograph as described before [63]. 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.

## 5.3. RESULTS AND DISCUSSION

### 5.3.1. INFLUENCE OF ACYLATION AND CO-POLYMERIZATION ON THE CATALYTICAL PROPERTIES OF EPOXIDE HYDROLASE (EH)

In order to crosslink the epoxide hydrolase (EH) via radical polymerization in organic solvents, the enzyme had to be vinylated first (Fig. 5.1). Itaconic anhydride which reacts with free amino-, hydroxyl- or sulfhydryl groups of proteins [30], was chosen as acylating agent. Initial experiments had shown that the optimum ratio (mg/mg) of itaconic anhydride to protein was 11.8 to 1. Typically, the resulting degree of protein derivatization was about 70% according to the TNBS assay (see material and methods). 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 crosslinking 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 estimated for native EH, derivatized EH and crosslinked EH (Table 5.1). Derivatization did hardly influence the activity and the enantioselectivity of EH.

However, crosslinking 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.

**TABLE 5.1. THE INFLUENCE OF PROTEIN DERIVATIZATION AND CROSSLINKING ON RELATIVE TOTAL INITIAL ACTIVITY (%) AND ENANTIOSELECTIVITY (E-RATIO) OF EH**

| Substrate | Free | | Derivatized | | Derivatized and crosslinked | |
|---|---|---|---|---|---|---|
| | Rel. init. act. (%) | E-ratio | Rel. init. Act. (%) | E-ratio | Rel. init. Act. (%) | E-ratio |
| 1,2-epoxyhexane | 100[a] | 3.7 (*R*) | 73 | 3.4 (*R*) | 62 | 3.1 (*R*) |
| 1,2-epoxyoctane | 100[b] | 3.0 (*R*) | 85 | 3.0 (*R*) | 27 | 2.1 (*R*) |
| Styrene oxide | 100[c] | 1.2 (*R*) | 100[c] | 1.0 | 53 | 1.0 |

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

### 5.3.2. 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 crosslinking. 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 [103]. 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) [61].

It should be mentioned here that crosslinking 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 crosslinking 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. 5.2. (p. 59), the time course studies of the hydrolysis of (±)-1,2-epoxyoctane by CrossLinked, ImPrinted EH (CLIP-EH) and by just crosslinked 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 in inverted enantioselectivity when compared to **B** and **R**.

**TABLE 5.2. RELATIVE TOTAL INITIAL ACTIVITIES (%) AND E-RATIOS OF NON-IMPRINTED AND IMPRINTED EH-POLYMERS HYDROLYZING 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*)** |

Table 5.2 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 above (see Fig. 5.2., p. 59) 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 *Rhodotorula glutinis*, was succesfully imprinted in aqueous phase and subsequently copolymerized 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. 5.2 and Table 5.2). Polymerizing the derivatized EH as such did show only minor effects on the enantioselectivity (Table 5.1), clearly leading to the conclusion that the strong effects 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.

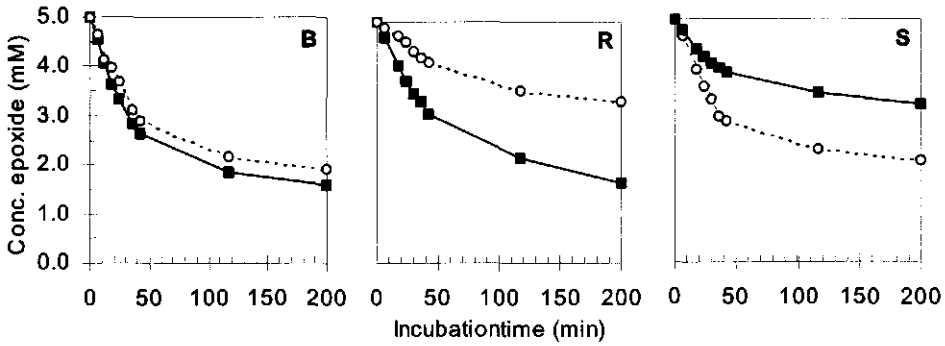


**FIG. 5.2. TIME COURSE STUDIES OF THE HYDROLYSIS OF (±)-1,2-EPOXYOCTANE USING NON-IMPRINTED AND IMPRINTED EH-POLYMERS**

**B**: Blank, without imprinting;
**R**: imprinted with (*R*)-1,2-epoxyoctane;
**S**: imprinted with (*S*)-1,2-epoxyoctane.

——■—— : (*R*)-enantiomer ----○---- : (*S*)-enantiomer

### 5.3.3. EFFECT OF DIFFERENT IMPRINTERS ON THE CATALYTICAL PROPERTIES OF EH-POLYMERS

As shown in Table 5.2, 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 5.3).

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.

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 hydrolyzing styrene oxide was 2.1 times better when compared with the blank (2.3 versus 1.1).

**TABLE 5.3. INFLUENCE OF VARIOUS IMPRINTERS ON THE TOTAL INITIAL ACTIVITY (%) AND ENANTIOSELECTIVITY (E-RATIO) OF NON-IMPRINTED AND IMPRINTED EH-POLYMERS**

| Substrate | Imprinter | Rel. total initial activity (%) | | E-ratio |
|---|---|---|---|---|
| | | *R* | *S* | |
| 1,2-epoxyhexane[a] | None | 100 | 100 | 3.1 (*R*) |
| | *R/S* | 106 | 89 | 3.3 (*R*) |
| 1,2-epoxyoctane[a] | 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*) |

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.

[a] Data presented were independently obtained from data in Table 5.2 (newly prepared EH-polymers)

### 5.3.4. 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 hydrolyze 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 5.4. 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 enantioselectivities 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.

**TABLE 5.4. OPERATIONAL STABILITY OF EH-POLYMERS (SUBSTRATE 1,2-EPOXYOCTANE)**

| Number of incubations | Imprinter | Initial specific activity (nmol $min^{-1}$ $mg^{-1}$) | | 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*)** |

The storage stability was tested in the time course of one 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 one week the remaining activities of the polymers were about 76% to 90%. The free enzyme almost completely lost its activity after four days when stored under the same conditions (without glycerol). The corresponding calculated half-lifes of the various EH preparations are presented in Table 5.5. 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.

**TABLE 5.5. HALF-LIFES OF NATIVE EH AND EH-POLYMERS (SUBSTRATE 1,2-EPOXYOCTANE)**

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

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 [106].

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, 106].

## 5.4. CONCLUSIONS

Immobilized protein imprinting was able to rationally modify the enantioselectivity of an EH. 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 [58]. 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. The increased stability is even more striking than the inversed enantioselectivity. However, more work has to be performed in order to optimize this procedure.

# CHAPTER 6

## CONCLUDING REMARKS, DISCUSSION AND RECOMMENDATIONS

## 6.1. MAIN GOALS OF THIS THESIS

The investigation of this thesis was carried out within the framework of the Innovation Oriented Research Program on Catalysis (as assigned by the Dutch Ministry of Economic Affairs). The objective was to develop clean biocatalytic routes to obtain optically active epoxides and diols by using kinetic resolution with yeast epoxide hydrolases. This project has both industrial and economic relevance because enantiopure epoxides are used as building blocks for the synthesis of a variety of pharmaceuticals. Chemical routes described, mostly yield racemic compounds together with relatively large amounts of waste products.

The main goal was to isolate and purify the epoxide hydrolase from the yeast *Rhodotorula glutinis*. We would subsequently characterize the purified enzyme and study the substrate specificity in comparison with other known epoxide hydrolases.

*1,2*-Epoxyhexane has been selected as a model substrate because hydrolysis of this epoxide was found to proceed with high enantioselectivity as well with high activity. In addition, the analysis of the enantiomers of *1,2*-epoxyhexane and its corresponding diol can easily be performed by chiral gas chromatography.

Finally, we wanted to develop an applicable separation method, in order to isolate the enantiopure epoxides and the diols.

## 6.2. EPOXIDE HYDROLASE FROM *RHODOTORULA GLUTINIS*

### 6.2.1. PURIFICATION AND CHARACTERIZATION

The EH of *R. glutinis* is a membrane-associated enzyme with a molecular weight of 46 kDa as based on SDS-PAGE (p. 26) as well as on its amino acid sequence (Fig. 6.1). At this moment, there is evidence that it is a homo-dimeric enzyme, as appeared from the approximately 80 kDa band in Superose-6 gelfitration, in contrast to an aggregation of detergent-protein as proposed by Thomas & McNamee, Lu *et al.* and Bentley & Oesch (see further below and paragraph 6.2.3.) [9, 74, 130]. Since the three-dimensional structure is not known yet, nothing more can be said about the way of association. The EH could be loosely associated with membranes, it could pass the membranes just once or even as much as six times [107]. In rats, the mEH 20 N-terminal amino acids function as a single membrane anchor signal sequence. This sequence is not involved in epoxide hydrolysis, because a truncated enzyme (the enzyme minus 20 amino acids of the N-terminal sequence) is as active as the non-truncated enzyme [32]. However, the N-terminal sequence of the RgEH does not predict a N-terminal membrane signal sequence. (cf. the sequence given in Fig. 6.1).

The EH from *Aspergillus niger* was also purified and characterized. This EH is soluble but also present as a dimeric enzyme [2, 84] with 29% identity to the primary structure of the *R. glutinis* EH. The N-terminal meander of the *A. niger* enzyme is involved in the formation of the homodimer [150]. Most probably, the N-terminal of the *R. glutinis* enzyme is also involved in this dimer formation (see also under 6.2.3).

The *R. glutinis* EH has an optimum temperature between 30 and 35°C. This range is in the vicinity of the optimum growth temperature of *Rhodotorula glutinis* (30°C). The pH optimum ranges from 7.5 to 9.0.

### 6.2.2. Improving the stability and enantioselectivity of the enzyme

Although the *R. glutinis* EH has no membrane-anchor, it is membrane-associated. High speed centrifugation of a cell free extract (CFE) showed that nearly all (75%) activity was found in the microsomal pellet (Table 3.1, p. 33). Solubilization of this pellet with detergents, followed by another high speed centrifugation step, resulted in a nearly complete solubilization of the EH in the supernatant. The increased enantioselectivity (E-ratio) of the EH containing supernatant will be the result of micelle-formation, as this enhancement was only seen at detergent concentrations above the CMC (critical micelle concentration). Non-ionic detergents showed the largest effects on both activity and enantioselectivity. For zwitterionic detergents the positive effects were less, whereas negatively charged detergents had a negative effect on both rate and enantioselectivity (Table 3.2. p. 34).

Apparently, a non-ionic detergent most closely resembles the enzyme's natural membrane lipid environment, resulting in stabilization of the enzyme.

Other stabilizing agents were found to be mannitol and glycerol. If 10% glycerol or 10% mannitol was added to the mixture before storage at –20°C, the activity and enantioselectivity remained stable for several weeks. This positive effect was not detectable when the enzyme-preparation was stored at 4°C. After a few days, the samples lost their activity completely.

A better way of stabilizing is to encapsulate the enzyme in a polymeric framework [62]. This enzyme-containing polymer could be stored at 4°C for at least two weeks without serious loss of activity (Table 5.5 p. 61) and could also easily be used to perform repetitive incubations (Table 5.4 p. 61). It may be noted that the immobilization method used by Kroutil et al. [65], stabilizing *Nocardia* EH, and Karboune et al. [51], stabilizing EH from *Aspergillus niger*, through ionic binding onto DEAE-cellulose could not be used as *R. glutinis* EH does not bind to the DEAE-cellulose material.

Apart from the stabilizing capabilities of the polymeric matrix, the active site of *R. glutinis* EH can be frozen in any desired conformation by adding appropriate imprinter molecules, leading to a reversal of enantioselectivity of the epoxide hydrolysis reactions (Fig. 5.2. p. 59).

### 6.2.3. Genetics

Recently, the complete nucleotide and deduced amino acid sequence of *Rhodotorula glutinis* epoxide hydrolase (*Rg*Eph1) was determined and they are given in Fig. 6.1. [137].

```
atggcgacacacacattcgcttcgcctcccacccgcttcaccgtcgacatcccacagtcggaactcgacgaa    72
 M  A  T  H  T  F  A  S  P  P  T  R  F  T  V  D  I  P  Q  S  E  L  D  E     24
cttcactcgcgactcgacaagacccgctggccggcgacagagatcgttccagaggatgggacggacgatccg   144
 L  H  S  R  L  D  K  T  R  W  P  A  T  E  I  V  P  E  D  G  T  D  D  P     48
acggcgtttgggctcggagcagggccgacgctgccgctcatgaaggaattggcgaagggttggcgcgagttc   216
 T  A  F  G  L  G  A  G  P  T  L  P  L  M  K  E  L  A  K  G  W  R  E  F     72
gactggaaaaaggcgcaggaccacctcaacacgtacgggggcttcccaccctctttcgccttgtagtctcgc   288
 D  W  K  K  A  Q  D  H  L  N  T[----------------intron 1----------------     83
tgacaggttctcataacccgcagcttcgagcactacatggtcgaaattgaggacctctcgatccacttcctc   360
----------------------]  F  E  H  Y  M  V  E  I  E  D  L  S  I  H  F  L     99
caccatcgctcgactcgcccgaacgctgttcccctcatcctctgccacggctggccaggccactttggcgag   432
 H  H  R  S  T  R  P  N  A  V  P  L  I  L  C  H  G  W  P  G  H  F  G  E    123
ttcctgaacgttatcccgctcttgacggagccgtcggacccctccgctcaggcgttccacgtcgtcgccgct   504
 F  L  N  V  I  P  L  L  T  E  P  S  D  P  S  A  Q  A  F  H  V  V  A  P    147
tcgatgcctggctatgcttggtctttgcctcctccgtcctccaagtggaacatgtgcgttcctttaattcaa   576
 S  M  P  G  Y  A  W  S  L  P  P  P  S  S  K  W  N  M[------------------    165
tcgtctcttctggcgtgactgatccatcccgtttgccaggcctgacaccgcgagggtcttcgacaagctcat   648
-----intron 2------------------------}  P  D  T  A  R  V  F  D  K  L  M    176
gaccgggcttggctacgagaagtgcgtctacccgttccgcgacgcttcttgagctttccccggacacgcaag   720
  T  G  L  G  Y  E  K[-----------intron 3-----------------------------     183
gcgatgctgatagtgcaactcggcaggtacatggcgcagggcggagactggggaagcatcgccgctcgctgc   792
-------------------------]  Y  M  A  Q  G  G  D  W  G  S  I  A  A  R  C    198
cttggatcgccgcacaaggaccattgcaaaggttcgtcccatcgggtcttcactcctcctcttcgtcttacc   864
 L  G  S  L  H  K  D  H  C  K  [--------------------intron 4------------    208
cttcacttgacgttaagcgctcgagctaatacctcgaagtcggcgcaacccgcagccgtccacctcaacttc   936
-----------------------------------------------------]A  V  H  L  N  F    214
ctccccgtcttcccacccgtcccgatgtggcttatcaacccgcacacgctccttgcctgggcaccgcgcttc  1008
 L  P  V  F  P  P  V  P  M  W  L  I  N  P  H  T  L  L  A  W  A  P  R  F    238
ctcgtgccggagaagcaggctgcgcgtatgaagcgcgggttggcgtaccttgagaaggggtgagtcgcagcg  1080
 L  V  P  E  K  Q  A  A  R  M  K  R  G  L  A  Y  L  E  K  G[------------    258
caggtctcgcgtttccgtagaaagtcctgacaggtcctcctcgtcttgcctcaacgcagctccgcctactac  1152
------intron 5-----------------------------------------}  S  A  Y  Y    262
gtcatgcagcagttgacggcaagctcagcgaagtctgcattgggaagcaaagctgaccctccgacgcccgca  1224
 V  M  Q  Q  L  T [-------------------intron 6-------------------------    268
gcctcgcacgcctgcgtacggcctgaccgacagtcccgtcggcttgctggcctggatcggcgagaaggtgcg  1296
]  P  R  T  P  A  Y  G  L  T  D  S  P  V  G  L  L  A  W  I  G  E  K [----  290
tcctttcctcatcagcacaagctcgaagctgattcgtgagcgtgcagttcgagccgaccattcaggaggcga  1368
------------------intron 7--------------------]  F  E  P  T  I  Q  E  A    298
gcaagcaagcccagccgacgtacgttctcgccagtagctgccgctcgccgtcttcgctaatgccccgctgcg  1440
S  K  Q  A  Q  P  T[-----------------intron 8----------------------------    305
cagcctgactcgcgacgagctctacttcacctgctcgctctactggttcagtgcgtcgactccgcacctccc  1512
--]  L  T  R  D  E  L  Y  F  T  C  S  L  Y  W  F  [---------------------    320
atcgtcgcgcccactgacactaagccgctcctcccaccacacagcccgctcaatcggcacctccttccttcc  1584
---------intron 9--------------------------]T  R  S  I  G  T  S  F  L  P    330
ctactcgctcaacccgcacttcaccaccttcctgaccgacagcaagtaccacctgcccaactttgccctctc  1656
  Y  S  L  N  P  H  F  T  T  F  L  T  D  S  K  Y  H  L  P  N  F  A  L  S    354
gctttacccaggcgagatctactgccccgccgagcgggacgccaagcgcaccggcaacctcaagtggatcaa  1728
  L  Y  P  G  E  I  Y  C  P  A  E  R  D  A  K  R  T  G  N  L  K  W  I  K    378
ggacgcgcctgagggaggacactttgctgcgctcgaaaagccggatgtgtttgtcgagcacctcagggaggc  1800
  D  A  P  E  G  G  H  F  A  A  L  E  K  P  D  V  F  V  E  H  L  R  E  A    402
gtttggcgtcatgtgggagaagtag                                                  1825
  F  G  V  M  W  E  K  *                                                     409
```

**FIG. 6.1. NUCLEOTIDE AND AMINO ACID SEQUENCE OF *RHODOTORULA GLUTINIS EPH1* (*RG*EPH1).** The *Rg*Eph1 nucleotide sequence is given in lower-case letters, the deduced amino acid sequence is given in the one-letter code. Introns have been numbered and are indicated in parentheses. An asterisk indicates the TAG stop codon. (from Visser et al. [137])

Based on this sequence, the molecular weight of the enzyme was calculated as 46,295 Da. This is in agreement with the earlier found molecular weight of approximately 46 kDa (p.26 and p. 64).

A comparison of this sequence with the partial amino acid sequence of the *R. glutinis* EH obtained earlier (P19) (Fig. 2.3. p. 28) and the sequences of mEH from both rabbit and rat is given in Fig. 6.2.

| | | | | |
|---|---|---|---|---|
| P19 | *1* | D A P E G **G H F** F A A L K | *19* | (Fig. 2.3) |
| RGEPH1 | *379* | D A P E G **G H F** - A A L E | *390* | (Fig. 6.3a) |
| RBMEH | *426* | Y M P R G **G H F** - A A F E | *437* | (Fig. 2.3) |
| RTMEH | *425* | Y M E R G **G H F** - A A F E | *436* | (Fig. 2.3) |

**FIG. 6.2. COMPARISON OF RGEHS AND RABBIT MEH (RBMEH) AND RAT MEH (RTMEH)**
In bold is the catalytic triad given.

Although the partial sequence found earlier (P19) was not completely correct, close similarities with other microsomal EHs are striking, especially the highly preserved GGHFAA motif near the C-terminal end of the enzyme [97]. The earlier found sequence has one F extra in this motif. The other partial sequences from chapter 2 were designated P16, P27, P29 and P30 (p. 27). These sequences were compared to the sequence given in Fig. 6.1 and summarized in Fig. 6.3. Since it is not always easy to assign the right amino acids, there are a few mistakes (indicated in bold). So all sequences found in Chapter 2 belong to the same enzyme: epoxide hydrolase.

| | | | |
|---|---|---|---|
| P16 | *1* | G P A Y G V M Q Q L T P | *12* |
| RGEPH1 | *258* | G S A Y Y V M Q Q L T P | *269* |
| P27 | *1* | Y H L H N F A | *7* |
| RGEPH1 | *346* | Y H L P N F A | *352* |
| P29 | *1* | W P A T E I V P E D Y T D | *13* |
| RGEPH1 | *34* | W P A T E I V P E D G T D | *46* |
| P30 | *2* | I G T S F L P V S L N P H F | *15* |
| RGEPH1 | *324* | I G T S F L P Y S L N P H F | *337* |

**FIG. 6.3. COMPARISON OF THE PARTIAL SEQUENCES AS FOUND IN CHAPTER 2 WITH THE RGEPH1**

The other mEH in Fig. 6.2. belong to the superfamily of α/β-hydrolase fold enzymes. The determined amino acid sequence contains enough evidence to confirm that the *R. glutinis* EH is also a member of this family. The catalytic triad of these α/β-hydrolase fold enzymes consists of a nucleophilic residue (Asp or Ser), an acidic residue (Asp or Glu) and a perfectly conserved histidyl (His) residue [97].

The nucleophile fits the conserved amino acid sequence motif sxnsxx, where s is a small amino acid, x any amino acid and n is the nucleophilic acid. This sequence is found in RgEPH1 in amino acids 188-193: GGDWGS with $D^{190}$ as the nucleophilic

Asp. It is also identical to the motif of the catalytic nucleophile of rat mEH (Arand et al. 1999). The catalytic acidic residue is found at $Glu^{359}$. This amino acid aligns with the rat mEH $Glu^{404}$.

However, no clear motifs are present for the catalytic triad and His-residues although this His is in mEHs situated in the amino acid sequence GGHFAA at the C-terminal end of the enzyme [69, 97]. In *R. glutinis* this highly preserved region is found at positions 383-388 near the C-terminus with the catalytic His at position 385. It resembles the rat mEH. The catalytic triad of the *R. glutinis* epoxide hydrolase consists therefore of $Asp^{190}$, $Glu^{359}$ and $His^{385}$.

A proposed mechanism based on the proposed mechanisms for other mEH is given in Fig. 6.4. The main difference of this mechanism compared with the known mechanism is the acidic residue. In the case of *R. glutinis* EH, this residue is Glu instead of Asp [70].

Another conserved amino acid sequence is the HGXP-motif containing the oxy-anion hole of the enzyme [69, 97]. In mammalian and plant sEHs, the X is a Phe-residue, whereas in mEHs and the bacterial sEHs, the X is a Trp-residue. This sequence is present at positions 115-118 with a Trp at the X-position (HGWP), see Fig. 6.1.

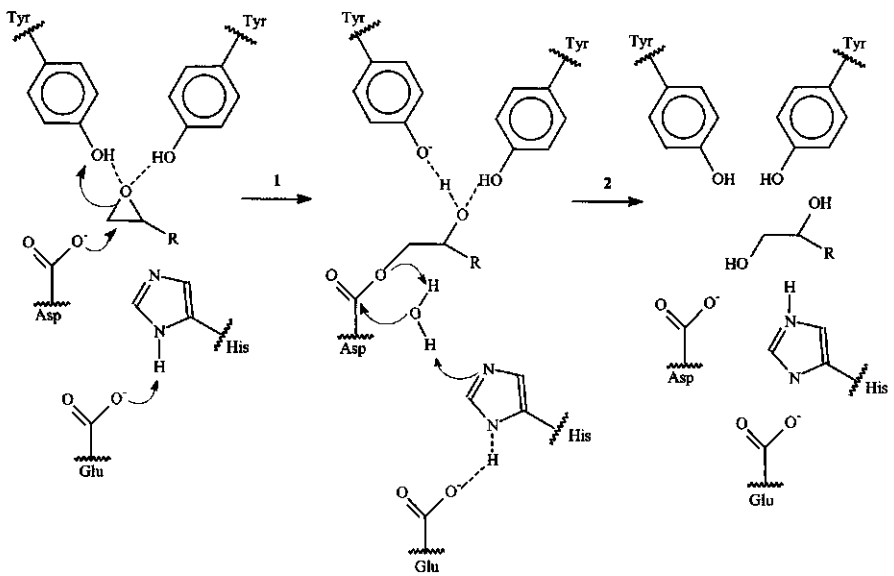


**FIG. 6.4. SCHEMATIC REPRESENTATION OF THE PROPOSED REACTION MECHANISM OF THE α/β HYDROLASE FOLD EH**

The epoxide will be held in position by the two Tyr residues. In the first step, the epoxide is attacked by the Asp nucleophile. An enzyme-substrate-ester intermediate is formed. Subsequently in the second step water, activated by the Glu-His pair, hydrolyzes the intermediate, resulting in the formation of a diol and the regenerated enzyme.

Visser et al. also overexpressed the RGEPH1 in *E. coli* using the pET-system (pET28a (+)). Plasmids were made which upon expression resulted in a fusion-protein of RGEH and an N-terminal histidine-tag (His-tag). This tag was added in order to facilitate the purification of the recombinant enzyme with metal affinity chromatography. Unfortunately, several attempts were made, but no one was successful. This is understandable if the N-terminus of the RGEH is indeed involved in the formation of the homodimer. The N-terminal His-tags are therefore not accessible to the Ni(II)-loaded column.

Another problem of this heterologous overexpression was the formation of inclusion bodies. These inclusion bodies contained a large amount of inactive epoxide hydrolase. Several attempts were made to decrease the level of inclusion bodies. However, the use of detergents like n-octyl β-glucoside in order to solubilize the inclusion bodies had no effect. Lowering the cultivation temperature of *E. coli* decreased the amount of inclusion bodies. Lowering the expression level of the T7-promotor resulted in a very low overall amount of produced EH, although more enzyme was found in the supernatant fraction. Overproduction of molecular chaperones (that are involved in the folding of enzymes) did result in a decrease of the amount of inclusion bodies. However, the amount in the soluble fraction hardly increased and the total yield was even reduced [134]. Finally, the amount of EH in the soluble fraction could be enhanced with a factor 800 by scaling up the cultivation volumes (200 times) and lowering the fermentation temperature. Visser gave as explanation for this effect the higher cell density in the fermentor [134].

## 6.3. EPOXIDE HYDROLASES AS BIOCATALYSTS

### 6.3.1. SOURCES OF EPOXIDE HYDROLASES

Epoxide hydrolases are ubiquitous enzymes and are found in a broad range of organisms. In this respect, it is worthwhile to consider whether or not the organism is suitable for large-scale production of the EH or for use as biocatalyst as a whole.

The first class of organisms are the mammals. The EH from these organisms are well studied and the enzyme in these animals is inducible for large production scale. However, large quantities of EH require extra slaughter of mammals like rabbits, dogs or cows or the use of slaughter waste for this purpose. Altogether, it is no realistic and acceptable source for EHs.

Several species of insects were found to have epoxide hydrolase activity. The EH of the mite *Rhizoglyphus robini* is capable of hydrolyzing styrene oxide, *trans*- and *cis*-stilbene oxide [24]. The juvenile hormone EH is found in eggs of *Manduca sexta* (the tobacco hornworm) [133]. Again, the low availability is a problem if large amounts of enzyme are required.

The last type of the higher organisms are the plants like soya-plants or *Arabidopsis* species. These plants are known to have an EH, but in small quantities. Although plants may not be suited, their EH-containing seeds, like soybeans, can be used as biocatalyst. A benefit is the ease of separation of biocatalyst and products. It

is possible to genetically modify the organism in order to overproduce the EH. The public opinion nowadays, however, is not in favor for genetically modified agricultural products. In addition, there remains the problem of the low enzyme content and the slow growth rate of plants before they start producing seeds.

The last group of organisms are the microorganisms: bacteria, yeast and fungi. These organisms are the most suitable for the production of EH as biocatalysts or as a whole. Large quantities are easily obtained and genetic modification is generally better accepted, providing that no pathogenic organism is developed and care is taken in handling of waste products. Different strains with their properties are described in the next paragraph.

### 6.3.2. KNOWN EPOXIDE HYDROLASES WITH INDUSTRIAL PERSPECTIVES

To become a versatile tool in the industry, enzymes must be able to hydrolyze either a large range of epoxides (oxiranes) or just one high volume epoxide. Depending on the substrate, the reaction should hydrolyze the epoxide enantioselectively (*i.e.* just one enantiomer is hydrolyzed). In some cases, two enzymes are used with an opposite enantiopreference together with a retention-versus-inversion type of water attack (see Fig. 1.3., p. 9) in order to obtain the product enantiopure. In this case, no epoxide is left [100].

Examples of methods used by biocatalysts:

1) Enantioselective hydrolysis of 2,2-disubstituted oxiranes: bacteria like *Rhodococcus* NCIMB 11216 followed by chemical steps [83, 101, 125]. Even high concentrations could be applied [46].

2) Enantioselective hydrolysis of aliphatic 1,2-epoxides: yeast like *Rhodotorula glutinis* and *Rhodosporidium toruloides* [14, 139]

3) The production of enantiopure diols by two microorganisms with opposite enantiopreferences. This process is also called deracemization or an enantioconvergent process:

> Combination of the fungi *Aspergillus niger* and *Beauvaria bassiana* (also known as *Beauvaria sulfurescens*) hydrolyzes both enantiomers of styrene oxide completely to the *R*-diol through retention and inversion of configuration, respectively [88, 100]. A similar combination of the bacteria *Nocardia* (recently described as *Rhodococcus ruber* [126]) and *Rhodococcus* ssp. hydrolyzed (*S*)-2,2-disubstituted oxiranes completely into the (*R*)-diol.

4) Enantioselective hydrolysis of symmetrically *cis*-2,3-disubstituted epoxides by the bacterium *Rhodococcus ruber* (previously known as *Nocardia* [126]) [8, 64, 102] resulted in the complete formation of the (2*R*, 3*R*)-diol [64, 128].

5) Enhancement of the yield of enantiopure diols from 50% to 100% could also be obtained by reracemation of the remaining epoxide followed by repeated kinetic resolution. After five cycles of this repeated resolution process, an overall yield of 95% enantiopure 1,2-diol product is possible upon complete conversion of the racemic epoxide [128].

### 6.3.3. *RHODOTORULA GLUTINIS* EH IN PRACTICE.

The epoxide hydrolase from *R. glutinis* has a preference in hydrolyzing the (*R*)-enantiomer of 1,2-aliphatic epoxides whereas the (*S*)-enantiomer remains nearly unaffected. Separation of the enzymes from the products is a problem since extraction of the reaction products with ethyl acetate inactivates the biocatalyst. Therefore, the use of the epoxide hydrolase containing polymer matrix (Chapter 5, p. 62) is advantageous for down stream processing. After the reaction, the polymer is collected by filtration or centrifugation while the converted substrates are available in the filtrate or supernatant. When the enzyme before polymerization is also imprinted, a versatile tool is obtained with a higher stability and a higher enantioselectivity.

An alternative approach for immobilization is the bioreactor developed by Choi et al [21, 22]. The formed diol is extracted with buffer (an aqueous phase) through the membrane and the remaining enantiopure epoxide is extracted with dodecane. Because this organic phase is in a separate membrane-reactor, no contact is made between the organism and the organic solvent.

## 6.4. RECOMMENDATIONS

### 6.4.1. ACHIEVED GOALS

The epoxide hydrolase from *Rhodotorula glutinis* has been isolated and purified. The EH belongs to the α/β-hydrolase fold family and is a membrane associated enzyme consisting of two identical subunits with a molecular weight of 46 kDa. Because the amount of purified enzyme was too small, the three-dimensional structure could not be determined. The EH activity and stability towards temperature and pH and the enantioselectivity has been established with crude extracts. Terminal aliphatic epoxides are the substrates of choice for this particular enzyme.

High epoxide concentrations could be applied and high yields obtained. Both epoxide and diol were obtained with acceptable enantioselectivities. Enzyme immobilization through encapsulation into an organic polymer matrix is beneficial for both stability and separation of substrate and product.

### 6.4.2. SUGGESTIONS FOR PRACTICAL ADJUSTMENTS

The expensive n-octyl β-D-glucoside used for isolation of the enzyme from *Rhodotorula glutinis* must be replaced by another (more cheaper) surfactant, *i.e.* Triton X-100. To remove Triton X-100 or other detergents with low CMCs successfully, Biobeads SM2 or Amberlite XAD-2 can be used (and re-used) [47, 105, 115] followed by filtration or centrifugation.

To overcome substrate or product inhibition in whole cells, a bioreactor can be used as proposed by Choi et al [21]. In this reactor, a cascade of three hollow-fiber membranes is used. The substrate in an organic solvent (dodecane) flows through the lumen side of the hollow-fiber and a cell suspension recirculates in opposite direction through the shell sides. The remaining epoxide is continuously extracted from the cell suspension with dodecane in a second reactor and the diol formed is subsequently extracted from the cell suspension in the third reactor with an aqueous buffer.

Cell-free systems (for example dialyzed second supernatant) can also be used when a second phase of epoxides is applied. This system can be optimized by dissolving the epoxide in another organic solvent (co-solvent) and applying the co-solvent as a second phase [73]. This can be used in a closed membrane-system as described by Baldascini et al [5] or in a flow-through bioreactor as described by Krieg et al [59, 60]. Both authors use an organic solvent: Baldascini used octane [5] and Krieg used acetonitril and ethanol [59].

In this respect, the use of the genetically engineered *E. coli* strain with the overexpressed EH in the reactor-system described by Choi et al [21] seems the most favorable option.

### 6.4.3. FUTURE WORK

The EH from *R. glutinis* is a membrane-associated enzyme and will be more active and stable in a lipid environment, as shown by the favorable effects of micelles [61]. This can also be effected by coating of the enzyme with lipids as shown for a number of enzymes in organic media or two-phase systems. For example, enzymes are coated successfully for the enantioselective esterification by lipase, transphosphatidylation of water-insoluble phospholipids by phospholipase D, hydrolysis of a lipophilic substrate by a catalytic antibody and transglycosylation by β-galactosidase [96].

In mammals, EHs are inducible enzymes. However, the EH from *R. glutinis* can not be induced. Several known inducers for mammalian EHs have been tested, such as *cis*-stilbene oxide, *trans*-stilbene oxide, β-methyl styrene oxide, thiazole, di-allylsulfide, pyrazine and chalcone. Although *cis*- and *trans*-stilbene oxide showed no activity at 1 mM, at 0.1 mM the activity was the same as in the blank. The other compounds showed no increase in activity and sometimes even a decrease in activity. This does not mean that the enzyme can not be induced, because the molecules themselves could have a slightly negative effect on the activity of the enzyme. For better understanding of induction, the RNA-levels should be monitored during growth.

Finally, to manufacture a stable, very enantioselective enzyme site-directed mutagenesis is a possibility. Although the way to success is somewhat longer than the earlier described method of bio-imprinting (Chapter 5), larger quantities of the modified enzyme can be obtained. By performing site-directed mutagenesis, Rink et al. altered the EH from *A. radiobacter* to obtain an enzyme with improved properties [111, 112, 113]. Also Arand et al. manufactured an enzyme with an increased turnover rate by site directed mutagenesis [1].

Another drawback from the EH from *R. glutinis* is the substrate range. Although many epoxides can be hydrolyzed by this enzyme, the EH has a preference for hydrolyzing aliphatic 1,2-epoxides with an acceptable rate and enantioselectivity. The aromatic epoxides are hydrolyzed at a much lower rate. The EH from *A. radiobacter*, however, has a preference for these aromatic epoxides. These two enzymes are complementary to each other. In order to cover a complete range of substrates, EH from different sources should be used.

It is recommended either to use whole cells (overexpressing EH or not) in a bioreactor to produce enantiopure epoxides and diols in large quantities or to use an immobilized-imprinted enzyme polymer for the conversion of smaller quantities of epoxides in an enantioselectivity of your choice. Further investigations to improve both methods (whole cells, the enzyme properties and the immobilization and imprinting procedures) are required to optimize this type of conversions for practical applications.

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# ABBREVIATIONS USED

AIBN: 2,2'-azobis-(2-methylpropionitril);
AOT: Aerosol dioctyl sodiumsulfosuccinate;
BSA: Bovine serum albumin;
CD: Cyclodextrin;
CFE: Cell free extract;
CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate;
CLIP-EH: Crosslinked imprinted epoxide hydrolase;
CMC: critical micellar concentration;
Cutscum: iso-octylphenoxypoly-ethanol;
DTT: dithiothreitol;
EDTA: Ethylene diamine tetraacetate;
EGDMA: Ethylene glycol dimethacrylate;
EH: Epoxide Hydrolase (EC 3.3.2.3)
Gal 12Ac: 6-O-(12-acetoxyoctadecanoyl)-galactose;
IPTG: isopropyl-β-D-thiogalactopyranoside
mEH: microsomal epoxide hydrolase
Mgal 12Ac: 6-O-(12-acetoxyoctadecanoyl)methylgalactose;
Mgal 12OH: 6-O-(12-hydroxy-octadecanoyl)methylgalactose;
N-Dodecylmaltoside: 1-O-n-Dodecyl-β-D-glucopyranosyl(1-4)α-D-glucopyranoside;
N-Octylglucoside: 1-O-n-Octyl-β-D-glucopyranoside;
OG: n-octylglucoside;
Non-idet P40: ethylphenolpoly (ethyleneglycolether)$_n$;
PAF: Platelet aggregation factor;
PAGE: Poly Acrylamide Gel Electrophoreses;
SDS: sodium dodecyl sulfate;
sEH: soluble or cytosolic epoxide hydrolase
SML: Sucrose Mono Laurate
Thesit: dodecylpoly(ethyleneglycolether)$_n$;
TNBS: 2,4,6-trinitrobenzene sulfonic acid;
Triton X-100: octylphenolpoly(ethyleneglycolether)$_n$;
Tween 20: poly(oxyethylen)n-sorbitan-monolaurate;
Zwittergent 3-12: N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate;

# SUMMARY

Epoxide hydrolases are ubiquitous enzymes that can be found in nearly all living organisms. Some of the enzymes play an important role in detoxifying xenobiotic and metabolic compounds. Others are important in the growth of organisms like the juvenile hormone in some insects. The role of these enzymes in some organisms is still not fully understood.

Epoxides are highly reactive valuable intermediates used by the pharmaceutical industry. Enantiopure epoxides are of high value in the production of pharmaceuticals like pain-killers or protease-inhibitors. There are a number of ways to produce enantiopure epoxides, but nowadays an environmentally friendly manner has a high preference. One such environmentally friendly method is the use of the epoxide hydrolases. These enzymes are able to enantioselectively hydrolyze one epoxide-enantiomer to its vicinal diol. By this so-called kinetic resolution, it is possible to obtain both the epoxide and diol enantiopure. Enantiopure diols are also of high value in the fine and pharmaceutical chemistry.

The first goal of this project is achieved: the epoxide hydrolase from *Rhodotorula glutinis* has been isolated and purified. The second goal (optimization of the reaction conditions) has been performed but it is still favorable to further optimize them. Initial experiments of enzyme stability towards temperature and pH has been performed with crude enzyme extracts, not with the purified enzyme. With respect to the third goal (a suitable method for the isolation and separation of epoxide and diol), the use of a recently described membrane reactor is recommended. The performance of this reactor, however, has not been verified for the EH studied.

The enzyme is partially characterized. The EH was found to be a membrane associated enzyme. Whether or not it is actually a membrane bound enzyme (and how many times it passes the membrane) is still unknown. The enzyme consists of two (most probably) identical subunits with a molecular mass of 45 kDa. Amino acid analysis revealed that the enzyme belongs to the $\alpha/\beta$-hydrolase fold family, because the characteristic catalytic histidine motive (GHF) can be found in the amino acid sequence. The N-terminal sequence, however, could not be detected. The amount of purified enzyme was too low to establish its the three-dimensional structure.

With partially purified enzyme sample, the specific activity and enantioselectivity could be enhanced when detergents were added. Non-ionic detergents had the largest positive effects, *e.g.* the specific activity for 1,2-epoxyhexane and styrene oxide was enhanced three and eight times, respectively. In the same way, the enantioselectivity for 1,2-epoxyhexane and styrene oxide could be enhanced over 10 and nearly 5 times, respectively. In addition, non-ionic detergents had an enzyme stabilizing effect. Anionic detergents had a very clear negative effect: enzyme activities were reduced to 20%.

Another method investigated to influence the stability, the activity and the enantioselectivity consists of polymerizing the epoxide hydrolase in a network. 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. 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 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

The effect of increasing epoxide amounts (up to 10 mmol per 10 mL of water, leading to phase separations) on both the activity and enantioselectivity has been studied, including the effect of detergents on such two-phase enzymatic conversions. It appeared that cell-free extracts without detergents gave the highest activity at 10 mmol epoxide per 10 mL of water added, without loss of enantioselectivity as compared to 1 mmol epoxide per 10 mL of water emulsions.

It is recommended either to use whole cells (overexpressing EH or not) in a bioreactor to produce enantiopure epoxides and diols in large quantities or to use an immobilized-imprinted enzyme polymer for the conversion of smaller quantities of epoxides in an enantioselectivity of your choice. Further investigations to improve both methods (whole cells, the enzyme properties and the immobilization and imprinting procedures) are required to optimize this type of conversions for practical applications.

# SAMENVATTING

Epoxide hydrolases zijn enzymen die overal in de natuur voorkomen: zoogdieren, insecten, planten en zelfs micro-organismen zoals bacteriën, schimmels en gisten bevatten deze enzymen. Dit enzym katalyseert de toevoeging van water aan de reactieve epoxide-ring. Deze epoxides zijn door hun structuur zeer reactieve verbindingen. Ze kunnen daardoor ook schade toebrengen aan het lichaam door bijvoorbeeld te reageren met DNA. Hierdoor kan kanker ontstaan. De epoxide hydrolases hebben in het menselijk lichaam dan ook voornamelijk een ontgiftende rol. Sommige epoxide komen per ongeluk het lichaam binnen maar anderen ontstaan als bijproduct tijdens de afbraak van vetzuren. De rol van de epoxide hydrolases in andere organismen is van vele nog niet volledig bekend. Van insecten is bekend dat een epoxide hydrolase een rol speelt bij het ontwikkelen van larve tot volwassen insect.

Ondanks dat epoxides als gevaarlijke stoffen bekend zijn, spelen ze toch ook een grote rol in de farmaceutische industrie en in de productie van fijnchemicaliën. Enantiozuivere epoxides (slechts 1 spiegelbeeld-vorm) worden gebruikt in de productie van pijnstillers en andere medicijnen zoals bijvoorbeeld AIDS-remmers. Er zijn een aantal verschillende manieren om deze epoxides enantiozuiver te produceren, maar tegenwoordig is het predikaat milieuvriendelijk ook belangrijk. Het gebruik van zware metalen of sterke zuren wordt dan ook afgeraden. En hier is een rol voor epoxide hydrolases weggelegd. Deze enzymen kunnen op een milieuvriendelijke manier een racemisch mengsel (mengsel van 2 spiegelbeelden) van epoxides scheiden. Dit doen ze door 1 spiegelbeeld om te zetten naar een diol. Het andere spiegelbeeld blijft achter. Deze methode wordt ook wel kinetische resolutie genoemd: scheiding (resolutie) door middel van een verschil in reactiesnelheid (kinetisch). Zo krijgt men een epoxide en een diol zuiver in handen, beiden van grote waarde in de fijnchemie.

Het eerste doel van dit project was om een epoxide hydrolase uit de gist *Rhodotorula glutinis* te isoleren en karakteriseren. Dit is gelukt. De gist bevat een membraangebonden epoxide hydrolase. Het is echter nog niet bekend in welk membraan van de gistcel dit enzym zich bevindt. Ook hoe het enzym zich in of aan het membraan bevindt, is nog onbekend. Het kan zijn dat het enzym slechts 1 keer door de membraan gaat, maar het kan ook zijn dat het enzym zich 6 keer door het membraan slingert. Het epoxide hydrolase bestaat uit 2, waarschijnlijk, identieke eenheden met een grootte van ongeveer 45 kDa. Verdere analyse gaf aan dat het enzym behoort tot een grote familie enzymen: de zogenaamde $\alpha/\beta$-hydrolase gevouwen enzymen. Deze enzymen zijn zo genoemd door hun karakteristieke patroon van $\alpha$-helices en $\beta$-platen (een vouwingsvorm van aminozuren in een eiwit). Een andere eigenschap van deze familie is de aanwezigheid van 3 opeenvolgende aminozuren (G H F) die het onder andere het katalytisch centrum van het enzym vormen, waarbij de Histidine (H) het belangrijkst is. Een andere eigenschap die veel over een enzym zegt is de N-terminale sequentie. Deze sequentie hebben we echter

niet kunnen ophelderen. Het zuiveren van het enzym was zodanig ingewikkeld dat er slechts een hele kleine hoeveelheid enzym beschikbaar was. Deze hoeveelheid was te laag om de ruimtelijke structuur van het eiwit te kunnen bepalen. Hierdoor kan er dan ook weinig gezegd worden over het actieve centrum en het reactiemechanisme van het enzym.

Het tweede doel van dit proefschrift was het optimaliseren van de reactiecondities. Dit is slechts uitgevoerd met een ruw celextract. Hieruit volgde dat een hoge activiteit en enantioselectiviteit bij een temperatuur van 35°C en een pH tussen de 7 en 8.5 lag. Zodra grotere hoeveelheden gezuiverd enzym beschikbaar zijn, dienen de reactieomstandigheden verder geoptimaliseerd te worden.

Met een gedeeltelijk gezuiverd enzym zijn de verdere experimenten uitgevoerd. Met name het verbeteren of veranderen van de specifieke activiteit en enantioselectiviteit is onderzocht. Tijdens de zuivering was al gebleken dat detergentia niet alleen in staat waren om het enzym van de membranen te scheiden, maar ook een invloed op activiteit en enantioselectiviteit te hebben. Er zijn verschillende soorten detergentia getest. De anionische (negatief geladen) detergentia hadden een negatief effect: de enzymactiviteit werd teruggebracht tot 20% van de beginactiviteit en daardoor liep de enantioselectiviteit ook terug. De grootste effecten vonden plaats als non-ionische (niet-geladen) detergentia gebruikt werden. De specifieke activiteit voor 1,2-epoxyhexaan en styreenoxide kon respectievelijk 3 en 8 keer verhoogd worden. De enantioselectiviteit kon zelfs met meer dan 10 en bijna 5 keer verhoogd worden voor respectievelijk 1,2-epoxyhexaan en styreenoxide. Doordat de detergentia in de gebruikte concentraties in staat waren om membraanstructuren na te bootsen, trad er een stabiliserend effect op wat dus een positief effect had op de omzettingen.

Een andere methode om de activiteit of de enantioselectiviteit te beïnvloeden, kan door het enzym te stabiliseren in een polymeernetwerk. Als tijdens het polymeriseren bijvoorbeeld substraten toegevoegd worden, dan wordt de actieve vorm van het eiwit "bevroren". Dit wordt "imprinten" genoemd en de toegevoegde moleculen zijn dan "imprinters". Op deze manier is het zelfs mogelijk om de voorkeur van het enzym te veranderen. De enantioselectieve omzetting van racemisch 1,2-epoxyoctaan werd omgekeerd: in plaats van voornamelijk de *R*-enantiomeer, werd nu de *S*-enantiomeer afgebroken. Dit is de eerste keer dat deze methode succesvol is toegepast op een membraan-geassocieerd enzym. De halfwaardetijd van het enzym werd op deze manier met circa 7 keer verlengd. Het meest opvallende was dat wassen met zoutzuur had minder effect op de activiteit dan op het vrije enzym. Het vrije enzym werd hierdoor volledig inactief, terwijl het geïmmobiliseerde enzym nog 50% activiteit over had.

Het effect van toenemende epoxide hoeveelheden (tot 10 mmol per 10 mL water, wat leidde tot fase-scheiding) op zowel de activiteit en enantioselectiviteit is onderzocht, alsmede het effect van detergentia op de enzymatische omzettingen in deze 2-fase systemen. Het bleek dat celvrije extracten (gedeeltelijk gezuiverd enzym) zonder detergentia de hoogste activiteit gaf bij 10 mmol epoxide per 10 mL water

zonder verlies van enantioselectivitei in vergelijking met 1 mmol per 10 mL water (epoxide-water emulsies).

Het laatste doel van dit project was het ontwikkelen van een bruikbare methode om de enantiozuivere epoxides en diolen van elkaar te scheiden. Het wordt aanbevolen om de onlangs beschreven membraanreactor te gebruiken. Deze reactor is hier niet verder getest.

Tot slot enige aanbevelingen: voor grote(re) hoeveelheden enantiomeer-zuivere epoxiden en diolen kan het beste gebruik gemaakt worden van hele cellen (die eventueel het epoxide hydrolase tot overexpressie brengen) in een bioreactor. Voor kleinere hoeveelheden of specifieke reacties, kunnen daarentegen ook het geïmmobiliseerde en ge-imprinte epoxide hydrolase gebruikt worden. Met de laatste methode kan dan ook de enantioselectiviteit naar eigen behoefte gekozen worden. Verder onderzoek om beide methodes (zowel de hele cellen, de enzym eigenschappen en de immobiliserings- en imprintingsmethodes) te verbeteren zijn nodig om dit soort conversies te optimaliseren voor praktische toepassingen.

# NAWOORD

Een proefschrift schrijven doe je alleen. Het werk verrichten voor dat proefschrift doe je echter niet alleen. Voor en achter de schermen hebben een hoop mensen meegeholpen om uiteindelijk tot dit boekje te komen. En een goede plaats om die mensen te bedanken is in het nawoord van datzelfde boekje.

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# CURRICULUM VITAE

Nicole Alexandra Esmeralda Kronenburg werd op 19 juli 1971 te Utrecht geboren. Na het behalen van het Gymnasium B diploma in 1989 aan de Openbare Scholengemeenschap Hendrik van der Vlist in Utrecht, ging zij Scheikunde studeren aan de Rijksuniversiteit Utrecht.

Ze specialiseerde zich in de richting biochemie en volgde een 6-maands bijvak in het Wilhelmina Kinder Ziekenhuis in Utrecht. Het onderwerp was het bestuderen van enoyl-CoA hydratase, een enzym wat betrokken is bij de vetzuurafbraak. Daarna volgde ze een hoofdvak van 9 maanden bij de vakgroep Biochemie van Membranen. Hier nam ze deel aan het onderzoek naar een nieuw te ontwikkelen anti-malariamiddel: de effecten van gramicidine en N-formylated gramicidine op de rode bloedlichamen. In augustus 1995 werd deze studie afgerond.

In 1996 werd begonnen als aio op het project "Een enantioselectieve epoxide hydrolase uit de gist *Rhodotorula glutinis*" bij de vakgroep Levensmiddelentechnologie en Voedingswetenschappen, leerstoelgroep Industriële Microbiologie van de Wageningen Universiteit. Van januari 2000 tot mei 2000 is zij werkzaam geweest als toegevoegd onderzoeker op hetzelfde onderwerp bij dezelfde leerstoelgroep.

Sinds september 2000 is zij werkzaam bij de inmiddels opgeheven leerstoelgroep Industriële Microbiologie als post-docteraal onderzoeker op het NWO/STW-project "Mushroom Oxidative Stress Tolerance as a novel strategy towards environmentally sound pathogen control". Vanaf media maart zal dit project worden voortgezet bij het Laboratorium voor Microbiologie van Wageningen Universiteit.

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