Cloning and application of epoxide hydrolases from yeasts

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Johannes Heinrich Visser

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Hans Visser

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Stellingen

1. Als een gistsoort een lage epoxidehydrolase activiteit bezit dan is de bepaling van de bijbehorende enantioselectiviteit pas mogelijk als er een recombinante gastheer wordt gebruikt waarin het desbetreffende epoxidehydrolase coderende gen in voldoende hoge mate tot expressie wordt gebracht.
   Dit proefschrift.

2. Het epoxidehydrolase coderende gen van *Xanthophyllomyces dendrorhous* is niet essentieel voor groei van deze gist onder laboratorium condities.
   Dit proefschrift.

3. Het oplosbare epoxidehydrolase van *Aspergillus niger* vertoont sterke gelijkenis met het membraan gebonden microsomale epoxidehydrolase (mEH) en niet met het oplosbare (soluble) epoxidehydrolase (sEH). Dit leidt tot verwarring over de aard en intracellulaire locatie van dit enzym.
   Arand *et al.* (1999). Biochem. J. 344: 273-280.

4. Het succes van de ontwikkeling van een op een specifieke omzetting toegespitst mutant enzym, via technieken zoals "error prone PCR" en "DNA shuffling", is in hoge mate afhankelijk van het voorhanden zijn van een geschikte screeningsmethode.

5. Leukoprotoxine is een betere naam voor leukotoxine aangezien leukotoxine zelf niet toxisch is maar het daaruit gevormde leukotoxine diol wel.
   Moghaddam *et al.* (1997). Nat. Med. 3: 562-566.

6. In landen die in staat zijn tot het reproductief klonen van mensen zou deze techniek bij wet verboden moeten worden.

7. Bij de overgang op de wintertijd zouden de televisieomroepen hun kinderprogramma's een uur eerder moeten laten beginnen.

8. De Rijn komt bij Spijk ons land binnen.

Stellingen behorend bij het proefschrift "Cloning and application of epoxide hydrolases from yeasts".

Hans Visser.
Wageningen, 1 maart 2002.

Contents

Chapter 1

Introduction

EPOXIDES

Epoxides are cyclic ethers that, due to the strong tension of the three-membered ring structure and the electronic polarization of the two C-O bonds, readily react with various nucleophilic compounds. Additionally, the epoxide ring of unsymmetrical epoxides can be opened at either the less hindered epoxide carbon atom (base-catalyzed reaction) or the more hindered epoxide carbon atom (acid-catalyzed reaction). Because of these properties, epoxides are versatile intermediates in organic synthesis (Swaving and de Bont, 1998). Consequently, epoxides are valuable building blocks for the synthesis of biologically active compounds such as clinical drugs or insecticides by the pharmaceutical and agrochemical industries.

Two enantiomeric forms (enantiomers) of an epoxide are possible if one of the epoxide carbon atoms is chiral. This means that such an epoxide is actually a racemic mixture of its enantiomers (Figure 1). For pharmaceutical companies it is very important to produce drugs that are as safe and effective as possible. It is therefore of great importance that, instead of the racemic epoxide mixtures, enantiopure epoxides are used in the chiral epoxide-dependent synthesis route of clinical drugs.

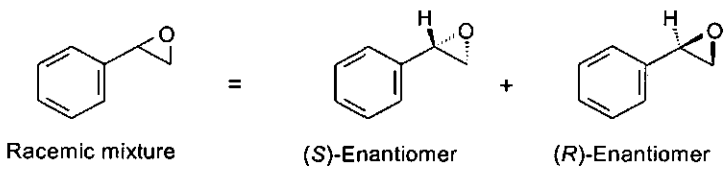

Figure 1. Chemical structures of both racemic and the separate enantiomers of phenylethylene oxide (styrene oxide).

Enantiopure epoxides can be obtained by using both chemical and biological procedures (for reviews see de Bont, 1993; Besse and Veschambre, 1994; Archelas and Furstoss, 1997). The chemical methods that are used usually introduce an epoxide moiety (epoxidation) at the position of a double bond in e.g. alkenes or allylic alcohols. Despite several successful asymmetrical epoxidations, these chemical methods have some drawbacks (Besse and Veschambre, 1994). First, the reactions are catalyzed by toxic heavy metals. Second, for some methods the catalyst (metalloporphyrines) has to be synthesized, which can be difficult. Third, a limited number of compounds can be used as substrate for epoxidation. Fourth, the yield of enantiopure epoxides can be low.

Biological procedures involve enzyme-catalyzed reactions, which have the advantage to allow stereospecific oxygenation reactions that are difficult to carry out chemically. For example, monooxygenases can be used in the stereospecific epoxidation of alkenes to generate enantiopure epoxides. Alternatively, enantiopure epoxides can be obtained by the enzyme-catalyzed kinetic resolution of a racemic epoxide mixture. One of the enzymes, which can be used for the kinetic resolution of

racemic epoxides is lipase. Ideally, lipase only modifies one of the two epoxide enantiomers e.g. by ester hydrolysis. This allows the separation of the remaining enantiopure epoxide enantiomer from the other (converted) enantiomer.

The use of both the chemical and biological (biocatalytic) methods, which are successful in the production of enantiopure epoxides are protected by patents. Therefore, from an industrial point of view it is of great interest to search for new catalysts that can be used to produce enantiopure epoxides. A biocatalyst that meets this demand is the epoxide hydrolase.

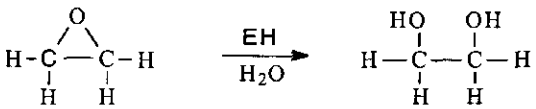

Figure 2. General scheme of the hydrolysis of a simple epoxide into its corresponding diol by an EH.

Epoxide hydrolases (EHs, E.C. 3.3.2.3) catalyze the hydrolysis of epoxides to the corresponding diols (Figure 2). They act cofactor independently using only water and they are ubiquitous in nature. Most interestingly, EHs can hydrolyze racemic epoxide mixtures in an enantioselective manner, which can result in enantiopure epoxides. Because of these properties, EHs might be promising candidate biocatalysts for the production of enantiopure epoxides from cheap and readily available racemic epoxides.

OUTLINE OF THIS THESIS

At the start of the work that is presented in this thesis, EHs from yeast origin were already subject of investigation in the group of Industrial Microbiology headed by Professor J. A. M. de Bont. The red-colored yeast *Rhodotorula glutinis* in particular showed the enantioselective hydrolysis of various structurally different epoxides leading to encouraging yields of enantiopure epoxides or diols (Weijers, 1997). Meanwhile, additional research has been performed on the biochemistry and application of EHs from basidiomycetous yeast species in both the analytical and preparative scale enantioselective hydrolysis of various epoxides (Botes, 1999; Botes *et al.*, 1998, 1999; Choi *et al.*, 1999, 2000; Kronenburg *et al.*, 1999; Weijers *et al.*, 1998).

The research described in this thesis was part of two successive research projects, which both focused on the potentials of yeasts EHs in enantioselective epoxide hydrolysis. The main objectives of these projects were to gain important fundamental knowledge on yeasts EHs and to develop biotechnological processes based on the use of these enzymes for the production of enantiopure epoxides.

To introduce EHs an overview of the molecular biology, biochemistry and potential application of these enzymes is given in chapter 2. In chapters 3 and 4 the isolation and characterization of the EH-encoding genes from the carotenoid producing yeast species

Xanthophyllomyces dendrorhous (perfect state of *Phaffia rhodozyma*, Golubev, 1995) and *R. glutinis* is described. The relationship of the EHs from these yeast species to other EHs was analyzed.

The EH from *R. glutinis* is a potential candidate to be applied in the enantioselective hydrolysis of epoxides. In order to develop an efficient EH-catalyzed epoxide hydrolysis system the EH-encoding gene of *R. glutinis* was over-expressed in *Escherichia coli*. When compared to homologous expression in *R. glutinis* itself, the recombinant expression system showed a strong increase in activity, due to the overproduction of EH, without loss of enantioselectivity. The utility of this system was demonstrated for the hydrolysis of 1-oxa-spiro[2.5]octane-2-carbonitrile, which is a versatile building block in organic synthesis.

Other yeast strains having high and low quality EH activities were reported (Botes *et al.*, 1999). The EH-encoding genes from a few of these strains were isolated and expressed in *E. coli*. The corresponding amino acid sequences and activities toward *trans*-1-phenyl-1,2-propane oxide were examined. A highly efficient recombinant EH was obtained for the enantioselective hydrolysis of *trans*-1-phenyl-1,2-propane oxide. The results of this study are described in chapter 5.

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Chapter 2

Molecular biology, biochemistry and potential application of epoxide hydrolases

INTRODUCTION

Epoxides are cyclic ethers that readily react with a number of reagents, nucleophiles in particular. Commonly used nucleophiles reacting with epoxides are oxygen, nitrogen, sulfur, halide and carbon compounds. This is one of the properties, which causes epoxides to be versatile intermediates in organic synthesis (Swaving and de Bont, 1998). However, due to their highly reactive nature epoxides can easily react and damage biologically important molecules such as proteins (enzymes) or nucleic acids (DNA). Therefore, on one hand epoxides are useful building blocks in organic chemistry, and on the other hand they are a threat to human health as a consequence of their potentially cytotoxic and carcinogenic properties.

Epoxide hydrolases (EHs; EC 3.3.2.3) are enzymes that catalyze the addition of water to epoxides, thereby usually generating the corresponding vicinal *trans*-diols (Figure 1). EHs have been found in many organisms, including mammals, insects, plants, fungi and bacteria and may therefore be regarded as ubiquitous enzymes. Several types of EHs are recognized that are involved in processes such as the detoxification of noxious epoxides, catabolism or biosynthetic pathways.

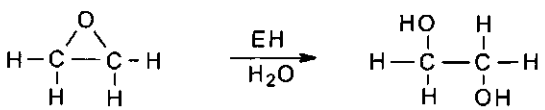


Figure 1. Epoxide hydrolysis by an EH resulting in the corresponding *trans*-diol.

In this chapter an overview will be given of EHs. First, different types and functions of epoxide hydrolases will be presented. Next, the relatedness of these EHs and their mechanisms of action will be evaluated. Finally, the use of EHs as biocatalyst in the enantioselective hydrolysis of epoxides is presented.

MAMMALIAN EHs

Microsomal EH

Microsomal EH (mEH) from rodents was the first epoxide hydrolase described (Oesch and Daly, 1971; Oesch *et al.*, 1971b). Microsomal EH activity has been detected in essentially all organs and tissues of rats and mice (Oesch, 1977). However, the enzyme is located primarily in the endoplasmic reticulum of hepatocytes (Oesch and Daly, 1971) and it has been suggested to use mEH as a marker enzyme for microsomal membranes (Oesch *et al.*, 1971a). Microsomal EH has been purified to homogeneity from livers and other tissues of several mammalian species revealing a minimum molecular weight ranging from approximately 46 to 54 kDa (Halpert *et al.*, 1979; Lu *et al.*, 1975, 1979; Papadopoulos *et al.*, 1994). Molecular studies indicated

that mEH is co-translationally inserted into the membrane of the endoplasmic reticulum by means of a single membrane signal anchor sequence, which is dispensable for catalytic activity (Okada *et al.*, 1982; Friedberg *et al.*, 1994).

Microsomal EH encoding-genes and cDNA's from several mammalian organisms have been isolated (Porter *et al.*, 1986; Falany *et al.*, 1987; Skoda *et al.*, 1988; Hassett *et al.*, 1989, 1994b). The mEH polypeptide from rabbit, rat and human comprises 455 amino acids with a calculated molecular mass of approximately 53 kDa (Heinemann and Ozols, 1984; Porter *et al.*, 1986; Skoda *et al.*, 1988). In human and rat single functional mEH-encoding genes per haploid genome have been proposed (Falany *et al.*, 1987). The human mEH gene is located to the long arm of chromosome 1 (Skoda *et al.*, 1988). The primary transcript of the human mEH gene extending from the start of transcription to the poly(A) addition site, is 20,271 nucleotides in length (Hassett *et al.*, 1994b). The rat mEH gene measures approximately 16,000 base pairs (Falany *et al.*, 1987). Despite the difference in size the human and rat mEH genes are organized very similar. Both genes consist of 9 exons, which are separated by 8 introns. In both species exon 1 does not encode protein. The sizes of introns 2 to 8 are identical, whereas the sizes of the human exons 1 and 9 are 100 nucleotides longer and 5 nucleotides shorter respectively than the rat exons 1 and 9. The difference of the latter is in the length of the non-coding tail (Hassett *et al.*, 1994b).

Two prominent genetic polymorphisms of human mEH exist (Hassett *et al.*, 1994a). The first one is found in exon 3 and results in the substitution of Tyr by His at amino acid position 113. The second is found in exon 4 and concerns the substitution of His by Arg at amino acid position 139. These polymorphisms have been associated with human diseases such as an altered risk to different types of cancer (Harrison *et al.*, 1999; Wu *et al.*, 2001) and emphysema (Smith and Harrison, 1997). *In vitro* studies indicate that the Tyr/His and His/Arg amino acid variations do not exert a primary influence on the mEH catabolic function, but may instead affect the mEH stability (Hassett *et al.*, 1994a).

Microsomal EH is regarded as the EH most contributing in the detoxification of potentially cytotoxic and carcinogenic epoxide metabolites of xenobiotic compounds, which are generated by the cytochrome P450 monooxygenase systems. Most mEH substrates are converted into less reactive and better water-soluble metabolites that can be readily conjugated or excreted, which explains the detoxifying role of mEH. It exhibits broad substrate specificity, ranging from simple short chain aliphatic epoxides to large polycyclic aromatic hydrocarbons such as benzo-(a)-pyrene-4,5-oxide (Figure 2) (Oesch *et al.*, 1971b; Jerina *et al.*, 1977). In addition to the epoxide derivatives of environmental chemicals e.g. polycyclic aromatic hydrocarbons, mEH is also involved in the metabolism of the epoxide derivatives of clinical drugs such as the anti-epileptic drugs carbamazepine and phenytoin (Eugster *et al.*, 1991; Riley *et al.*, 1988).

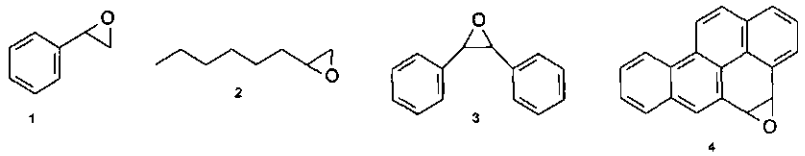


Figure 2. Chemical structures of mEH substrates. 1, Styrene oxide. 2, 1,2-Epoxyoctane. 3, *cis*-Stilbene oxide. 4, Benzo-(a)-pyrene-4,5-oxide.

In contrast to its protective role, mEH is also involved in the bio-activation of the ultimate carcinogenic metabolite 7,12-dimethylbenz(a)antracene-3,4-diol-1,2-epoxide (Miyata *et al.*, 1999), which is capable of producing DNA adducts (Dipple and Nebzydoski, 1978).

Furthermore, mEH was identified as a subunit of the anti-oestrogen-binding site (Mésange *et al.*, 1998) and it is active in endogenous steroid epoxide metabolism (Vogel-Bindel *et al.*, 1982) and sodium-dependent bile acid transport (von Dippe *et al.*, 1996). However, Honscha *et al.* (1995) showed that heterologously expressed mEH was unable to mediate the transport of the bile acids taurocholate and cholate. Additionally, in mice mEH is not essential for reproduction and physiological homeostasis (Miyata *et al.*, 1999).

Several mEH inhibitors have been described. Among these are 1,1,1-trichloro-2,3-propene oxide (Oesch *et al.*, 1971c), cycloalkene oxides e.g. cyclohexene oxide (Oesch *et al.*, 1973; Magdalou and Hammock, 1988), the anti-epileptic drug valpromide (Kerr *et al.*, 1989) and pentachlorophenol (Moorthy and Randerath, 1996). More recently metals such as zinc and mercury (Draper and Hammock, 1999) and ureas, amides and amines (Morisseau *et al.*, 2001) were reported to inhibit mEH.

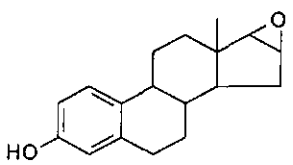


Figure 3. Chemical structure of the steroid epoxide estroxide, which is an endogenous substrate of rat mEH (Fändrich *et al.*, 1995).

A number of chemicals induce rat mEH gene expression e.g. phenobarbital (Hardwick *et al.*, 1983), nitrosamines (Craft *et al.*, 1988), diazines (Cho and Kim, 1998), 2-acetylaminofluorene (Åström *et al.*, 1987), *trans*-stilbene oxide (Schmassmann and Oesch, 1978) and others. In addition to these exogenous compounds, rat mEH is also induced by its endogenous substrate, which is the steroid

epoxide estroxide (Figure 3) (Fändrich *et al.*, 1995). Human mEH expression in primary hepatocyte cultures indicated that mEH gene expression is only modestly induced by common mEH inducers (Hassett *et al.*, 1998). Dexamethasone, a glucocorticoid, was reported to repress mEH gene expression (Bell *et al.*, 1990).

Soluble EH

Soluble EH (sEH, formally known as cytosolic EH) is an enzyme, which is like mEH involved in the metabolism of epoxides derived from both exogenous and endogenous sources (Meijer and DePierre, 1988). Soluble EH activities have been demonstrated in different tissues from mammals such as mice, rats and rabbits. In general, liver, heart and kidney tissues show the highest sEH activities (Gill and Hammock, 1980; Schladt *et al.*, 1986). The sEH protein has been purified from e.g. rat and human livers. Native sEH exists as a homodimeric protein with an estimated molecular mass of 120 kDa and a subunit mass of 58 to 61 kDa (Schladt *et al.*, 1988; Wang *et al.*, 1982). In addition to the protein, sEH-encoding genes and cDNAs have been isolated (Beetham *et al.*, 1993; Grant *et al.*, 1993; Knehr *et al.*, 1993; Sandberg and Meijer, 1996). The human sEH gene measures approximately 45 kbp in length, contains 19 exons (Sandberg and Meijer, 1996) and is located on chromosome 8 (Larsson *et al.*, 1995). The open reading frames of sEH cDNA clones predict corresponding polypeptides of 554 amino acids with a calculated molecular mass of 62 kDa (Beetham *et al.*, 1993; Knehr *et al.*, 1993).

Initially, sEH was localized to the cytosolic and mitochondrial fraction of mammalian cells (Gill and Hammock, 1980, 1981). Additional studies showed that sEH is present in the cytosol and in peroxisomes (Waechter *et al.*, 1983; Messing Eriksson *et al.*, 1991). The C-terminus of sEH contains the amino acid sequence Ser-Lys-Ile, which is highly homologous to the peroxisomal targeting sequence Ser-Lys-Leu. It was suggested that this difference might turn the signal sequence into an impaired peroxisomal targeting sequence, which causes the presence of sEH in both peroxisomes and the cytosol (Arand *et al.*, 1991). However, Mullen *et al.* (1999) presented evidence that the dual localization of sEH in the cytosol and peroxisomes of rodents is not due to an inefficient peroxisomal targeting sequence. Instead, they suggest the existence of two sEH isozyme variants (cytosolic and peroxisomal) in mammalian cells.

Also soluble EH exhibits a broad substrate range. This range partly overlaps with and is complementary to that of mEH. In general, *trans*-substituted epoxides are the better sEH substrates (Fretland and Omiecinski, 2000). Examples of xenobiotic epoxides accepted by sEH are *trans*-8-ethylstyrene 7,8-oxide (Wang *et al.*, 1982) and *trans*-β-methylstyrene oxide (Ota and Hammock, 1980). *Trans*-stilbene oxide (TSO) is the typical sEH model-substrate to distinguish sEH activity from mEH activity (Figure 4). The broad range of xenobiotic epoxides metabolized by sEH suggests a protective role of this enzyme against these harmful compounds (Grant *et al.*, 1996).

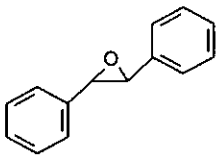


Figure 4. Chemical structure of *trans*-stilbene oxide, a typical sEH substrate.

Another role of sEH is found in the metabolism of fatty acid epoxides. In mammals, cytochrome P450 epoxygenase mediated conversion of arachidonic acid results in the formation of a class of biologically active metabolites known as *cis*-epoxyeicosatrienoic acids (EETs) (Capdevila *et al.*, 2000). These EETs are hydrolyzed by sEH to their corresponding dihydroxyeicosatrienoic acids (Fang *et al.*, 2001). In mice, the sEH-dependent metabolism of arachidonic acid was identified to play an important role in the control of blood pressure regulation and renal function (Sinal *et al.*, 2000). Additionally, linoleic acid is a polyunsaturated lipid, which is a very abundant fatty acid in the average American diet. It can be oxidized by autooxidation or P450 enzymatic action to epoxides known as leukotoxin and isoleukotoxin. Both epoxides are believed to be protoxins that upon hydrolysis by sEH are converted to the cytotoxic isoleukotoxin- and leukotoxin diols (Figure 5) (Moghaddam *et al.*, 1997).

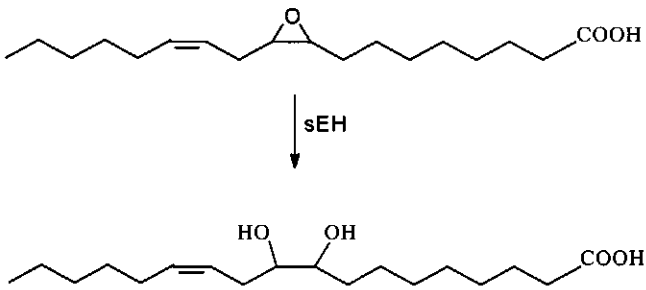


Figure 5. Hydrolysis of leukotoxin into leukotoxin diol by sEH.

Two genetic polymorphisms, altering the sEH amino acid sequence, were identified and characterized. The first concerns a substitution of an Arg at amino acid position 287 by a Glu. The second involves the insertion of an Arg following Ser at position 402 resulting in two adjacent Arg residues at positions 403 and 404 instead of the more common Arg at position 403. Soluble EH proteins containing the Arg insertion exhibit strikingly lower activity, which may have important physiological implications (Sandberg *et al.*, 2000).

In rodents, sEH is induced by a diverse group of compounds collectively termed peroxisome proliferators. Among these are clofibrate, di(2-ethylhexyl)phthalate, *p*-chlorophenoxyacetic acid and 2-ethylhexanoic acid. In general, peroxisome proliferation is accompanied by increased sEH activity (Meijer and DePierre, 1988).

Chalcone oxide and derivatives thereof e.g. 4-phenylchalcone oxide are very potent inhibitors of sEH (Mullin and Hammock, 1982). In addition to these, sEH activity is also inhibited by *trans*-3-phenylglycidols (Dietze *et al.*, 1991), ureas and carbamates (Morisseau *et al.*, 1999b) and metals such as Zn^{2+}, Cd^{2+}, Hg^{2+} and Cu^{2+} (Draper and Hammock, 1999).

Cholesterol 5,6-oxide hydrolase

Cholesterol 5,6-epoxide hydrolase is an enzyme, which is, like mEH, predominantly present in liver microsomes but has also been detected in all other tissues tested (Aström *et al.*, 1986). There is no protein purification protocol reported for cholesterol 5,6-epoxide hydrolase, nor has the gene or cDNA encoding this enzyme been isolated. It hydrolyzes cholesterol 5,6α-epoxide and cholesterol 5,6β-epoxide to cholestane 3β, 5α, 6β-triol solely (Figure 6) (Watabe *et al.*, 1981; Nashed *et al.*, 1985). However, the α-epoxide is a better substrate than the β-epoxide (Nashed *et al.*, 1985) and enzymatic activity is inhibited by the product of the reaction through a competitive mechanism (Sevian and McLeod, 1986).

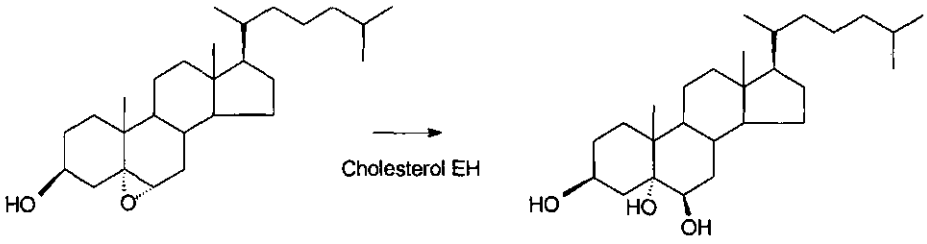


Figure 6. Conversion of cholesterol 5,6α-epoxide into cholestane 3β, 5α, 6β-triol by cholesterol EH.

Furthermore, cholesterol 5,6-epoxide hydrolase is inhibited by iminocholestanols (Watabe *et al.*, 1981; Nashed *et al.*, 1985), 7-ketocholesterol and 6- or 7-ketocholestanol (Sevanian and McLeod, 1986), 7-dehydrocholesterol 5,6β-oxide (Nashed *et al.*, 1986) and detergents such as Emulgen 108 and Lubrol PX (Watabe *et al.*, 1986).

In rodents, cholesterol 5,6-epoxide hydrolase is induced by clofibrate, which is a clinical drug commonly prescribed to patients unable to control serum cholesterol (Finley and Hammock, 1988).

The oxidation of cholesterol proceeds as part of the lipid peroxidation proccess in membranes. Among the most typical cholesterol oxidation products are the, already mentioned, enantiomeric (α and β) cholesterol 5,6-epoxides, which have been shown to be weak direct-acting mutagens. Therefore, it is likely that the cholesterol epoxide hydrolase functions to detoxify the mutagenic cholesterol epoxides (Fretland and Omiecinski, 2000).

However, cholestane 3β,5α,6β-triol is more toxic and a more potent inhibitor of DNA synthesis than the epoxide. In case of low epoxide hydrolase activity mutagenicity, caused by the cholesterol epoxide, is favored. On the other hand, a fast conversion to the cholestanetriol favors cytotoxicity (Sevanian and Peterson, 1984, 1986).

Leukotriene A_4 hydrolase

Leukotrienes are important chemical signaling molecules in a variety of inflammatory and allergic conditions. Leukotriene B_4 is a powerful chemotactic agent that seems to play a role in the recruitment of inflammatory cells to the site of tissue injury (Samuelsson, 1983; Haeggström, 2000). Leukotriene A_4 hydrolase is a cytosolic EH that catalyzes the last step in the biosynthesis of LTB_4: it converts LTA_4 ((5*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) into LTB_4 ((5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid) (Figure 7). However, it does not hydrolyze styrene oxide or *trans*-stilbene oxide (McGee and Fitzpatrick, 1985).

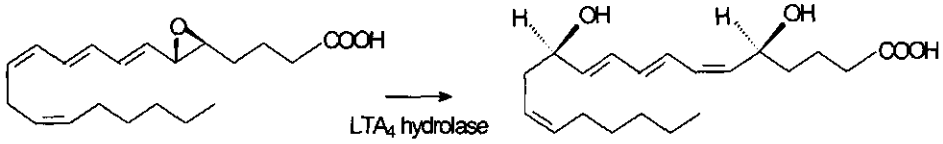


Figure 7. Conversion of LTA_4 into LTB_4 by LTA_4 hydrolase.

LTA_4 hydrolase displays some typical features. First, it is a bifunctional zinc metalloenzyme that, in addition to its EH activity, also possesses an aminopeptidase activity (Haeggström *et al.*, 1990a, 1990b; Minami *et al.*, 1990). It contains a zinc-binding domain, which is required for both enzymatic activities (Haeggström, 2000). Second, in contrast to hydrolysis of epoxides by other EHs, hydrolysis of LTA_4 into LTB_4 by LTA_4 hydrolase does not result in a vicinal diol. Instead, the introduced hydroxyl groups are 8 carbon atoms apart from one another. Third, LTA_4 hydrolase is covalently modified and inactivated by its endogenous substrate LTA_4 during catalysis. After initial binding of LTA_4 to the active site, the hydroxyl group of Tyr378 may attack the reactive epoxide to cause covalent modification and enzyme inactivation (Haeggström, 2000).

LTA_4 hydrolase has been purified from a number of mammalian cells and tissues and the molecular masses of the purified protein varies between 67 and 70 kDa (Evans

et al., 1985; Haeggström *et al.*, 1988; Iversen *et al.*, 1995). Isolated LTA_4 hydrolase-encoding cDNA encodes a protein of 610 amino acids with a corresponding molecular weight of 69 kDa (Funk *et al.*, 1987; Medina *et al.*, 1991; Minami *et al.*, 1995). The human LTA_4 hydrolase-encoding gene is larger than 35 kb and contains 19 exons. A single copy of the gene is present, which was localized to chromosome 12 (Mancini and Evans, 1995).

LTA_4 hydrolase contains 1 mole of zinc, which primary function is catalytic, per mole of protein. However, at zinc concentrations exceeding a 1:1 (metal:enzyme) ratio, zinc is inhibitory to the epoxide hydrolase activity. In addition to zinc, other divalent cations such as Cd^{2+} and Hg^{2+} are effective inhibitors of LTA_4 hydrolase (Wetterholm *et al.*, 1994). The homology of LTA_4 hydrolase to zinc proteases led to the analysis of the inhibitory effect of protease inhibitors on LTA_4 hydrolase activity. As a result of this, bestatin and captopril, inhibitors of aminopeptidases and angiotensin-converting enzyme respectively, were found to inhibit LTA_4 hydrolase too (Orning *et al.*, 1991). Furthermore, more powerful and selective synthetic inhibitors (α-keto-β-amino ester, thioamine and hydroxamate compounds) of LTA_4 hydrolase have been developed in several laboratories (Haeggström, 2000).

Hepoxilin A_3 hydrolase

Arachidonic acid is converted by 12-lipoxygenase to yield (12*S*)-hydroperoxyeicosatetraenoic acid ((12*S*)-HPETE). Hypoxilins A_3 and B_3 are hydroxy epoxide metabolites formed through the rearrangement of (12*S*)-HPETE by a putative hepoxilin synthetase enzyme activity. Changes in intracellular calcium and potassium concentrations as well as changes in secondary messenger systems appear to be the basic biological actions achieved by hepoxilins (Pace-Asciak, 1994).

Hepoxilin A_3 hydrolase is a cytosolic enzyme, which has been purified from the rat liver (Pace-Asciak and Lee, 1989). The molecular mass of the purified enzyme was 53 kDa. The presence of hypoxilin A_3 hydrolase activity in crude homogenates of rat kidney, ovaries, spleen, liver, lung, skin and other tissues indicates the ubiquity of this enzyme in the rat. Both hypoxilin A_3 and B_3 are converted by hypoxilin A_3 hydrolase to their corresponding triols trioxilin A_3 and B_3 (Figure 8). However, hepoxilin A_3 is the better substrate (Pace-Asciak *et al.*, 1983). In addition, hypoxilin A_3 hydrolase is only marginally active toward other epoxides such as leukotriene A_4 and styrene oxide (Pace-Asciak and Lee, 1989).

Trichloropropene oxide, an inhibitor of mEH, also inhibits the hepoxilin epoxide hydrolase from intact human platelets (Margalit *et al.*, 1993). Human neutrophils contain specific hepoxilin A_3 binding sites, which might implicate a receptor-mediated action of this epoxide (Reynaud *et al.*, 1996).

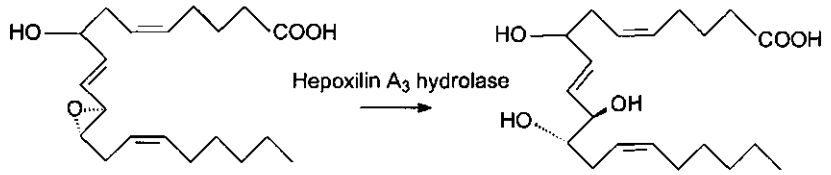


Figure 8. Conversion of hepoxilin A_3 into trioxilin A_3 by hepoxilin A_3 hydrolase.

The knowledge of hepoxilin A_3 hydrolase is rather limited when compared to the other mammalian epoxide hydrolases. Isolation and characterization of hepoxilin A_3 hydrolase-encoding cDNAs or genes would increase the knowledge of this epoxide hydrolase and its relation to the other epoxide hydrolases.

PLANT EH

One of the first reports on epoxide hydrolase activity in plants described the hydrolysis a fatty acid epoxide, 18-hydroxy-9,10-epoxystearic acid, to 9,10,18-trihydroxystearic acid by a 3000 × *g* precipitate from an apple skin homogenate (Croteau and Kolattukudy, 1975). The common precursor of the former compound, 9,10 epoxystearic acid, is formed by the epoxidation of oleic acid (Blée and Schuber, 1990; Pinot *et al.*, 1992). In soybean, 9,10-epoxystearic acid is a better EH substrate than 18-hydroxy-9,10-epoxystearic acid. Furthermore, the enantioselective hydrolysis of *cis*-9,10-epoxystearic acid results exclusively in the formation of *threo*-(9*R*,10*R*)-dihydroxystearic acid (Figure 9), which is a naturally occurring metabolite in higher plants (Blée and Schuber, 1992b). All the fatty acid compounds mentioned above are major components of the plant protective biopolymer cutin (Kolattukudy, 1981). Therefore, plant EH is likely to be involved in the biosynthesis of this plant envelope polymer. In addition, soybean EH also hydrolyzes linoleic acid mono-epoxides. An example of such an epoxide is 9,10-epoxy-12-octadecenoate, which is also known as the leukotoxin found in mammals (Blée and Schuber, 1992a).

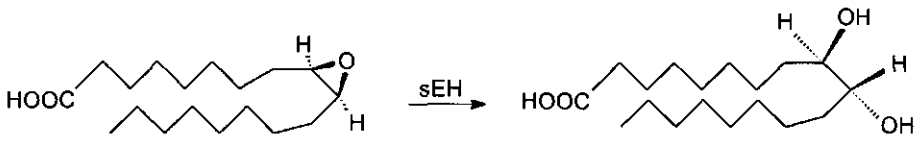


Figure 9. Hydrolysis of (9*R*,10*S*)-epoxystearic acid into *threo*-(9*R*,10*R*)-dihydroxystearic acid by soybean sEH.

In addition to the involvement in cutin biosynthesis, fatty acid epoxides as well as the EH generated hydroxylated derivatives play an important role in the plant defense mechanism against plant pathogens i.e. plant pathogenic fungi. For example, a trihydroxy-octadecenoic acid is produced in plant suffering from rice blast disease and it exerts a growth inhibitory effect against the rice blast fungus *Pyricularia oryzae* (Kato *et al.*, 1985).

Furthermore, an enzyme similar to soybean EH is involved in the biosynthesis of dodecano-4-lactone in ripening nectarines. Therefore, plant EH might also be important in the biosynthesis pathway of fruit aroma components (Schöttler and Boland, 1996).

In addition to these natural lipid epoxides, plant EH is also able to hydrolyze benzyl- or phenyl- substituted epoxides such as *trans*-1,3- diphenylpropene oxide and *trans*-stilbene oxide (Morisseau *et al.*, 2000).

Fatty acid epoxide hydrolase activity is present in numerous plant species (Stark *et al.*, 1995). The enzyme has been purified from soybean seedlings as a soluble 64-kDa dimeric protein with an apparent subunit mass of 32 kDa (Blee and Schuber, 1992c). However, the soluble EH purified from soybean seed was present as a monomeric enzyme with a molecular mass of 36 kDa (Arahira *et al.*, 2000). Furthermore, recombinant soluble EHs from cress and potato were expressed as monomeric proteins with masses of 36 and 39 kDa respectively. In addition to the proteins, plant EH-encoding genes and cDNA sequences have been isolated too (Stapleton *et al.*, 1994; Kiyosue *et al.*, 1994; Guo *et al.*, 1998; Arahira *et al.*, 2000). The EH cDNAs encode proteins of 312-321 amino acids in length with corresponding molecular masses of 35-39 kDa. Amino acid sequence comparison studies indicate that these plant soluble epoxide hydrolases are very similar to mammalian sEH. The main difference is the absence of the first approximately 235 N-terminal amino acids of mammalian sEH in plant sEH, i.e. plant sEHs are missing the N-terminal domain of the mammalian sEHs (Kiyosue *et al.*, 1994; Stapleton *et al.*, 1994; Guo *et al.*, 1998).

The expression of soluble plant EH-encoding genes is induced after exposure to stress-like situations. The cress sEH gene is induced by plant hormones such as auxin and by draught stress (Kiyosue *et al.*, 1994). Potato sEH mRNA accumulates on wounding and application of the plant hormone methyl jasmonate (Stapleton *et al.*, 1994). The tobacco sEH gene is induced during the resistance response to tobacco mosaic virus (Guo *et al.*, 1998) and the soybean sEH gene is induced by the phytohormone ethylene (Arahira *et al.*, 2000).

Plant sEH is inhibited by 4-fluorochalcone-oxide and *trans*-3-phenylglycidol in a similar way as mammalian sEH (Kiyosue *et al.*, 1994; Stapleton *et al.*, 1994). Other substituted chalcone oxides are potent inhibitors of plant sEH. However, 4-phenyl-chalcone-oxide does not inhibit plant sEH, while it is an excellent inhibitor of mammalian sEH (Morisseau *et al.*, 2000). Furthermore, the mEH inhibitor 1,1,1-trichloropropene-2,3-epoxide only weakly inhibits plant sEH (Pinot *et al.*, 1997a).

A few reports discuss the existence of an additional plant EH located in the microsomal fraction of plant cells (Blée and Schuber, 1992c; Pinot *et al.*, 1997a).

Despite these indications, more research is necessary to confirm the presence of a mEH-type plant EH.

INSECT EH

Juvenile hormones (JHs) I, II and III are epoxide-containing sesquiterpenoids which regulate embryogenesis, larval growth and development, metamorphosis and reproduction in insects. The corpora allata secrete JHs into the hemolymph of the insect and during development and reproduction hemolymph JH concentrations change. JHs biosynthesis and degradation determine the JH titer in the hemolymph. Two metabolic enzymes, JH esterase (JHE) and JH epoxide hydrolase (JHEH), are involved in the degradation of JHs and thereby regulating JH titers (Figure 10) (de Kort and Granger, 1996).

In addition, insect EH activity has been associated with the clearance of pheromone concentrations from insect sensory tissues. Analogues of disparlure, an epoxide-containing pheromone, have been shown to be inhibitory to gypsy moth EH and therefore to pheromone metabolism (Graham and Prestwich, 1994).

EH activity, both soluble and microsomal, has been demonstrated in insects such as tobacco hornworm (*Manduca sexta*) (Touhara and Prestwich, 1993), southern armyworm (*Spodoptera eridania*) (Mullin and Wilkinson, 1980), bulb mite (*Rhizoglyphus robini*) (Cohen *et al.*, 1993), fruit flies (*Drosophila melanogaster*) (Harshman *et al.*, 1991) and the cabbage looper (*Trichoplusia ni*) (Hanzlik and Hammock, 1988). The enzyme associated to the microsomal compartment (JHEH) has been purified and contains a molecular weight of 46-50 kDa (Touhara and Prestwich, 1993; Mullin and Wilkinson, 1980).

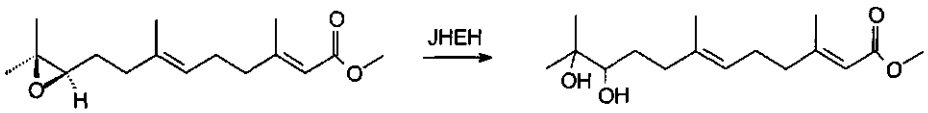


Figure 10. Conversion of juvenile hormone III into its corresponding diol by JHEH.

Although JHs are its endogenous substrates, insect EH shows high activity toward monosubstitued epoxides such as 1,2-epoxyoctane and styrene oxide and lower activity toward *cis*-1,2-distubstituted epoxides such as cyclohexene oxide (Mullin and Wilkinson, 1980). Therefore, an additional role for the insect EH might be the detoxification of harmful epoxides. Interestingly, EH activity is considerably induced (4-5 fold) in mites ingesting *trans*-stilbene-oxide-impregnated filter papers or plant material from onions and garlic. Nevertheless, tobacco hornworm JHEH showed highest activity toward JH III. The relative activities for other substrates such as JH I,

JH II, *trans*- and *cis*-1,3-diphenylpropene oxide, *trans*- and *cis*-stilbene oxide and *cis*-9,10-epoxystearic acid were all less than 16% compared to JH III (100%) (Debernard *et al.*, 1998).

Phytochemicals such as sinigrin, flavone, menthol, *trans*-β-carotene, chalcone and *trans*-cinnamic acid decrease bulb mite EH activity (Cohen *et al.*, 1993). In addition, several epoxides such as 1,1,1-trichloro-2,3-epoxypropane and mixed-function oxidase inhibitors inhibit southern armyworm EH (Mullin and Wilkinson, 1980). Recently, two classes of inhibitors, a glycidol-ester series and an epoxide-ester series, of JHEH from the cabbage looper were developed and found to be highly effective (Linderman *et al.*, 2000).

Insect JHEH-encoding cDNAs have been isolated from tobacco hornworm (Wojtasek and Prestwich, 1996) and cabbage looper (VanHook Harris *et al.*, 1999). These cDNA sequences encode proteins of 462 and 463 amino acids respectively. The respective predicted amino acid sequences share 44% and 39% sequence identity to human mEH. Southern blot analysis suggests that multiple epoxide hydrolase genes are present in the cabbage looper (VanHook Harris *et al.*, 1999).

FUNGAL EH

Fungi

The plant pathogenic fungus *Fusarium solani pisi* was one of the first fungi found to contain an epoxide hydrolase activity (Kolattukudy and Brown, 1975). A cell-free extract prepared from cutin grown *F. solani pisi* cells catalyzed the hydrolysis of a cutin epoxide-containing monomer, 18-hydroxy-9,10-epoxyoctadecanoic acid (18-hydroxy-9,10-epoxystearic acid), to 9,10,18-trihydroxyoctadecanoic acid. Interestingly, less than 6 % of this activity was observed when cells were grown on glucose instead of cutin. Therefore, the epoxide hydrolase of this fungus is induced by cutin (monomers). Moreover, it might take care of the detoxification of toxic epoxy acids, which are released from the plant by fungal hydrolysis of the cutin-polymer thus making a successful invasion possible. However, 9,10-epoxystearic acid, another cutin monomer, as well as styrene oxide were not readily hydrolyzed by this fungus (Kolattukudy and Brown, 1975).

Other fungal epoxide hydrolase activities have been reported and are involved in the metabolism of phenanthrene by white rot fungi (Bezalel *et al.*, 1996; Sutherland, 1991) or coincide with secondary metabolite pigment production in dematiaceous fungi (Grogan *et al.*, 1996).

In addition, multiple EHs are believed to be related to the production of host-specific toxins by the tomato pathogenic fungus *Alternaria alternata f. sp. lycopersici* (Morisseau *et al.*, 1999c). With molecular masses of 20-25 kDa, these EHs are different from sEH and mEH. One of the EH activities was located in the soluble fraction of a cell extract and hydrolyzes its possible physiological substrate 9,10-epoxystearic acid more efficiently than the model sEH and mEH substrates *trans*- and *cis*-stilbene oxide

and *trans*- and *cis*-diphenylpropane oxide. *In vivo* treatment of the fungal culture with the peroxisome proliferator clofibrate stimulated EH activity by 83% and enhanced toxin production 6.3 fold. Potent inhibitors of mammalian sEH e.g. 4-fluorochalcone oxide also inhibited this fungal sEH activity (Pinot *et al.*, 1997b).

The EH from *A. niger* has been purified to homogeneity using *p*-nitrostyrene oxide as substrate. This soluble enzyme is a tetramer composed of four identical subunits with a molecular mass of 45 kDa. A DNA probe for the enzyme has been obtained by genomic PCR using degenerate primers derived from internal peptide sequences (Morisseau *et al.*, 1999a). Subsequently, the gene has been cloned and characterized. It encodes a polypeptide of 398 amino acids with a calculated molecular weight of 44.5 kDa. The deduced amino acid sequence of this soluble *A. niger* EH shares significant sequence similarity to membrane bound mammalian mEH. However, the mEH N-terminal membrane anchor sequence is missing in the fungal enzyme, which explains its soluble character (Arand *et al.*, 1999).

Yeasts

Information on biological functions of yeast EHs is very limited. Nevertheless, an EH activity has been demonstrated in the biosynthesis route of the aroma compounds γ-decalactone and γ-dodecalactone in the lactone producing yeast *Sporidiobolus salmonicolor*. The EH involved catalyzes the hydrolysis of 9,10- and 11,12-epoxystearic acid esters, which is the first step in this pathway (Haffner and Tressl, 1998).

Following research on the potential of fungal and bacterial EH activities, a number of different yeast strains have been screened for the hydrolysis of styrene oxide (Weijers and de Bont, 1999). From this study, *Rhodotorula glutinis* CIMW 147 was selected for further analysis. It exhibits a remarkably broad substrate range including aryl-, alkyl- and alicyclic epoxides. High enantioselectivity was demonstrated in the hydrolysis of methyl substituted aryl and aliphatic epoxides, whereas lower selectivity was seen with terminal epoxides (Weijers, 1997). *Meso*-compounds 2,3-epoxybutane, 1,2-epoxycyclopentane and 1,2-epoxycyclohexane are also accepted, which is of special interest since enantiopure *trans* diols could be obtained with a theoretical yield of 100% (Weijers and de Bont, 1999). In general, the asymmetric hydrolysis of *meso*-epoxides by microbial epoxide hydrolases is rare (Archer, 1997). The hydrolysis of a homologous range of unbranched 1,2-epoxyalkanes (Figure 11) revealed that both enantioselectivity and reaction rate are strongly influenced by the chain length of the epoxide. The enantioselectivity was optimal in the hydrolysis of 1,2-epoxyhexane, while 1,2-epoxyheptane was converted at the highest initial reaction rate (Weijers *et al.*, 1998).

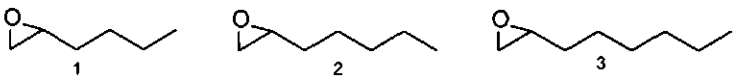


Figure 11. 1,2-Epoxyalkanes, substrates of the *R. glutinis* EH. 1, 1,2-Epoxyhexane. 2, 1,2-Epoxyheptane. 3, 1,2-Epoxyoctane.

Low enantioselectivity in the hydrolysis of 1,2-epoxyoctane by many EHs prompted Botes *et al.* (1998) to screen for a 1,2-epoxyoctane enantioselective EH containing yeast strain. A total number of 187 yeast strains from 25 genera were analyzed. Of these, 8 yeast strains belonging to the basidiomycetes genera *Rhodotorula*, *Rhodosporidium* and *Trichosporon* hydrolyzed 1,2-epoxyoctane in an asymmetric way. *Rhodotorula araucariae* CBS 6031 and *Rhodosporidium toruloides* CBS 349 exhibited excellent enantioselectivity for this aliphatic epoxide. Taken together, it is evident that substrate specificity and enantioselectivity in basidiomycetes yeasts are strain dependent (Botes *et al.*, 1998).

The EH from *R. glutinis* CIMW 147 is membrane associated and has been purified after solubilization by Triton X-100. The native enzyme exists as an homodimeric protein with a subunit molecular mass of 45 kDa. Peptide analysis revealed an amino acid sequence very similar to that of mEH (Kronenburg *et al.*, 1999). Additionally, the membrane associated EH from *R. toruloides* CBS 349 was purified to homogeneity. The enzyme has an apparent monomeric molecular mass of 54 kDa. A carbohydrate content of over 42% indicates heavy glycosylation of this yeast EH (Botes, 1999). In addition to the EH activities of *R. glutinis* CIMW 147 and *R. toruloides* CBS 349, the EH activities of other related *Rhodotorula* and *Rhodosporidium* as well as *Trichosporon* species were membrane associated (Botes *et al.*, 1999b).

The *R. toruloides* enzyme is inactivated by chemical residue-specific modification of Asp/Glu, His and Ser residues. In addition, metals such as Co^{2+}, Hg^{2+}, Ag^{2+}, Mg^{2+} and Ca^{2+} inhibited enzyme activity, whereas EDTA increased enzyme activity (Botes *et al.*, 1999a).

Detergents have been shown to affect the *R. glutinis* EH. Several non-ionic detergents stimulated both the specific activity of the enzyme as well as the enantioselectivity. Thesit and sucrosemonolaurate had the most pronounced effect (Kronenburg and de Bont, 2001).

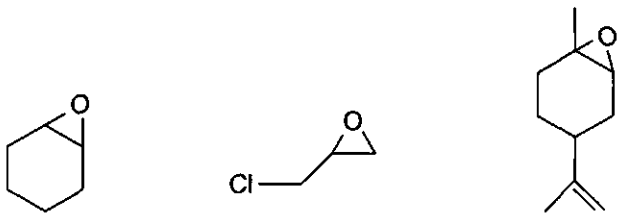
Kull *et al.* (1999) have cloned and characterized a bifunctional leukotriene A_4 hydrolase from *S. cerevisiae*, which is 42% identical to the human LTA_4 hydrolase. The human LTA_4 hydrolase converts LTA_4 into LTB_4. However, *S. cerevisiae* LTA_4 hydrolase does not. In the hydrolysis of LTA_4, a compound identified as (5*S*,6*S*)-dihydroxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid was produced instead of LTB_4 ((5*S*,12*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid). It has been suggested that, as no leukotriene biosynthesis and LTA4 formation has been described in yeast,

the *S. cerevisiae* LTA_4 hydrolase active site accommodates some other lipid, which is structurally related to LTA_4 (Kull *et al.*, 1999).

BACTERIAL EH

Initially, research on bacterial epoxide hydrolases was focused on the use of these enzymes as asymmetric biocatalysts in the process of obtaining enantiopure epoxides and/or vicinal diols. Therefore, most EH-containing bacteria were isolated in epoxide hydrolase screening programs for the hydrolysis of certain types of epoxides (Mischitz *et al.*, 1995; Zocher *et al.*, 2000). Especially the group of K. Faber (Graz, Austria) analyzed a great number of different bacteria for their abilities to hydrolyze epoxides. Among these bacterial species, which showed interesting EH activity, are several *Rhodococci* (Hechtberger *et al.*, 1993; Faber *et al.*, 1996), *Corynebacterium* sp. UPT 9 (Mischitz *et al.*, 1995), *Mycobacterium paraffinicum* NCIMB 10420 and *Nocardia* species (Kroutil *et al.*, 1996). In general, 2,2- and 2,3-disubstituted epoxides are hydrolyzed with good to excellent enantioselectivity by these bacterial enzymes (Orru and Faber, 1999).

Contrary to the "random" screening of bacterial strains in order to find an EH-containing bacterium, other bacteria were isolated by selection for growth on an epoxide as sole source of carbon and energy. Thus indicating a specific metabolic function of these enzymes. *Corynebacterium* sp. C12 was isolated on cyclohexene oxide (Carter and Leak, 1995), while *Agrobacterium radiobacter* AD1 was isolated on epichlorohydrin (van den Wijngaard *et al.*, 1989) (Figure 12).



1 2 3

Figure 12. Chemical structures of cyclohexene oxide (1), epichlorohydrin (2) and limonene-1,2-epoxide (3).

The enzymes purified from *Corynebacterium* sp. C12 and *A. radiobacter* AD1 have subunit sizes of 32 and 35 kDa respectively. While the *Corynebacterium* sp. C12 EH was found to be a multimeric (probably tetrameric) protein, the *A. radiobacter* AD1 EH was isolated as a monomeric protein (Misawa *et al.*, 1998; Jacobs *et al.*, 1991).

The genes encoding the *Corynebacterium* sp. C12 and *A. radiobacter* AD1 enzymes have been isolated and encode polypeptides of 287 amino acids (32 kDa) and 294 amino acids (34 kDa) respectively. Amino acid sequence comparison to protein

databases indicates that these bacterial enzymes are similar to one another and to the sEHs found in mammals and plants (Misawa *et al.*, 1998; Rink *et al.*, 1997).

While bacterial sEHs are expressed constitutively, the expression level is induced to a much higher level by the growth substrates cyclohexene oxide and *trans*-dihydroxycyclohexane as was observed for the *Corynebacterium* sp. C12 epoxide hydrolase (Carter and Leak, 1995).

Recently, a novel degradation pathway of the monoterpene limonene, a plant secondary metabolite, by *Rhodococcus erythropolis* DCL14 was reported and includes the conversion of limonene-1,2-epoxide (Figure 12) to limonene-1,2-diol (van der Werf *et al.*, 1999b). The enzyme involved, limonene-1,2-epoxide hydrolase (LEH), is induced when *R. erythropolis* DCL14 is grown on monoterpenes. LEH was purified to homogeneity and found to be a monomeric cytoplasmic enzyme of only 17 kDa. LEH exhibits narrow substrate specificity, i.e. of several compounds tested only limonene-1,2-epoxide, 1-methylcyclohexene oxide, cyclohexene oxide and indene oxide were substrates. LEH belongs to a novel class of epoxide hydrolases (van der Werf *et al.*, 1998).

EHs AND THE α/β HYDROLASE FOLD FAMILY OF ENZYMES

Soluble and microsomal EH belong to the α/β hydrolase fold family of enzymes

Amino acid sequence comparison of mammalian sEH and mEH reveals no clear similarity or relation between these two enzymes. However, both proteins share significant sequence similarity to the bacterial haloalkane dehalogenase (Arand *et al.*, 1994), which is a member of the α/β hydrolase fold family (Franken *et al.*, 1991). Based on the sequence similarity to haloalkane dehalogenase, it was hypothesized that sEH and mEH are distantly related enzymes that have evolved, together with haloalkane dehalogenase, from a common ancestral protein. Moreover, sEH and mEH probably belong to the α/β hydrolase fold family of enzymes, which hydrolyze epoxides via an enzyme-substrate-ester intermediate (Arand *et al.*, 1994).

Consequently, the related structures of these enzymes contain a highly similar α/β sheet, which consists of β-sheets connected by α-helices (Figure 13) (Ollis *et al.*, 1992). The best-conserved structural features of the fold are the loop-borne elements of a catalytic triad. The order of the catalytic amino acid residues in the primary sEH and mEH structure is always a nucleophilic residue, an acidic residue and a perfectly conserved His residue. The nucleophilic residue, Asp, is situated in a nucleophile "elbow" and fits in the conserved motif: s-x-n-x-s-s, where "s" is a small residue, "x" is any residue and "n" is the nucleophile. The acidic residue is either a Glu or Asp.

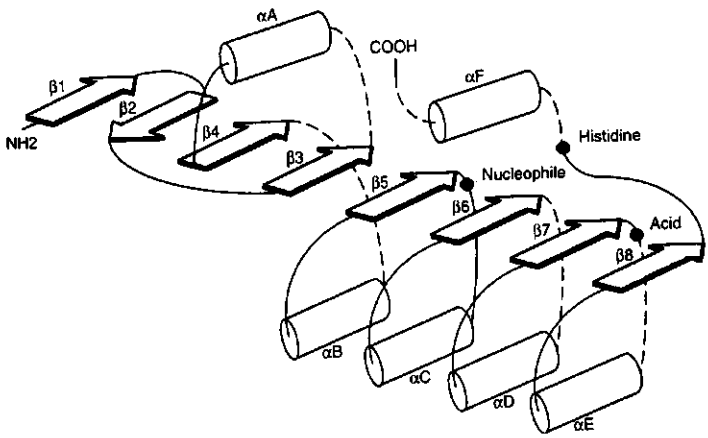


Figure 13. Schematic representation of the α/β hydrolase fold. β1-β8, β-sheets. αA-αF, α-helices. The positions of the members of the catalytic triad are indicated (Nucleophile, Acid and Histidine).

The hydrolysis reaction occurs in two steps starting with the formation of a covalent intermediate formed between the nucleophile and the substrate. This intermediate is stabilized by another conserved motif, the oxyanion hole. The covalent intermediate is hydrolyzed in the second step by a water molecule, which is activated by the Asp/Glu-His pair.

The gene evolution of epoxide hydrolases was further studied by Beetham *et al.* (1995). The amino acid sequence relationships of mammalian sEH to plant sEH, mEH, haloalkane dehalogenase and haloacid dehalogenase were analyzed. Homology was found between the carboxy-terminal residues (230-554) of mammalian sEH and haloalkane dehalogenase, plant sEH and mammalian mEH. The amino-terminal residues (1-229) of mammalian sEH are homologous to haloacid dehalogenase.

The homology between haloalkane dehalogenase and haloacid dehalogenase does not indicate relatedness between these enzymes. Mammalian sEH was suggested to derive from a gene fusion based on the homology of the amino- and carboxy-terminal residues to the respective dehalogenases. Additionally, the homology of mammalian sEH and mEH suggest that they derive from a gene duplication, probably of an ancestral bacterial (epoxide) hydrolase gene (Beetham *et al.*, 1995).

The relatedness of mammalian sEH, mEH, JHEH, plant sEH, bacterial sEH and fungal EH is illustrated in figure 14.

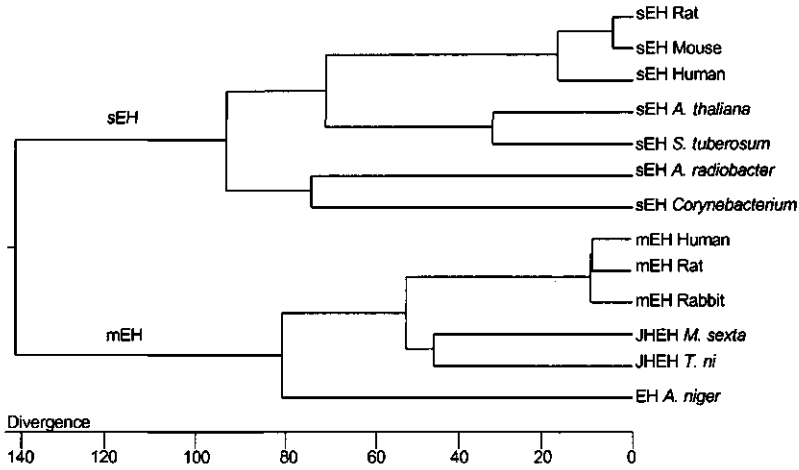


Figure 14. Phylogenetic tree of several α/β hydrolase fold EHs. EH, epoxide hydrolase. sEH, soluble EH, mEH, microsomal EH. JHEH, juvenile hormone EH. *A. thaliana, Arabidopsis thaliana. S. tuberosum, Solanum tuberosum. A. radiobacter, Agrobacterium radiobacter. M. sexta, Manduca sexta. T. ni, Trichoplusia ni. A. niger, Aspergillus niger.*

The enzymatic mechanism of α/β hydrolase fold EHs

Initially, conversion of epoxides by EHs was thought to occur by direct hydrolysis. In this mechanism a specific histidine residue of the enzyme activates a water molecule by proton abstraction. Subsequently, the activated water hydrolyzes the epoxide by nucleophilic attack (Hanzlik *et al.*, 1976; DuBois *et al.*, 1978). However, an alternative two-step-mechanism was proposed (Armstrong *et al.*, 1980). In this mechanism, an acidic amino acid residue of the EH performs a nucleophilic attack at the epoxide ring, which results in a covalent intermediate between substrate and enzyme by means of an ester bond. Then, a histidine activated water molecule hydrolyzes this bond resulting in the diol product and the regenerated enzyme.

The first experimental results supporting this mechanism came from single-turnover experiments showing the transfer of radio-labeled oxygen (^{18}O) from mEH to 1,10-phenanthroline 5,6-oxide, which indicates the formation of a substrate-enzyme ester intermediate (Lacourciere and Armstrong, 1993). Additionally, the results of other biochemical experiments suggests the covalent linkage of Asp^{333} of murine sEH to its

substrate (Borhan *et al.*, 1995; Pinot *et al.*, 1995). Furthermore, a putative covalent enzyme-substrate intermediate was isolated by the brief exposure of murine sEH to radio-labeled JHIII (Hammock *et al.*, 1994). Definitive proof for the formation of an enzyme-substrate-intermediate was reported by Müller *et al.* (1997). An enzyme-substrate-ester intermediate between rat mEH and ^{14}C-labeled *cis*-9,10-epoxystearic acid was visualized by denaturing SDS protein gel electrophoresis and subsequent fluorography. The radio-active signal of the mEH-substrate intermediate could be suppressed by inclusion of the competitive inhibitor 1,1,1-trichloropropene oxide in the mEH/*cis*-9,10-epoxystearic reaction mixture. In a similar way, binding of ^{14}C-labeled *cis*-9,10-epoxystearic acid to rat sEH was demonstrated.

Based on homology to haloalkane dehalogenase the members of the catalytic triad for sEH (Asp^{333}-Asp^{495}-His^{523}) and mEH (Asp^{226}-Glu^{404}-His^{431}) were predicted (Arand *et al.*, 1994; Beetham *et al.*, 1995). These candidate catalytic residues have been changed in the respective enzymes by site directed mutagenesis. As predicted, murine sEH mutants with changes in the positions of Asp^{333}, Asp^{495}, and His^{523} were completely inactive (Pinot *et al.*, 1995; Arand *et al.*, 1996). Similarly, in several other reports site specific mutants of mEH revealed Asp^{226}, Glu^{404} and His^{431} to constitute the catalytic triad (Bell and Kasper, 1993; Laughlin *et al.*, 1998; Tzeng *et al.*, 1998; Arand *et al.*, 1999). Members of the catalytic triad of other α/β hydrolase fold EHs have been identified and align with the catalytic residues of the respective sEH and mEH enzymes (Rink *et al.*, 1997; Arand *et al.*, 1999).

Enzyme kinetics studies show that for most EHs the first step of the reaction proceeds significantly faster than the second step, which is therefore rate limiting (Tzeng *et al.*, 1996; Rink *et al.*, 1998; Arand *et al.*, 1999). For example, the mEH rate constant of the first step is three orders of magnitude higher than the rate constant of the second step with glycidyl-4-nitrobenzoate as substrate (Tzeng *et al.*, 1996).

X-ray structures of α/β hydrolase fold EHs

Recently, crystal structures of the EHs from *A. radiobacter* AD1, murine liver cytosol and *A. niger* were reported (Nardini *et al.*, 1999; Argiriadi *et al.*, 1999; Zou *et al.*, 2000). The information of the enzyme's structure confirms previous found data and also shows new aspects of the way EHs operate.

The first three-dimensional EH structure was reported for *A. radiobacter* AD1 EH (Nardini *et al.*, 1999). This enzyme shows a two-domain structure: a core domain showing the typical α/β hydrolase fold topology and a second mainly α-helical domain, which lies like a cap on top of the core domain. The active site cavity, with the catalytic residues Asp^{107} and His^{275}, is located in a predominantly hydrophobic environment between these two domains. However, the acidic member of the catalytic triad, Asp^{246}, is forced away from the active site due to crystal packing forces. A side chain of Gln^{134} takes the vacant space and thereby mimics a bound substrate. A tunnel, connecting the back of the active site cavity and the surface of the enzyme, provides access to the active site for the catalytic water molecule. The nitrogen atoms of Phe^{108} and Trp^{38} form the oxyanion hole.

Importantly, two previously unanticipated tyrosine residues (Tyr^{152} and Tyr^{215}) are the only acidic functional groups present in the active site (Nardini *et al.*, 1999, 2001). These cap domain located tyrosine residues polarize and activate the epoxide ring by hydrogen bond formation, which facilitates the nucleophilic attack by Asp^{107}. As a result of the formation of the covalent bond between the primary carbon atom of the epoxide and the carboxylic side chain of Asp^{107} a negative charge emerges on the epoxide oxygen. This newly formed oxyanion is stabilized by dislocation of the negative charge to one of the tyrosines by transferring a proton (Rink *et al.*, 1999, 2000).

The crystal structure of murine sEH revealed that the sEH is a dimer (Argiriardi *et al.*, 1999). Each sEH monomer is composed of an N-terminal (Arg^{4}-Gly^{218}) and a C-terminal domain (Val^{235}-Ala^{544}), which are connected by a 16-residue proline-rich linker (Thr^{219}-Asp^{234}). The C-terminal domain contains the active site for epoxide hydrolysis. Its structure is similar to that of haloalkane dehalogenase and *A. radiobacter* AD1 EH, which have α/β hydrolase folds. The N-terminal domain contains a vestigial active site, which is similar to that of haloacid dehalogenase. The vestigial domain plays an important structural role by stabilizing the dimer, but does not participate in epoxide hydrolysis. The active site of sEH is located at the base of a, hydrophobic pocket-containing, L-shaped cavity. In addition to the catalytic triad (Asp^{333}-Asp^{495}-His^{523}), a tyrosine residue (Tyr^{465}) was identified, which may function in epoxide activation and opening (Argiriardi *et al.*, 1999). Indeed, both Tyr^{381} and Tyr^{465} are required for full catalytic activity (Yamada *et al.*, 2000). Additionally, the x-ray structures of alkylurea inhibitors complexed with murine sEH show hydrogen bond interactions between the inhibitors and Tyr^{381} and Tyr^{465}. This suggest that these tyrosine residues serve as general acid catalysts that facilitate epoxide ring opening in the first step of the hydrolysis reaction (Argiriadi *et al.*, 2000).

A. niger EH is a soluble enzyme with an amino acid sequence, which is similar to the amino acid sequence of the membrane bound enzyme mEH. However, it lacks the N-terminal membrane anchor sequence of mEH. The catalytic triad has been identified as Asp^{192}, Asp^{348} and His^{374}. The acid residue of the charge relay is an Asp residue (Asp^{348}), whereas the corresponding residue in all other mEHs is a Glu residue. The presence of Asp^{348} contributes to the high epoxide turnover number of this fungal enzyme when compared to other EHs. In the hydrolysis of 4-nitrostyrene oxide by *A. niger* EH the first step in the reaction procedure is rate limiting, which is in contrast to the common second step of hydrolysis of the ester intermediate (Arand *et al.*, 1999). Despite these differences, the x-ray structure of *A. niger* EH is of special interest because of its relationship to the very important mammalian epoxide-metabolizing enzyme mEH.

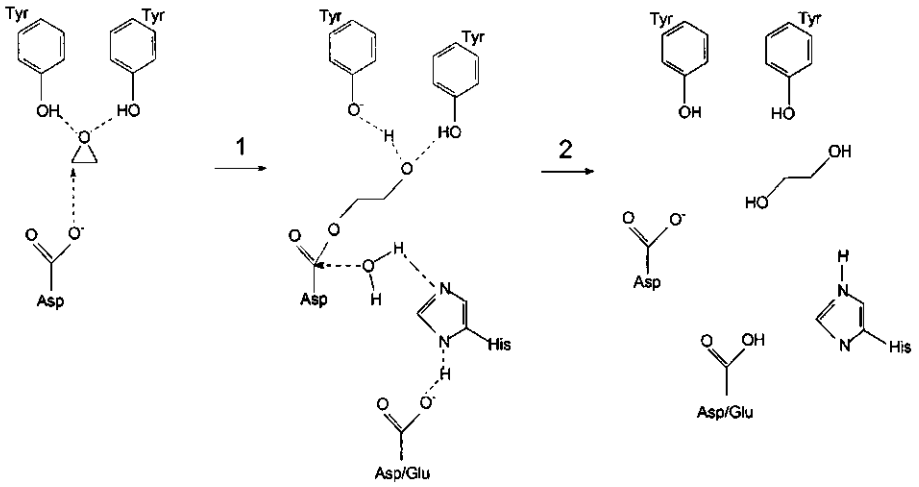


Figure 15. Schematic presentation of the reaction mechanism of α/β hydrolase fold EHs. In the first step, the epoxide, which is positioned by tyrosine residues, is attacked by the Asp nucleophile. An enzyme-substrate-ester intermediate is formed. The intermediate is hydrolyzed in the second step by an Asp/Glu-His pair-activated water molecule. This results in the formation of the diol and the regenerated enzyme.

The x-ray structure of *A. niger* EH revealed a dimer consisting of two 44 kDa subunits. Each subunit is composed of three parts. First, there is a core α/β hydrolase fold, which contains the active site residues. Second, a lid or cap is present, which caps the active site and protrudes from the α/β hydrolase fold. The third part is an N-terminal meander that in turn caps the lid. The dimer interface results primarily from the interaction of the lids and the tips of the N-terminal meanders. The catalytic triad Asp^{192}, Asp^{348} and His^{374} have been found on expected positions in the crystal structure. In analogy to the other EH crystal structures, two tyrosine residues, Tyr^{251} and Tyr^{314}, have been identified which probably act as proton donors for the epoxide ring, thereby facilitating ring opening in the first step of the reaction (Zou *et al.*, 2000).

The structure of *A. niger* EH has been suggested to be representative for the class of mEHs (Zou *et al.*, 2000). Therefore, new insights could be given on several unanswered questions concerning mEH. For example, the inability of mEH to hydrolyze bulky trans-substituted epoxides could be explained by the shape of the substrate-binding site: the catalytic nucleophile waits for the substrate at the end of a relative narrow hydrophobic tunnel. Inhibition of mEH by fatty acid amides could be explained by the binding of these inhibitors to the two proton donating tyrosine residues. The N-terminal part of mEH that precedes the core α/β hydrolase fold is unique to mEHs and could offer the possibility of mEH to interact with other xenobiotic metabolizing enzymes such as cytochrome P450-dependent

monooxygenases. However, based on the *A. niger* EH structure a possible role of mEH in bile acid transport was rejected (Zou *et al.*, 2000).

The reaction mechanism of α/β hydrolase fold EHs can be summarized as follows: In the first step, the epoxide moiety of the substrate is bound and activated by active site located tyrosine residues to facilitate ring opening. A covalent intermediate between enzyme and substrate is formed upon nucleophilic attack of de epoxide ring by the nucleophilic residue. In the second step, the histidine and acidic residue of the so-called charge relay activate a water molecule by proton abstraction, which in turn hydrolyzes the covalent enzyme-substrate ester intermediate. This step results in the diol-product and regenerated enzyme (Figure 15).

Aspects concerning the catalytic mechanisms of non α/β hydrolase fold EHs

Detailed insight into the catalytic mechanism of the zinc metalloenzyme LTA_4 hydrolase has been obtained by determination of its crystal structure. The structure does not show the specific topology of an α/β hydrolase fold. Based on the LTA_4 structural data a reaction mechanism of LTA_4 hydrolysis has been proposed, which is different from that of the α/β hydrolase fold EHs (Thunnissen *et al.*, 2001).

Contrary to LTA_4 hydrolase, no information is available on the catalytic mechanism of hepoxilin hydrolase. Therefore, no comparison of this enzymatic activity to other EH activities can be made.

Microsomal EH catalyzes epoxides by a base-catalyzed mechanism, whereas cholesterol 5,6-oxide hydrolase operates by an acid-catalyzed mechanism (Nashed *et al.*, 1986). Müller *et al.* (1997) have visualized the covalent intermediates between two EHs, mEH and sEH, and their substrates, which is typical for α/β hydrolase fold EHs. However, the same experimental approach could not reveal covalent binding of cholesterol epoxide to cholesterol 5,6-oxide hydrolase. Therefore, the enzymatic mechanism and structure of cholesterol 5,6-oxide hydrolase are different from those of sEH and mEH.

LEH from *R. erythropolis* DCL14 belongs to a novel class of EHs, which is different from the α/β hydrolase fold EH class. For example, the nucleophilic attack of LEH on 1-methylcyclohexene oxide takes place at the more substituted carbon atom, which suggests an acid catalyzed reaction mechanism (van der Werf *et al.*, 1999a).

Table 1. Overview of the diversity and properties of cloned epoxide hydrolases known to date.

Organisms	Epoxide hydrolase	α/β hydrolase fold[a]	Enzyme character	Typical substrates
Mammals	Microsomal EH (mEH)	Yes	Membrane bound	Polycyclic hydrocarbon epoxides
	Soluble EH (sEH)	Yes	Soluble	Fatty acid epoxides, *trans*-stilbene oxide
	Leukotriene A_4 hydrolase	No	Soluble	Leukotriene A_4
Plants	EH[b]	Yes	Soluble	Fatty acid epoxides
Insects	Juvenile hormone EH[c]	Yes	Membrane bound	Juvenile hormones I, II and III
Fungi	*Aspergillus niger* EH[c]	Yes	Soluble[d]	Styrene oxide derivatives
	Rhodotorula glutinis EH[c] (this thesis)	Yes	Membrane associated	1,2-Epoxyalkanes, styrene oxide derivatives
	Xanthophyllomyces dendrorhous EH[c] (this thesis)	Yes	Membrane associated	1,2 Epoxyhexane, 1-methyl-cyclohexene oxide
	Saccharomyces cerevisiae leukotriene A_4 hydrolase	No	Soluble	Leukotriene A_4
Bacteria	*Agrobacterium radiobacter* EH[b]	Yes	Soluble	Epichlorohydrin
	Corynebacterium sp. EH[b]	Yes	Soluble	Cyclohexene oxide
	Limonene-1,2-epoxide hydrolase	No	Soluble	Limonene-1,2-epoxide

[a], Based on literature data such as crystal structures, enzymatic mechanisms and amino acid sequence similarity studies. [b], similar to mammalian sEH. [c], similar to mammalian mEH. [d], the *A. niger* EH is a soluble enzyme despite of its similarity to membrane bound mammalian mEH. References are in the text.

APPLICATION OF MICROBIAL EHs IN FINE ORGANIC CHEMISTRY

Production of enantiopure epoxides using EHs

Due to their chemical versatility, enantiopure epoxides are recognized as high-value intermediates in fine organic chemistry. This is especially true for the synthesis of biologically active compounds such as clinical drugs. Indeed, in order to produce safer drugs, these products have to be prepared as single enantiomers (Archelas and Furstoss, 2001).

The methods used for the production of enantiopure epoxides are based on chemical or biological catalysts. Chemically, epoxides are synthesized by e.g. epoxidation of alkenes catalyzed by metals. Biologically, enzymes or cells are used in e.g. the direct or indirect epoxidation of alkenes by monooxygenases or peroxidases, respectively. Alternatively, enzymatic resolution of chemically produced racemic epoxides by e.g. lipases and epoxide hydrolases is an attractive alternative methodology for the production of an enantiopure epoxide (Figure 16). These chemical and biological methodologies have been reviewed by several authors (de Bont, 1993; Besse and Veschambre, 1994; Archelas and Furstoss, 1997, 1999).

EHs are interesting promising biocatalysts for the production of enantiopure epoxides for a number of reasons. They are cofactor-independent enzymes and only need water for hydrolysis. Consequently, EH-catalyzed production of enantiopure epoxides is a clean alternative for the chemical procedure involving toxic metal catalysts. EHs are ubiquitous in nature. Therefore, if a specific epoxide conversion is desired it seems possible to screen for the epoxide hydrolase, which can perform this conversion. Furthermore, microbial EHs can be produced in large amounts from various microorganisms. Consequently, research on the application of EHs for the synthesis of enantiopure epoxides and diols has focused on microbial EHs and not on e.g. mammalian EH due to the low amounts of available enzyme. EHs can be partly purified and used as an enzymatic powder without significant loss of activity upon storage. They are active in the presence of organic solvents, which allows the use of hydrophobic substrates. They often lead to excellent enantiopurity of the remaining epoxide and also in some cases of the formed diol, which itself can be used as a chemical building block (Archelas and Furstoss, 1997; Swaving and de Bont, 1998).

A disadvantage of the enzymatic resolution of a racemic epoxide is the maximum yield of 50% for the remaining enantiopure epoxide. Nevertheless, EHs are interesting biocatalysts and may enable the preparation of enantiopure epoxides starting from cheap and readily available racemic epoxides (Archelas and Furstoss, 2001).

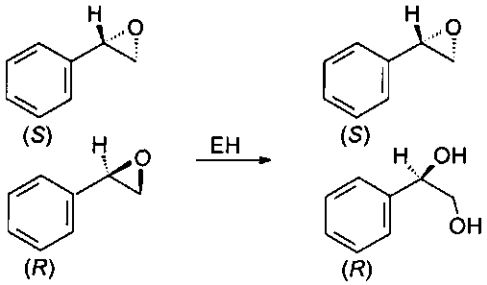


Figure 16. Example of the enantioselective hydrolysis of an epoxide by an EH. The (*R*)-enantiomer of a racemic styrene oxide mixture is hydrolyzed into (*R*)-1-phenyl-1,2-ethanediol. The (*S*)-enantiomer of styrene oxide is not converted. Consequently, enantiopure (*S*)-styrene oxide remains at a maximal yield of 50% of the racemic epoxide mixture.

Microbial EHs in epoxide biocatalysis

A general correlation between certain microbial EHs and the substitutional pattern of various types of epoxides has been reported (Orru and Faber, 1999). Yeast strains of the genera *Rhodotorula* and *Rhodosporidium* give the best enantioselectivities with monosubstituted epoxides. Fungi such as *A. niger* and *B. bassiana* give best results with styrene oxide type substrates. Bacterial EHs, especially *Actinomycetes* such as *Rhodococcus* and *Nocardia* sp. give the best enantioselectivities with highly 2,2- and 2,3-disubstituted epoxides (Orru and Faber, 1999). These data might be used as a general guideline to make a choice for the potentially best EH for the enantioselective hydrolysis of a certain epoxide of interest. In addition, new EH activities are reported continuously (van der Werf *et al.*, 1999b; Zocher *et al.*, 2000).

Enantiocomplementary EH activities have been reported for both fungal and bacterial strains (Archelas, 1998; Krenn *et al.*, 1999). For example, the residual epoxide of hydrolysis of *trans*-2-methyl-1-pentyloxirane by the fungus *Syncephalastrum racemosum* had the (1*R*,2*R*) configuration, while the opposite enantiomer was isolated using *Chaetomium globosum*. Another example concerns the enantiocomplementary EH activities of *A. niger* and *B. bassiana* toward styrene oxide and *para*-substituted styrene oxides (Archelas, 1998). Bacterial EHs of matching opposite enantiopreference have been found among several species. While *Rhodococcus* and *Nocardia* spp. hydrolyze (*S*)-2,2-disubstituted epoxides, methylotrophic bacteria such as *Mycoplana rubra* and *Methylobacterium* spp. exhibited a preference for the (*R*)-enantiomers. Thus, the stereochemical course of the reaction can be controlled by choosing the appropriate microorganism (Krenn *et al.*, 1999).

Most reports on EH-containing fungi are related to the use of these organisms in the production of enantiopure epoxides and or diols. For example, Zhang *et al.* (1995) screened 80 fungal strains to find an EH activity capable of catalyzing the kinetic

resolution of racemic indene oxide into (1*S*,2*R*)-indene oxide, which is a precursor to a side chain of the HIV protease inhibitor MK 639. *Diplodia gossipina* ATCC 16391 was found to be the best catalyst (Zhang *et al.*, 1995). Instead of the conversion of a specific epoxide, Moussou *et al.* (1998) screened 42 fungal strains using racemic 1,2-epoxyhexane and 1-methyl-1,2-epoxypentane as substrates. Seven strains, *Aspergillus niger* LCP 521, *Aspergillus terreus* CBS 116-46, *Beauveria bassiana* ATCC 7159, *Chaetomium globosum* LCP 679, *Cunninghamella elegans* LCP 1543, *Mortierella isabellina* ATCC 42613 and *Syncephalastrum racemosum* MUCL 28766, were of interest. The group involved (R. Furstoss, Marseille, France) further analyzed *A. niger* LCP 521 and *B. bassiana* ATCC 7159 (formerly named *Beauveria sulfurescens*) with a focus on the asymmetric synthesis of enantiopure epoxides and or diols. They showed the first examples of several-gram-scale enantioselective hydrolysis of geraniol epoxide and limonene epoxide derivatives (Chen *et al.*, 1993) as well as substituted styrene oxide derivatives (Pedragosa-Moreau *et al.*, 1996) using these fungi.

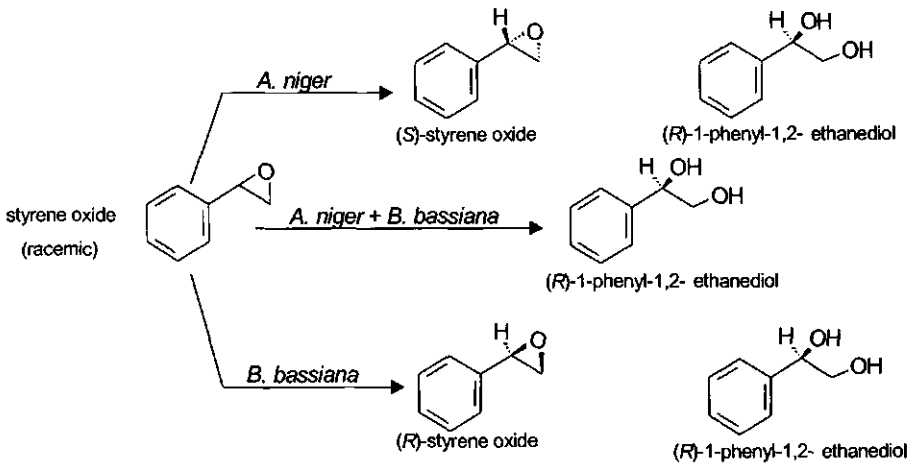


Figure 17. Enantioselective styrene oxide hydrolysis by *A. niger* and *B. bassiana*. For details, see text.

Interestingly, enantiocomplementary styrene oxide hydrolysis was demonstrated by *A. niger* and *B. bassiana* (Figure 17). *A. niger* hydrolyzes (*R*)-styrene oxide while *B. bassiana* hydrolyzes (*S*)-styrene oxide. Moreover, both fungi give rise to the (*R*)-diol. Therefore, *A. niger* and *B. bassiana* could be used to obtain these respective compounds with high enantiopurity (Pedragosa-Moreau *et al.*, 1993).

Alternatively, lyophilized cells of *Nocardia* EH1 gave almost enantiopure (2*R*,3*R*)-heptane-2,3-diol as the sole product in a 79% yield in the hydrolysis of racemic *cis*-2,3-epoxyheptane (Kroutil *et al.*, 1996). These data indicate the versatility and usefulness of microbial EHs in the synthesis of enantiopure epoxides and diols.

Additional research has been performed to stabilize, improve and / or manipulate EH activity and enantioselectivity. For example, immobilization of a partially purified EH from *Nocardia* EH1 through ionic binding onto DEAE-cellulose stabilized the enzyme resulting in a 225% increase in activity when compared to free enzyme. Moreover, the stabilized immobilized enzyme could be successfully employed in repeated batch conditions, which was not the case for whole cell reactions (Kroutil *et al.*, 1998). Non-ionic detergents such as thesit and sucrosemonolaurate have been shown to affect the *R. glutinis* EH. Both the specific activity of the enzyme as well as the enantioselectivity were stimulated (Kronenburg and de Bont, 2001). Another way of stabilizing EH activity and, additionally, improving enantioselectivity has been reported by means of immobilized imprinting of *R. glutinis* EH (Kronenburg *et al.*, 2001). In this technique, EHs are derivatized with itaconic anhydride, imprinted by substrates (imprinters) and co-polymerized with ethylene glycol dimethacrylate to form a bioplastic. After removal of the imprinter an enzyme with rationally modified properties is obtained, which resulted in e.g. reversion of enantiopreference.

Preparative scale production of enantiopure epoxides

Extra points of attention need to be considered when enantiopure epoxides are prepared on a larger scale. Analytical scale EH-catalyzed epoxide hydrolysis is usually performed in aqueous media. A drawback of these aqueous media is that most epoxides are poorly water soluble and that epoxides are unstable in aqueous buffer systems. The chemical non-enantioselective hydrolysis of epoxides can result in a drop in yield of the enantiopure residual epoxide. Furthermore, chemical epoxide hydrolysis hampers the production of enantiopure diols. To avoid these problems organic solvents have been applied to improve both epoxide stability and solubility (Nellaiah *et al.*, 1996; Choi *et al.*, 1999). However, organic solvents have a toxic effect and are consequently inhibitory to EH activities (Goswami *et al.*, 1999; Choi *et al.*, 1999).

Despite this, several preparative-scale EH-catalyzed hydrolytic resolutions, reviewed by Archelas and Furstoss (2001), are conducted under various conditions with epoxide concentrations of up to 2 M, in batch reactors with one or two phases and even in plain water. Choi *et al.* (1999, 2000) reported a very interesting system. In their cascade two-phase hollow-fiber membrane bioreactor the aqueous cell suspension of the biocatalyst *R. glutinis* is circulated through the shell sides of each hollow-fiber membrane module. The organic solvent dodecane, which was used to dissolve 1,2-epoxyhexane, circulates through the lumen side. Therefore, solvent toxicity is minimized. Two additional modules are used to extract enantiopure (*S*)-1,2-epoxyhexane and inhibitory levels of formed 1,2-hexanediol. The continuously operated system allows long-term operation, which leads to mass production of (*S*)-1,2-epoxyhexane. Based on the calculated volumetric productivity (633 $\mu mol^{-1} \cdot l^{-1} \cdot h^{-1}$) the authors suggest that this system is potentially useful for large-scale industrial applications since a volumetric productivity of at least 200 ($\mu mol^{-1} \cdot l^{-1} \cdot h^{-1}$) is needed to be economically viable (Choi *et al.*, 2000).

However, due to the increasing environmental pressure on fine chemical industries, the employment of environmentally friendly processes is encouraged. The use of toxic chemicals such as organic solvents should be reduced or even eliminated. In line with this, several reports describe the hydrolysis of high amounts of epoxides (second phase) in plain water instead of buffer / organic solvent solutions (Genzel *et al.*, 2001; Manoj *et al*, 2001).

Nonetheless, EHs have been shown to be applicable in the chemoenzymatic synthesis of valuable target-compounds. Among these are (*S*)-Ibuprofen, which is a strong non-steroidal anti-inflammatory drug, (*R*)-Nifénalol, which is a β-adrenergic blocker, and Eliprodil, which is a neuroprotective agent (Cleij *et al.*, 1999; Pedragosa-Moreau *et al.*, 1997; Manoj *et al.*, 2001).

Recombinant DNA technology in EH research

Research on the use of EHs in biocatalysis has mainly been focused on microbial EHs, because microorganisms can be cultivated in large amounts. Consequently, an "unlimited" source of biocatalyst is available. EHs from e.g. mammalian sources were simply not available in sufficient quantities. However, the isolation of EH encoding genes from various organisms (including microorganisms) and the over-expression of these genes in suitable hosts opened the way to evaluate the use of EHs from all sources in biocatalysis as well as the biological research focused on the characterization of the EH protein and function.

For example, enantiopure (2*R*,3*R*)-3-phenylglycidol has been prepared with a 31% yield using human sEH, which was produced in the baculovirus expression system (Williamson *et al.*, 2000). The preparation of (S)-2-pyridyloxirane has been established using a recombinant *A. niger* strain in which the *A. niger* LCP521 EH encoding gene was over-expressed (Genzel *et al.*, 2000). *Escherichia coli* has been used to express EH genes to e.g. analyze enantioselective epoxide hydrolysis (Lutje Spelberg *et al.*, 1998) or to obtain sufficient EH protein for crystallographic studies (Nardini *et al.*, 1999; Zou *et al.*, 2000) or to analyze site specific EH mutants (Arand *et al.*, 1999a).

The elucidation of EH structures and enzymatic mechanisms are a major step towards the design of mutant EHs with improved characteristics. Some interesting observations concerning EH activity and enantioselectivity have already been reported. The replacement of the mEH amino acid residue Glu^{404} with Asp leads to a strongly increased the V_{max} of the enzyme. A 23-fold increase was found with styrene oxide, while a 39-fold increase was observed with 9,10-epoxystearic acid (Arand *et al.*, 1999b). Additionally, substitution of Tyr^{215} by Phe in the *A. radiobacter* EH results in a 2 to 4 fold increase in enantioselectivity toward styrene oxide and derivatives thereof (Rink *et al.*, 1999). Thus recombinant DNA technology has already proven to be very useful in EH research and it will facilitate both the development of mutant EHs with improved characteristics and the production of sufficient EH for biocatalytic purposes in the near future.

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Chapter 3

Isolation and Characterization of the Epoxide Hydrolase Encoding Gene from *Xanthophyllomyces dendrorhous*

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ABSTRACT

The epoxide hydrolase-encoding gene (*EPH1*) from the basidiomycetous yeast *Xanthophyllomyces dendrorhous* was isolated. The genomic sequence has an open reading frame of 1236 basepairs, interrupted by 8 introns encoding a polypeptide of 411 amino acids with a calculated molecular mass of 46.2-kDa. The amino acid sequence is similar to that of microsomal epoxide hydrolase and belongs to the α/β hydrolase fold family. The *EPH1* gene was not essential for growth of *X. dendrorhous* in rich medium under laboratory conditions. The Eph1 encoding cDNA was functionally expressed in *Escherichia coli*. A six-fold increase in specific activity was observed using resting cells as compared to *X. dendrorhous*. The epoxides 1,2-epoxyhexane and 1-methylcyclohexene oxide were substrates for both the native and the recombinant Eph1. The isolation and characterization of the *X. dendrorhous* EH-encoding gene is an essential step in developing a yeast EH based epoxide biotransformation system.

INTRODUCTION

Epoxide hydrolases (EHs) catalyze the hydrolysis of epoxides to their corresponding diols. These enzymes have potential industrial uses in fine chemistry as both enantiomerically pure epoxides and 1,2-diols are central building blocks in the asymmetric synthesis of biologically active molecules (25, 40). They are found in a wide variety of both prokaryotic and eukaryotic organisms where they perform different functions. The best studied mammalian EHs are involved in the detoxification of epoxides derived from both xenobiotic compounds and endogenous substrates (2, 22, 30). Plant EHs are believed to be involved in the biosynthesis of cutin (4) and components of plant defense mechanisms (17). Insect EH might be involved in the regulation of juvenile hormone titers by degrading the hormone in concert with juvenile hormone esterase (8). Microbial EHs are found in bacteria and fungi where they perform catabolic functions (5, 6).

Two types of mammalian EH, soluble EH (sEH) and microsomal EH (mEH), are involved in the detoxification of noxious epoxides. Several plant (15, 32) and bacterial (18, 28) EHs are similar to sEH whereas the insect juvenile hormone EH sequence is similar to mEH (42). Both type of EHs belong to the α/β hydrolase fold family of enzymes (1, 28), a large group of proteins that are structurally and mechanistically related (24). Hydrolysis of epoxides by these enzymes occurs via an enzyme-ester-substrate intermediate (1, 16, 19). In addition to the α/β hydrolase fold enzymes, a few EHs belong to other classes of enzymes e.g. leukotriene A_4 hydrolase (10), cholesterol EH (38) and limonene-1,2-epoxide hydrolase (34). EH activity has been described from several fungi e.g. *Rhodotorula glutinis* (39), *Aspergillus niger* (20) and *Beauveria sulfurescens* (26), but no EH-encoding gene from an eukaryotic microorganism has been reported yet.

The broad substrate ranges, high enantioselectivities and reaction rates (40) make yeast EHs promising candidates for industrial applications. However, in most cases wildtype yeast EHs do not meet the exact demands for a specific epoxide conversion in an industrial process. Therefore, our ultimate goal is to develop a yeast EH based biocatalyst with tailor-made substrate specificity and enantioselectivity. Isolation and characterization of a yeast EH encoding gene, thereby obtaining information about the gene and gene product which is needed for the construction of an improved enzyme via genetic engineering, would be a first step to take. The EH activity and the availability of molecular genetic tools (41) prompted us to isolate and characterize the EH-encoding gene from the basidiomycetous yeast *Xanthophyllomyces dendrorhous*.

MATERIALS AND METHODS

Strains, plasmids and DNA manipulations

X. dendrorhous, perfect state of *Phaffia rhodozyma* (12), strain CBS 6938 was used throughout this study. *E. coli* XL1-Blue-MRF' (Stratagene, La Jolla, CA, USA) was used for transformation and expression experiments. The vectors pGEM-T Easy (Promega Benelux BV, Leiden, The Netherlands) and the prokaryotic expression vector pKK223-3 (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) were used for cloning and expression experiments respectively in *E. coli*. For the construction of pEHKO a 750 bp *EPH1* fragment, corresponding to the C-terminal half of the enzyme, with a unique *Eco*RV restriction site was amplified in a PCR using the primers PXD2 and PXD3 and cDNA as template. This PCR product was cloned into pGEM-T Easy and the truncated *EPH1* fragment was released as a 776 bp *Eco*RI fragment from the plasmid. The fragment was purified and ligated into the unique *Eco*RI restriction site of the *X. dendrorhous* transformation vector pPR1T. This newly constructed vector was designated pEHKO. Plasmid pPR1T was constructed by ligating a 0.3 kb *Bam*HI-*Hin*dIII PCR product, corresponding to the glyceraldehyde-3-phosphate dehydrogenase terminator sequence of *X. dendrorhous* (36), into the corresponding restriction sites of the *X. dendrorhous* transformation vector pPR1 (41). If not stated otherwise standard molecular cloning techniques were used (29). Chromosomal DNA was isolated from SDS-lysed protoplasts of *X. dendrorhous* and extracted with phenol/chloroform as described previously (36). Isolation of plasmid DNA from *E. coli* was performed using Qiagen columns (Westburg BV, Leusden, The Netherlands). Isolation of DNA trapped in agarose gel was performed using the QIAEX II Gel Extraction Kit. The non-radioactive DIG DNA Labeling and Detection Kit (Roche Diagnostics Nederland BV, Almere, The Netherlands) was used to label and detect probes in Southern blots or colony hybridizations. The construction of the *X. dendrorhous* genomic and cDNA libraries was described previously (36). The standard nomenclature for yeast genes and proteins was considered to denominate the *X. dendrorhous* epoxide hydrolase gene and protein (33).

Transformation of *X. dendrorhous* and *E. coli* by electroporation

Electrocompetent cells of *X. dendrorhous* were prepared as described by Wery *et al* (41). Electrocompetent cells were mixed with 5 μg of linearized DNA and the mixture was transferred to a precooled electroporation cuvette (0.2 cm). A pulse was given of 0.8 kV, 1000 Ω and 25 μF using a Bio Rad gene pulser (Bio Rad Laboratories BV, Veenendaal, The Netherlands). Immediately 0.5 ml of YePD medium, containing 1% yeast extract, 2% bactopeptone and 2% glucose, was added and the mixture was transferred to a sterile 1.5 ml Eppendorf tube. After incubation for 2.5 hours at 21°C, 100 μl aliquots were spread onto solid YePD plates containing 40 μg/ml G418. Plates were incubated at 21°C until colonies appeared.

E. coli was cultivated in LB (29) medium lacking NaCl to an OD_{600} of 0.5. Cells were washed twice in an equal volume of ice cold demineralized water and once in an equal volume of 10% glycerol in demineralized water. After centrifugation (1,000 × *g*, 10 minutes) cells were resuspended in 0.05 volume of 10% glycerol in demineralized water. Hundred μl of cells were mixed with plasmid DNA and transferred to an electroporation cuvette (0.2 cm). A pulse was given of 2.5 kV, 200 Ω and 25 μF. Immediately 1 ml of SOC medium (29) was added. The transformation mixture was transferred to a 1.5 ml Eppendorf tube and incubated at 37°C for 1 hour. Aliquots were spread onto solid LB agar plates containing 50 μg/ml ampicillin.

Polymerase Chain Reaction

Standard reactions were carried out in an automated thermal cycler (Perkin-Elmer Nederland, Nieuwerkerk a/d IJssel, The Netherlands). The reaction conditions were: 5 min 94°C, followed by 30 cycles: 1 min 94°C, 2 min 45 or 50°C, 1 or 2,5 min 72°C, ending with 10 min 72°C. The oligonucleotide primers used are:

PANEH2: 5'- TCNCTBCCYGGHTAYACNTTYTC-3' (encoding SLPGYTFS of *A. niger* EH).

PANEH4A: 5'- TCVAGWGCWGCRAARTGDCCDCC-3' (encoding antisense GGHFAALE of *A. niger* EH).

PXD2: 5'-*CACTGCCCGGCTATACGTTCTC*CTCTGGTCCGCAACG -3' (encoding LPGYTFSSGPQR of *X. dendrorhous* EH).

PXD3: 5'- *CAAGAGCAGCAAAGTGTCCGCC*TCGAGCATG -3' (encoding antisense HARGGHFAAL of *X. dendrorhous* EH).

Nucleotides printed in italics correspond to a part of the degenerate oligonucleotides PANEH2 and PANEH4A found in pxdeh1.

PROKATG: 5'-TTTGAATTCATGACGTCTGCGACATTCCTAC-3'.

PROKTAA: 5'-AGCTCTGCAGTTAAAGCTCGGAATGATAGTTC-3'.

Restriction sites introduced for subcloning purposes are underlined.

EH enzyme assay

X. dendrorhous strains were cultivated to a high cell density culture for 48 hours at 21°C in 400 ml YePD medium. *E. coli* [pKK223-3] and *E. coli* [precEph1] were grown overnight in 100 ml LB medium supplemented with ampicillin (50 μg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.84 mM). Cells were harvested by centrifugation (1,000 × *g*, 10 minutes) and washed in 50 mM potassium phosphate buffer (pH 7.0). Cell pellets were resuspended in 3 ml 50 mM potassium phosphate buffer (pH 7.0) to obtain a concentrated suspension of resting cells. One ml of these suspensions were mixed with 5 mM of racemic substrate by vortexing in sealed tubes or bottles and incubated shaking at 35°C in a waterbath. To monitor epoxide degradation headspace samples were taken over a period of time and were analyzed using GC analysis on a Chrompack CP9000 gaschromatograph equipped with an α- or β-cyclodextrin 120 chiral column (α- or β-DEX respectively, Supelco Inc.). Racemic 1,2-epoxyhexane (Aldrich, Zwijndrecht, The Netherlands) was analyzed using the β-DEX (detection limit: 50 μM). Racemic 1-methylcyclohexene oxide was analyzed using the α-DEX (detection limit: 50 μM). To determine the protein content, 1 ml of a diluted sample was added to 0.5 ml of 0.5 N NaOH and incubated at 100°C for 30 minutes. Subsequently 100 μl of this preparation was applied in the Bio-Rad *DC* Protein Assay (Bio-Rad).

Nucleotide sequence accession number

The nucleotide sequence of the *EPH1* gene has been deposited in the GenBank database under accession no. AF166258.

RESULTS

Synthesis of a specific EH probe of *X. dendrorhous* by PCR

Initial enzyme localization studies showed that most EH activity was located in the membrane fraction of a cell free extract of *X. dendrorhous* (results not shown). Therefore, we surmised that *X. dendrorhous* contains a mEH. We aligned amino acid (aa) sequences of mEHs from human (31), rabbit (14), rat (27) and *Manduca sexta* (42) and identified conserved regions. Several conserved peptide sequences were used to design degenerate oligonucleotides which could serve as primers in a PCR using *X. dendrorhous* chromosomal DNA as template, but no *X. dendrorhous* EH fragment was amplified. We then used the conserved portion of the amino acid sequence of *Aspergillus niger* EH (11) to design degenerate oligonucleotides using *X. dendrorhous* preferred codons (36). A 1.2 kb fragment was amplified in a PCR using oligonucleotides PANEH2 and PANEH4. This fragment was cloned into pGEM-T Easy, yielding pxdeh1, and the nucleotide sequence of the PCR fragment was determined. Comparison of the deduced aa sequence to protein databases revealed homology to the C - terminal part of eukaryotic mEHs e.g. rat, mouse and rabbit. This

result indicated that a 1.2 kb fragment of the putative *X. dendrorhous* EH encoding gene, denominated *EPH1*, was cloned.

Isolation of the *X. dendrorhous EPH1* gene

The 1.2 kb fragment of pxdeh1 was used to screen a cDNA and a genomic cosmid library of *X. dendrorhous*. The *EPH1* cDNA contained an open reading frame (ORF) of 1236 bp corresponding to a polypeptide of 411 aa with a calculated molecular mass of 46,185 Da. The two highest scores of a comparison of the total Eph1 aa sequence to the swissprot protein database corresponded to pig mEH, 32.5% identity, and rabbit mEH, 32.2% identity. Comparison of the pxdeh1 nucleotide sequence with cDNA sequences revealed the presence of several introns.

Cosmid pEHcos8 hybridized to pxdeh1 in a colony hybridization screen, indicating that this cosmid carries a chromosomal *X. dendrorhous* DNA fragment, containing a part or the entire *EPH1* gene. The nucleotide sequence of the *EPH1* gene was determined by sequencing pEHcos8 initially with specific oligonucleotides designed from the pxdeh1 sequence and subsequently by a primer walking strategy. We sequenced 2931 bp including the *EPH1* gene, 716 bp upstream of the putative translation initiation codon, and 219 bp downstream of the putative TAA stop codon. The *EPH1* ORF is interrupted by 8 introns varying in size between 61 and 110 bp. The promoter region contains putative elements such as a CCAAT box, TATA-box and CAP signal. The 3' nontranslated region contains the sequence TATGTATG...TATGT...TTT, which is similar to 3' nontranslated regions of several *S. cerevisiae* genes (21, 43).

Inactivation of the *EPH1* gene in *X. dendrorhous* by transformation-mediated gene disruption

We inactivated the chromosomal *EPH1* by transformation-mediated gene disruption. We linearized pEHKO (Fig. 1) by digestion with *Eco*RV and introduced it into *X. dendrorhous* CBS6938 by electrotransformation (41). Transformants were selected by G418 resistance. Both *X. dendrorhous* CBS6938 and putative *EPH1*$^-$ deletion mutants, further designated CBS6938[pEHKO], were cultivated for 48 h at 21°C in rich medium (YePD) to reach the stationary growth phase. Apparently, the mutation has no lethal effect on *X. dendrorhous* and *EPH1* is not essential for growth under these laboratory conditions.

Both a racemic aliphatic, 1,2-epoxyhexane, and a racemic alicyclic, 1-methylcyclohexene oxide (MCHO), epoxide were used as substrates to demonstrate Eph1 activity. *X. dendrorhous* hydrolyzed 1,2-epoxyhexane with low enantioselectivity at an initial reaction rate of 4.8 nmol/min/mg protein. MCHO was enantioselectively hydrolyzed at 0.17 nmol/min/mg protein by the wildtype strain. No epoxide hydrolase activity was seen on either substrate using transformants CBS6938[pEHKO]1 and CBS6938[pEHKO]2.

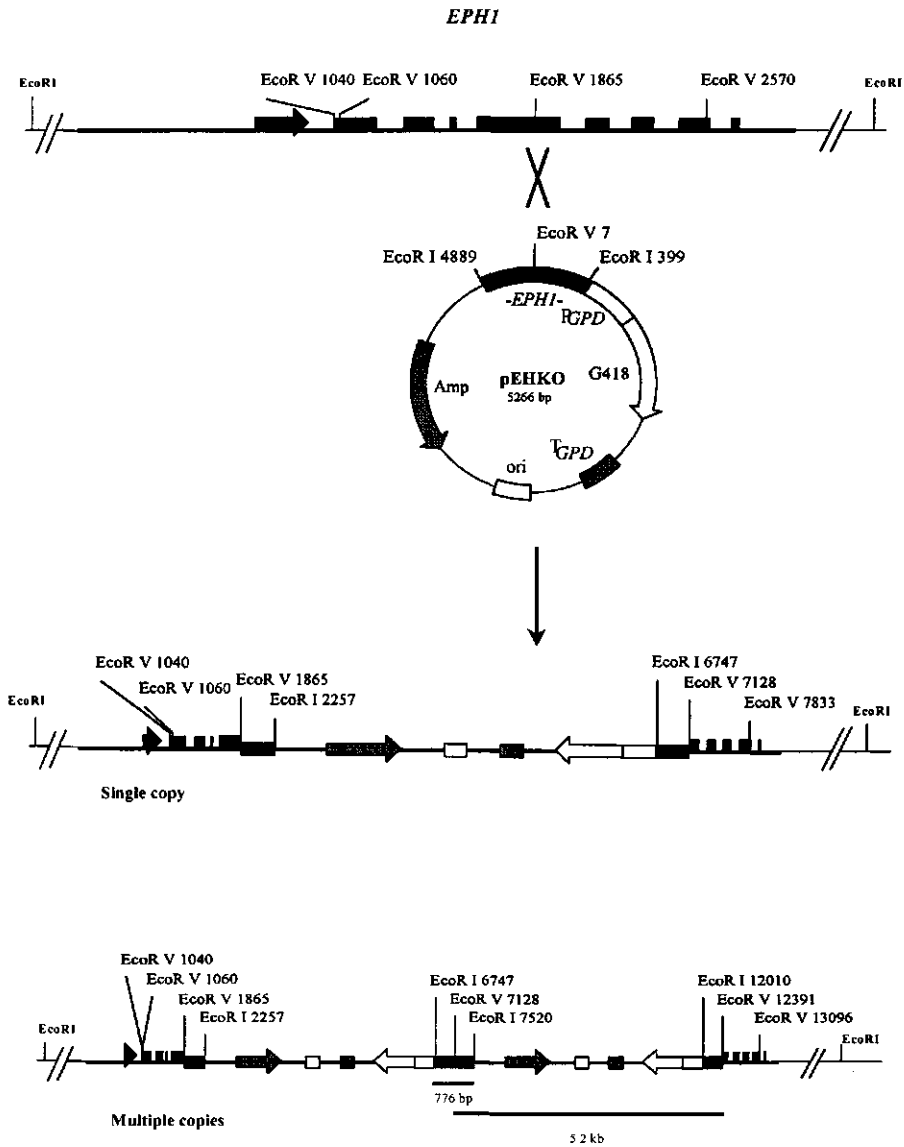

Figure 1. Transformation-mediated inactivation of the *EPH1* gene. Schematic representation of a single copy and multiple copies of plasmid pEHKO integrated into the *EPH1* gene. The first *EPH1* exon is shown by a black box with an arrowhead to indicate the *EPH1* direction. Plasmid pEHKO was constructed by cloning of a 776 bp *Eco*RI PCR fragment, containing the truncated *EPH1* gene copy (corresponding to aa 138-388), in the *X. dendrorhous* transformation vector pPR1T. Prior to electroporation plasmid pEHKO was linearized by digestion with *Eco*RV.

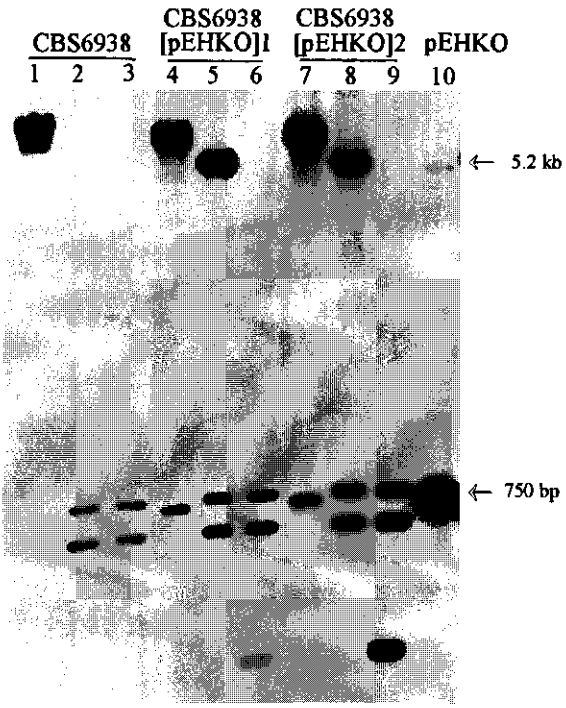

Figure 2. Transformation-mediated inactivation of the *EPH1* gene. Autoradiograph of the Southern blot analysis of chromosomal DNA of wildtype CBS6938 and transformants CBS6938[pEHKO]1 and 2. Lanes 1, 4, 7, chromosomal DNA digested with *Eco*RI. Lanes 2, 5, 8, chromosomal DNA digested with *Eco*RV. Lanes 3, 6, 9, chromosomal DNA digested with *Eco*RI and *Eco*RV. Lane 10, plasmid pEHKO DNA digested with *Eco*RI. The hybridizing DNA fragments corresponding to the size of plasmid pEHKO (5.2 kb) in lanes 5 and 8 and the approximately 750 bp, truncated *EPH1* containing, *Eco*RI fragment of pEHKO in lanes 4, 7 and 10 are indicated by arrows.

We probed DNA from the two transformants on Southern blots with the 1.2 kb *Eco*RI fragment of pxdeh1 (Fig. 2). Wildtype *X. dendrorhous* CBS6938 DNA digested with *Eco*RI resulted in a large (> 10 kb) hybridizing fragment. Digestion of CBS6938[pEHKO] with *Eco*RI resulted in three fragments. The presence of the approximately 750 bp *Eco*RI fragment from CBS6938[pEHKO] could be explained by multiple copy integration of pEHKO in the *EPH1* gene (Fig. 1). This was confirmed by the hybridization patterns of genomic DNA digested with *Eco*RV or *Eco*RI/*Eco*RV. CBS6938 DNA digested with *Eco*RV had two fragments of 705 and 805 bp, whereas CBS6938[pEHKO] DNA digested with *Eco*RV had the expected wildtype pattern plus an additional fragment of 5.2 kb corresponding to size of the linear form of pEHKO. An additional fragment of approximately 385 bp was noticed corresponding to the equally large *Eco*RI-*Eco*RV fragments of pEHKO. A similar pattern was observed by restriction of pEHKO with the same endonucleases.

Functional expression of recombinant Eph1 in *E. coli*

We expressed the Eph1 encoding cDNA in *E. coli*. Therefore, Eph1 encoding cDNA was amplified in a PWO polymerase (Roche Diagnostics Nederland BV, Almere, The Netherlands) catalyzed PCR using the forward primer PROKATG and the reverse primer PROKTAA with *EPH1* cDNA as template. The 1255 bp PCR product was digested with *Eco*RI and *Pst*I and ligated into the corresponding sites of the prokaryotic expression vector pKK223-3 yielding precEph1. *E. coli*[precEph1] was subjected to the EH enzyme assay. The initial reaction rates of *E. coli*[precEph1] towards 1,2-epoxyhexane and MCHO were 31 and 1 nmol/min/mg protein respectively, approximately six fold higher than the values from wildtype *X. dendrorhous*. As expected, no EH activity could be detected using *E. coli*[pKK223-3]. Unlike 1,2-epoxyhexane, MCHO was selectively degraded by both native Eph1 and recEph1 (Fig. 3); 1R,2S-MCHO was degraded faster than 1S,2R-MCHO. The yield of 1S,2R-MCHO, after 1R,2S-MCHO is completely degraded, is 30%.

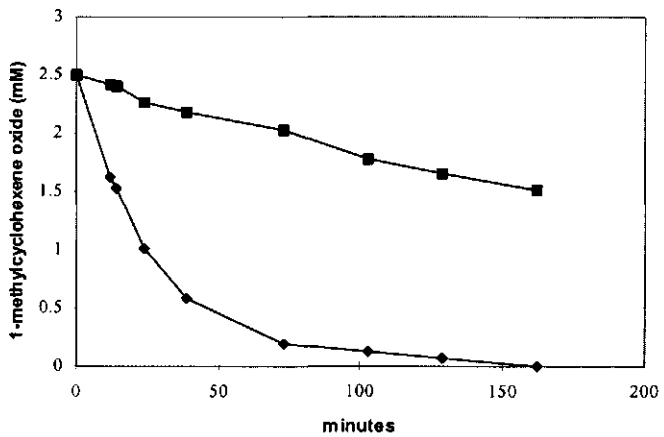

Figure 3. Functional expression of *X. dendrorhous EPH1* in *E. coli*. The hydrolysis of 5 mM of racemic 1-methylcyclohexene oxide was monitored over a period of time. ■, 1S,2R-MCHO (35). ◆, 1R,2S-MCHO (35).

DISCUSSION

Eph1 from the basidiomycetous yeast *X. dendrorhous* resembles that of mammalian mEHs and belongs to the family of α/β hydrolase fold of enzymes which consists of a large number of enzymes including sEH and mEH (1, 24). Several conserved sequence motifs have been found in this family of enzymes especially in the regions of the highly

conserved nucleophile-histidine-acid catalytic triad (24). The consensus of the nucleophile motif is sm-x-nu-x-sm-sm, where sm is a small aa, x is any aa and nu is the nucleophile. The HGXP motif contains the oxyanion hole of the enzyme (1, 16, 24). The three putative members of the Eph1 catalytic triad are an aspartate residue at position 181, corresponding to the nucleophile motif, a glutamate residue at position 359, which aligns to the catalytic acidic residue, and a histidine residue at position 385 could be the catalytic histidine (Fig. 4).

```
Eph1  M----------------------TSANIP--------------------TPFQVSFAQQDVDRMMAKIRDTRLPTAPI   36
RBmEH MLLELLLASVLGFVIYWFVSGDKEESLPLEDGWWGPGSRPVGLEDESIRPFKVETSDEEINDLHQRI--DRIRLTPP   75
RTmEH MWLELVLASLLGFVIYWFVSRDKEETLPLGDGWWGPGSKPSAKEDESIRPFKVETSDEEIKDLHQRI--DRFRASPP   75
      *                          ''''''*                          **'*' '''''' ' ''*  '*'' '*

Eph1  VPGASWDYGIDLDWLTELHKYWANEWSWEETEKRINKYPHFRVDIEEISLHFVHIKSKQ----PDAIPLILSHGWPS  109
RBmEH LENSRFHYGFNSNYLKKILSYWRHEFDWKKQVEILNSYPHFKTKIEGLDIHFIHVKPPQVPPGRTPKPLLMVHGWPG  152
RTmEH LEGSRFHYGFNSNYMKKVVSYWRNEFDWRKQVEILNQYPHFKTKIEGLDIHFIHVKPPQLPSGRTPKPLLMVHGWPG  152
      ' ''''''**'' ''''''' '** '*''* '  ' '* ****''''**''''**'*'*' *    ''' **'' ****'
                                                                                    N
Eph1  SFLEFWEVIDELVDPTKAG---QPAFHVVIPSMPGYTFSSGPQRKGWTVVDTARVYNSLMVNVLGYKTYTCGAGDWGS  184
RBmEH SFFEFYKIIPLLTDPKSHGLSDEHIFEVICPSIPGYGFSQASSKKGFNSVSTARIFYKLMLR-LGFQEFYIQGGDWGA  229
RTmEH SFYEFYKIIPLLTDPKSHGLSDEHVFEVICPSIPGYGYSEASSKKGLNSVATARIFYKLMTR-LGFQKFYIQGGDWGS  229
      ** **'''*  *'***'' *   '' *'*' **'***''* '' '** ' *'***'' '** ' **'''' '''***'

Eph1  WITAQILHDYSEFAVVAHFTMIKASVPILNPIYSLPILLGK-----IPFVPKGVARWLQSLVYTEAEINGLERTDKF  256
RBmEH LVCTNMAQLVPSHVKGLHLNM----ALILRNHYTLTLLLGRRIGGLLGYTERDMELLYP---FKEKVFYSLMRES--  297
RTmEH LICTNMAQMVPNHVKGLHLNM----AFISRSFYTMTPLLGQRFGRFLGYTGKDIELLYP---YKEKVFYSIMRES--  297
       ' ''' '  '' '   *''*    ' * '  *''' ***'      ' '' '''' '  '''* *'  ''*''

Eph1  WKEGLGYQKIQGSKPMTLGAALFDSPVGILSWIGEKYHGWSDPR-----APSAPSQVTPNHIVTVTALYFLTGSIHT  328
RBmEH -----GYMHIRATKPDTVGCALNDSPVGLAAYILEKFSTWTNSEFRDLEDGGLERKFSLQDLLTNIMIYWTTGSIVS  369
RTmEH -----GYLHIQATKPDTVGCALNDSPVGLAAYILEKFSTWTKSEYRELEDGGLERKFSLDDLLVNIMIYWTTGTIVS  369
        ** '**''** *'* *  *****' ''* **' '*''' '  ' '' ' '''' ' '*' **'* '
                                          A                   H
Eph1  SFLPYKEY----TLSPMAVAVGKKRPIGLSIFPAEITQYPRSWVASSC-KLVNYKVHARGGHFAAVDNPGAYVEDIR  400
RBmEH SQRYYKENLGQGFMAHKHERLKVHVPTGFAAFPCEIMHVPEKWVRTKYPQLISYSYMPRGGHFAAFEEPELLARDIC  446
RTmEH SQRYYKENLGEGIMVHKHEGMKVFVPTGFSAFPSELLHAPEKWVKVKYPKLISYSYMERGGHFAAFEEPKLLAQDIR  446
      *  ***         ''  '   *'*.. ** *' ' * '**  '' '*''*'   ******* ''*   ' ** '

Eph1  ETIGKNYHSEL 411
RBmEH KFVG--LVERQ 455
RTmEH KFVS--LAELQ 455
      ' ''    '
```

Figure 4. Amino acid comparison of Eph1 to microsomal EHs. Alignment of the amino acid sequences of Eph1, *X. dendrorhous* epoxide hydrolase (this study), RBmEH, rabbit mEH (14) SwissProt datafile P04068 and RTmEH, rat mEH (27) P07687. *, character to show that a position is perfectly conserved. ', character to show that a position is well conserved. The residues forming the catalytic triad are indicated by N, nucleophile; A, acidic residue; and H, histidine. The aa sequence corresponding to the nucleophile motif is single underlined. The aa sequence corresponding to the HGXP motif is double underlined.

The predicted Eph1 polypeptide (411 aa) of *X. dendrorhous* is shorter than most mammalian mEHs (455 aa) and the juvenile hormone EH (462 aa). These differences are mainly found in the N-terminal regions of these enzymes (Fig. 4). The mEH is cotranslationally inserted into the endoplasmic reticulum (13, 23) using a single membrane anchor segment in the N-terminus of the enzyme (9). This segment is missing from Eph1 and its absence may partly explain the difference in length between the mammalian mEHs and Eph1. Even though Eph1 lacks a membrane anchor segment,

it appears to be membrane associated since most of the epoxide hydrolase activity was found in the membrane fraction of a *X. dendrorhous* cell free extract.

Protein engineering, in which both specific and random amino acids were substituted, has been shown to be a powerful tool for the improvement of enzyme characteristics (7). For mEH it was shown that the replacement of the catalytic acidic residue, Glu at position 404, with an aspartate residue greatly increased the Vmax of this enzyme (3). We demonstrated the expression of *EPH1* cDNA in *E. coli*. This expression system is a powerful tool for the analysis of Eph1 mutants (e.g. Glu359Asp) created by protein engineering. Furthermore, it is a simple system for studying the substrate range of mutant Eph1. In addition, the Eph1 aa sequence information may be helpful to clone other yeast EH-encoding genes.

Recently, we have isolated the EH-encoding gene from the yeast *Rhodotorula glutinis* using the *X. dendrorhous* Eph1 sequence information (37). Comparison of aa sequences and substrate specificities might give new insights for protein engineering experiments.

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Chapter 4

Cloning and characterization of an epoxide hydrolase encoding gene from *Rhodotorula glutinis*

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ABSTRACT

We cloned and characterized the epoxide hydrolase gene, *EPH1*, from *Rhodotorula glutinis*. The *EPH1* open reading frame of 1230 bp was interrupted by 9 introns and encoded a polypeptide of 409 amino acids with a calculated molecular mass of 46.3-kDa. The amino acid sequence was similar to that of microsomal epoxide hydrolase which suggests that the epoxide hydrolase of *R. glutinis* also belongs to the α/β hydrolase fold family. *EPH1* cDNA was expressed in *Escherichia coli* and resting cells showed a specific activity of 200 nmol min^{-1} (mg protein)$^{-1}$ towards 1,2-epoxyhexane.

INTRODUCTION

Epoxide hydrolases (EHs) catalyze the hydrolysis of epoxides to their corresponding diols without the need for a cofactor. EHs have great potential in bio-organic synthesis of fine chemicals. The enantioselective hydrolysis of racemic epoxides by EHs can result in enantiopure epoxides and vicinal diols which are both valuable intermediates in the pharmaceutical and agrochemical industries. These biocatalysts from microbial origin have already been shown to be applicable in fine organic chemistry (Archelas and Furstoss 1998).

Mammalian EHs have been studied in great detail because of their central role in the detoxification of xenobiotic compounds in the liver (Oesch 1973). Genes encoding EHs have been cloned from various sources e.g. mammals, plants, insects and bacteria. Based on amino acid sequence homology studies, these EHs were classified as members of the α/β hydrolase fold family of enzymes which are structurally, functionally and mechanistically related (Ollis et al. 1992, Arand et al. 1994). Moreover, the X-ray structure of bacterial soluble EH (sEH) confirmed that this enzyme is an α/β hydrolase fold enzyme (Nardini et al. 1999). Furthermore, the members of the catalytic triads from soluble (sEH) and microsomal (mEH) EHs have been determined (Arand et al. 1996, Rink et al. 1997, Arand et al. 1999).

In contrast to the EHs described above, the information about EHs from yeasts (Weijers and de Bont 1999) is limited at the molecular level. Botes and co-workers (1998) screened 187 yeast strains from 25 genera for the hydrolysis of 1,2-epoxyoctane. From the 54 EH positive yeast strains only 8 strains, belonging to the basidiomycetous genera *Trichosporon*, *Rhodotorula* and *Rhodosporidium*, showed asymmetric hydrolysis of the epoxide. *Rhodotorula glutinis*, a red pigmented yeast, is able to convert a broad range of epoxides of which some with high enantioselectivity (Weijers 1997). The purification of an EH from *R. glutinis* strain CIMW 147 was recently reported by Kronenburg et al. (1999).

The properties of the *R. glutinis* EH make this enzyme a candidate biocatalyst for the preparative-scale epoxide hydrolysis in fine chemistry. Its application is even more likely if the EH-encoding gene is cloned and over-expressed in a suitable host. In

addition, mutant EHs showing optimal activity and enantioselectivity for an epoxide of interest can be developed via protein engineering. Therefore, we isolated and characterized the EH encoding gene from *R. glutinis* strain CIMW 147 and over-expressed it in *E. coli*.

MATERIALS AND METHODS

Strains, plasmids and DNA manipulations

R. glutinis strain ATCC 201718 (formerly known as strain CIMW 147, Weijers 1997) was used as a source for the EH gene. *E. coli* strain XL1-Blue-MRF' (Stratagene) was used for transformation and expression experiments. The vector pGEM-T Easy (Promega) and the prokaryotic expression vector pKK223-3 (Pharmacia) were used for cloning and expression experiments respectively in *E. coli*. If not stated otherwise standard molecular cloning techniques were used (Sambrook *et al.* 1989). Isolation of plasmid DNA from *E. coli* was performed using Qiagen columns (Westburg BV, Leusden, The Netherlands). Isolation of DNA trapped in agarose gel was performed using the QIAEX II Gel Extraction Kit (Westburg BV, Leusden, The Netherlands).

Total RNA isolation from *R. glutinis*

R. glutinis was cultivated at 30°C in 200 ml glucose containing medium (Weijers 1997) to an OD_{660} = 1. Cells were harvested by centrifugation (16,300 × *g*, 5 minutes, 4°C) and washed in cold sterile water. The cell pellet was resuspended in a minimal volume of cold sterile water. This cell suspension was transferred to a mortar containing liquid nitrogen. Cells were broken by grinding the frozen cell suspension. Total RNA was extracted from the broken cells using RNAzol (Cinna/Biotecx Laboratories International inc., Texas, USA) and dissolved in diethyl pyrocarbonate (DEPC) treated water.

Chromosomal DNA isolation from *R. glutinis*

R. glutinis was cultivated at 30°C in 200 ml glucose containing medium (Weijers 1997) to an OD_{660} = 7. Cells were harvested by centrifugation (16,300 × *g*, 5 minutes, 4°C) and washed in cold sterile water. The cell pellet was resuspended in a minimal volume of cold sterile water. This cell suspension was transferred to a mortar containing liquid nitrogen and 1 gram of alumina type-A5 (Sigma). Cells were broken by grinding the frozen cell suspension. The broken cells were added to a centrifuge tube containing 5 ml of DNA extraction buffer (50 mM Tris, 10 mM $MgCl_2$, 50 mM NaCl, 1% (W/V) SDS, pH 7.4). Chromosomal DNA was isolated from this mixture by repeated phenol/chloroform extractions and an ethanol precipitation step. The co-purified RNA was degraded by incubation with DNase free RNase A (10 µg/ml). A final phenol/chloroform extraction resulted in the purified chromosomal DNA preparation.

PCR and 5'/3' RACE experiments

Polymerase chain reactions were carried out in an automated thermal cycler (Perkin-Elmer). The oligonucleotide primers used in this study are listed in table 1. The reaction conditions were: 5 min 94°C, followed by 30 cycles: 1 min 94°C, 1.5 min 45, 50 or 55°C, 1 or 2.5 min 72°C, ending with 10 min 72°C. The 5'/3' RACE Kit (Roche Diagnostics) was applied for the isolation of partial cDNA sequences of the *R. glutinis* EH.

EH enzyme assay

E. coli [pKK223-3] and *E. coli* [pKKRgEph1] were grown overnight at 37°C in 50 ml Luria-Bertani (LB) medium supplemented with ampicillin (50 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG) (0.84 mM). Cells were harvested by centrifugation (1,000 × *g*, 10 minutes, 4°C) and washed in 50 mM potassium phosphate buffer (pH 7.0). Concentrated cell suspensions were mixed with 20 mM 1,2-epoxyhexane (Aldrich) by vortexing in sealed tubes and incubated shaking at 35°C in a waterbath. To monitor epoxide degradation headspace samples were taken over a period of time and were analyzed using GC analysis on a Chrompack CP9000 gaschromatograph equipped with a β-cyclodextrin 120 chiral column (Supelco). Protein contents were determined using the Bio-Rad *DC* Protein Assay.

Sequence submission

The *EPH1* nucleotide sequence has been deposited in the GenBank database under accession number: AF172998.

Table 1 Oligonucleotide primers used in the PCR experiments.

Primer	Sequence (5'→3')
PEH1	CTBCAYGGWTGGCCNGGHTCNTT
PP19.1	AARTGVCCVCCCTCSGGVGCGTC
SP3.1	CGTCGCCCCTTCGATGCCTG
SP5.1	TCATGAGCTTGTCGAAGACCC
SP5.2	CCAGGCATCGAAGGGGCGACG
PdT	CCCCCCCTCGAGTTTTTTTTTTTTTTTT
PEHATG	TTTTGAATTCATGGCGACACACACATTCGC
PEHTAG	AGCTCTGCAGCTACTTCTCCCACATGACGCC

Restriction sites for subcloning purposes are underlined. B: C, T or G. Y: C or T. W: A or T. N: A, C, T or G. H: A, C or T. R: A or G. V: A, C or G. S: C or G.

RESULTS

Isolation of a chromosomal EH encoding DNA fragment of *R. glutinis* by PCR

Oligonucleotide primer PP19.1 was derived from the amino acid sequence from *R. glutinis* EH peptide P19 (Kronenburg et al. 1999) and oligonucleotide primer PEH1 was derived from the *A. niger* EH amino acid sequence (R. Furstoss and M. Arand, personal communication). A 1.3 kb fragment was amplified in a PCR using primers PP19.1 and PEH1 and chromosomal DNA of *R. glutinis* as template. This fragment was cloned into pGEM-T Easy, yielding pRGEH1, and the nucleotide sequence was determined. The deduced amino acid sequence contained sequences resembling those of peptides P16: GPAYGVMQQLTP(E)(D)F, P27: YHLHNFASK(S)(G)(R) and P30: (S or T or Y)IGTSFLPVSLNPHF from the purified *R. glutinis* EH (Kronenburg et al. 1999). These results suggested that a 1.3 kb fragment of the putative *R. glutinis* EH encoding gene, denominated *EPH1*, was cloned.

Isolation of the *EPH1* gene

Specific *R. glutinis* EH primers, SP3.1, SP5.1 and SP5.2 were designed based on the protein encoding nucleotide sequence of pRGEH1. These primers were applied in the method of rapid amplification of cDNA ends (RACE) using the 5'/3' RACE kit and total *R. glutinis* RNA. The Oligo dT-Anchor primer (5'/3 RACE Kit) and primer SP5.1 were used in a first round of PCR in the 5' RACE reaction. Subsequently, a 479 bp fragment was amplified in a second round of PCR using nested primer SP5.2 and an aliquot of the first round PCR reaction mixture as template. The 3' RACE reaction using primers SP3.1 and PdT resulted in the amplification of a 908 bp DNA fragment. These fragments were cloned into pGEM-T Easy resulting in pEH479 and pEH908 respectively and their nucleotide sequences were determined. The overlapping cDNA fragments in pEH479 and pEH908 were used to reconstruct a full length EH encoding cDNA. It contained an open reading frame (ORF) of 1230 bp corresponding to a polypeptide of 409 amino acids. The calculated molecular mass of 46,295 Da was in agreement with the molecular mass of approximately 45 kDa observed by Kronenburg et al. (1999).

To clone the chromosomal copy of the *R. glutinis* EH gene oligonucleotides PEHATG and PEHTAG were designed based on the terminal sequences of the *EPH1* ORF. A 1825 bp fragment was amplified in a PCR using primers PEHATG and PEHTAG and chromosomal DNA as template. This fragment was ligated into pGEM-T Easy yielding pRGEH2. Comparison of the pRGEH2 nucleotide sequence with that of *EPH1* cDNA revealed that the *EPH1* ORF was interrupted by 9 introns varying in size between 52 and 95 bp (Figure 1).

The two highest scores of a comparison of the deduced Eph1 amino acid sequence to the SwissProt protein database by BLAST search corresponded to human mEH (Skoda et al. 1988), accession number (acc. no.): P07099, 32% identity and rat mEH (Porter et al. 1986), acc. no.: P07687, 31% identity.

Functional expression of *EPH1* in *E. coli*

The first-strand cDNA synthesis reaction mixture from the 3' RACE experiment was used as a template in a PCR using the primers PEHATG and PEHTAG in order to overexpress a full length *EPH1* cDNA in *E. coli*. A fragment of 1250 bp was amplified, purified, digested with endonucleases *Eco*RI and *Pst*I and ligated in the corresponding restriction sites of the prokaryotic expression vector pKK223-3 resulting in pKKRgEph1. Plasmid pKKRgEph1 was introduced into *E. coli* by electroporation and transformants, designated as *E. coli*[pKKRgEph1], were analyzed for epoxide hydrolase activity using 1,2-epoxyhexane as a substrate. Resting cells of *E. coli*[pKKRgEph1] converted 1,2-epoxyhexane at an initial reaction rate of 200 nmol min^{-1} (mg protein)$^{-1}$. As expected, no EH activity could be detected using *E. coli*[pKK223-3]. Comparison of cell free extracts of *E. coli*[pKK223-3] and *E. coli*[pKKRgEph1] by SDS-PAGE showed no accumulation of a 46 kDa protein (results not shown).

DISCUSSION

***R. glutinis* Eph1 is a putative α/β hydrolase fold EH**

The conserved catalytic triad of α/β hydrolase fold enzymes consist of a nucleophilic residue (Asp or Ser), an acidic residue (Asp or Glu) and a perfectly conserved histidyl residue (Ollis et al. 1992). The nucleophile fits to the conserved amino acid sequence motif: sxnsxx, where s is a small amino acid, x any amino acid and n the nucleophilic amino acid (Ollis et al. 1992). However, no clear motifs are present for the catalytic acidic and histidyl residues although in mEHs the catalytic His is situated in the amino acid sequence GGHFAA at the C-terminal end of the enzyme. Another conserved amino acid sequence is the HGXP motif containing the oxyanion hole of the enzyme (Ollis et al. 1992, Lacourciere and Armstrong 1994). In mammalian and plant sEHs the X of the HGXP motif is a Phe residue whereas in the mEHs and bacterial sEH it is a Trp residue.

Figure 1 (opposite page). Nucleotide sequence and deduced amino acid sequence of *R. glutinis EPH1*. The *EPH1* nucleotide sequence is given in lowercase letters. The deduced amino acid sequence is given in the one letter code below the nucleotide sequence. Introns have been numbered and are indicated between brackets. The TAG stop codon is indicated by an asterisk. The regions corresponding to oligonucleotides SP5.1 and SP5.2 are indicated by arrows below the nucleotide sequence. The region corresponding to oligonucleotide SP3.1 is indicated by an arrow above the nucleotide sequence.

```
atggcgacacacacattcgcttcgcctcccacccgcttcaccgtcgacatcccacagtcg     60
 M  A  T  H  T  F  A  S  P  P  T  R  F  T  V  D  I  P  Q  S      20
gaactcgacgaacttcactcgcgactcgacaagacccgctggccggcgacagagatcgtt    120
 E  L  D  E  L  H  S  R  L  D  K  T  R  W  P  A  T  E  I  V      40
ccagaggatgggacggacgatccgacggcgtttgggctcggagcagggccgacgctgccg    180
 P  E  D  G  T  D  D  P  T  A  F  G  L  G  A  G  P  T  L  P      60
ctcatgaaggaattggcgaagggttggcgcgagttcgactggaaaaaggcgcaggaccac    240
 L  M  K  E  L  A  K  G  W  R  E  F  D  W  K  K  A  Q  D  H      80
ctcaacacgtacgggggcttcccaccctctttcgccttgtagtctcgctgacaggttctc    300
 L  N  T[----------------------intron 1----------------------      83
ataacccgcagcttcgagcactacatggtcgaaattgaggacctctcgatccacttcctc    360
----------]  F  E  H  Y  M  V  E  I  E  D  L  S  I  H  F  L      99
caccatcgctcgactcgcccgaacgctgttcccctcatcctctgccacggctggccaggc    420
 H  H  R  S  T  R  P  N  A  V  P  L  I  L  C  H  G  W  P  G     119
cactttggcgagttcctgaacgttatcccgctcttgacggagccgtcggacccctccgct    480
 H  F  G  E  F  L  N  V  I  P  L  L  T  E  P  S  D  P  S  A     139
                (SP3.1)
caggcgttccacgtcgtcgccccttcgatgcctggctatgcttggtctttgcctcctccg    540
                (SP5.2)
 Q  A  F  H  V  V  A  P  S  M  P  G  Y  A  W  S  L  P  P  P     159
tcctccaagtggaacatgtgcgttcctttaattcaatcgtctcttctggcgtgactgatc    600
 S  S  K  W  N  M[----------------------intron 2-------------     165
catcccgtttgccaggcctgacaccgcgagggtcttcgacaagctcatgaccgggcttgg    660
                                  (SP5.1)
--------------]  P  D  T  A  R  V  F  D  K  L  M  T  G  L  G     180
ctacgagaagtgcgtctacccgttccgcgacgcttcttgagctttccccggacacgcaag    720
  Y  E  K[-------------------------------intron 3-----------     183
gcgatgctgatagtgcaactcggcaggtacatggcgcagggcggagactggggaagcatc    780
--------------------------]  Y  M  A  Q  G  G  D  W  G  S  I     194
gccgctcgctgccttggatcgctgcacaaggaccattgcaaaggttcgtcccatcgggtc    840
 A  A  R  C  L  G  S  L  H  K  D  H  C  K  [---------------     208
ttcactcctcctcttcgtcttaccttcacttgacgttaagcgctcgagctaatacctcg    900
-----------------------------intron 4-------------------------
aagtcggcgcaacccgcagccgtccacctcaacttcctccccgtcttcccacccgtcccg    960
------------------]A  V  H  L  N  F  L  P  V  F  P  P  V  P     222
atgtggcttatcaacccgcacacgctccttgcctgggcaccgcgcttcctcgtgccggag   1020
 M  W  L  I  N  P  H  T  L  L  A  W  A  P  R  F  L  V  P  E     242
aagcaggctgcgcgtatgaagcgcgggttggcgtaccttgagaaggggtgagtcgcagcg   1080
 K  Q  A  A  R  M  K  R  G  L  A  Y  L  E  K  G[------------     258
caggtctcgcgtttccgtagaaagtcctgacaggtcctcctcgtcttgcctcaacgcagc   1140
------------------intron 5-------------------------------]
tccgcctactacgtcatgcagcagttgacggcaagctcagcgaagtctgcattgggaagc   1200
 S  A  Y  Y  V  M  Q  Q  L  T [--------------intron 6--------     268
aaagctgaccctccgacgcccgcagcctcgcacgcctgcgtacggcctgaccgacagtcc   1260
------------------------] P  R  T  P  A  Y  G  L  T  D  S  P     280
cgtcggcttgctggcctggatcggcgagaaggtgcgtcctttcctcatcagcacaagctc   1320
  V  G  L  L  A  W  I  G  E  K [----------intron 7----------     290
gaagctgattcgtgagcgtgcagttcgagccgaccattcaggaggcgagcaagcaagccc   1380
----------------------] F  E  P  T  I  Q  E  A  S  K  Q  A      302
agccgacgtacgttctcgccagtagctgccgctcgccgtcttcgctaatgccccgctgcg   1440
Q  P  T[--------------------intron 8-------------------------     305
cagcctgactcgcgacgagctctacttcacctgctcgctctactggttcagtgcgtcgac   1500
--] L  T  R  D  E  L  Y  F  T  C  S  L  Y  W  F  [----------     320
tccgcacctcccatcgtcgcgcccactgacactaagccgctcctcccaccacacagcccg   1560
-------------------------intron 9------------------]T  R      322
ctcaatcggcacctccttccttccctactcgctcaacccgcacttcaccaccttcctgac   1620
  S  I  G  T  S  F  L  P  Y  S  L  N  P  H  F  T  T  F  L  T    342
cgacagcaagtaccacctgcccaactttgccctctcgctttacccaggcgagatctactg   1680
  D  S  K  Y  H  L  P  N  F  A  L  S  L  Y  P  G  E  I  Y  C    362
ccccgccgagcgggacgccaagcgcaccggcaacctcaagtggatcaaggacgcgcctga   1740
  P  A  E  R  D  A  K  R  T  G  N  L  K  W  I  K  D  A  P  E    382
gggaggacactttgctgcgctcgaaaagccggatgtgtttgtcgagcacctcagggaggc   1800
  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
```

```
Eph1   M-------ATHTF-----------------------ASPPTR-------FTVDIPQSELDELHS  28
RTmEH  MWLELVLASLLGFVIYWFVSRDKEETLPLGDGWWGPGSKPSAKEDESIRPFKVETSDEEIKDLHQ  65

Eph1   RLDKTRWPATEIVPEDGTDDPTAFGLGAGPTLPLMKELAKGWR-EFDWKKAQDHLNTFEHYMVEI  93
RtmEH  RIDRFR----ASPPLEGS--RFHYGFNSN----YMKKVVSYWRNEFDWRKQVEILNQYPHFKTKI 120

                                  HGXP
Eph1   EDLSIHFLHHRSTR----PNAVPLILCHGWPGHFGEFLNVIRLLTEPSDPS---AQAFHVVAPSM 151
RtmEH  EGLDIHFIHVKPPQLPSGRTPKPLLMVHGWPGSFYEFYKIIPLLTDPKSHGLSDEHVFEVICPSI 185

                                                 sxnxss
Eph1   PGYAWSLPPPSSKWNMPDTARVFDKLMTGLGYEKYMAQGGDWGSIAARCLGSLHKDHCKAVHLNF 216
RtmEH  PGYGYSEASSKKGLNSVATARIFYKLMTRLGFQKFYIQGGDWGSLICTNMAQMVPNHVKGLHLNM 250

Eph1   LPVFPPVPMWLINPHTLLA-WAPRFLV-PEKQAARM---KRGLAY-LEKGSAYYVMQQLTPRTPA 275
RtmEH  --AFISRSFYTMTP--LLGQRFGRFLGYTGKDIELLYPYKEKVFYSIMRESGYLHIQATKPDTVG 311

Eph1   YGLTDSPVGLLAWIGEKFEP-TIQEASKQAQPTLTR----DELYFTCSLYWFTRSIGTS--FLPY 333
RtmEH  CALNDSPVGLAAYILEKFSTWTKSEYRELEDGGLERKFSLDDLLVNIMIYWTTGTIVSSQRYYKE 376

                                        A                          H
Eph1   SLNPHFTTFLTDSKYHLPNFALSLYPGEIYCPAERDAK-RTGNLKWIKDAPEGGHFAALEKPDVF 397
RtmEH  NLGEGIMVHKHEGMKVFVPTGFSAFPSELLHAPEKWVKVKYPKLISYSYNERGGHFAAFEEPKLL 441

Eph1   VEHLREAFGVMWEK 411
RtmEH  AQDIRKFVSLAELQ 455
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Figure 2 Amino acid comparison of *R. glutinis* EH and rat mEH. The amino acids are given in the one letter code. Eph1, *R. glutinis* Eph1. RTmEH, rat mEH. |, identical amino acid residues at that position. HGXP, HGXP motif. sxnxss, nucleophile motif (see text). A, catalytic acidic residue. H, catalytic histidine.Other α/β hydrolase folded microbial EH encoding genes have been cloned from *Agrobacterium radiobacter* AD1 (Rink et al. 1997) and a *Corynebacterium* sp. (Misawa et al. 1998). These genes encode sEHs with subunit sizes of 34 and 32 kDa respectively. The sequence similarity of Eph1 to mEHs (this study) and enzyme localization studies (Kronenburg et al. 1999) indicate that the 46 kDa EH of *R. glutinis* is a membrane-associated EH.

Several of these characteristics were also found in the Eph1 amino acid sequence (Figure 2). The Eph1 amino acid sequence $GGD^{190}WGS$ fits perfectly to the nucleophile motif and is identical to the amino acid sequence flanking the catalytic nucleophile of rat mEH (Arand et al. 1999). Eph1 Glu^{359} aligns to the catalytic acidic residue of rat mEH. At the Eph1 C-terminus the sequence $GGH^{385}FAA$ is found which resembles the rat mEH sequence containing the catalytic histidine. Therefore, the

putative catalytic triad of Eph1 might consist of: Asp^{190}, Glu^{359}, His^{385}. A Trp residue was found at the X position in the Eph1 HGXP motif. These results suggest that the *R. glutinis* EH is an α/β hydrolase fold enzyme.

A novel non α/β hydrolase folded bacterial EH, limonene-1,2-epoxide hydrolase, was found in *Rhodococcus erythropolis* DCL14 (van der Werf et al. 1998). It was estimated that a minimal molecular mass of 25 kDa is necessary to accommodate the reaction mechanism as used by the α/β hydrolase folded EHs.

Therefore, the 17 kDa limonene-1,2-epoxide hydrolase of *R. erythropolis* DCL14 is too small to belong to the α/β hydrolase folded EHs (van der Werf et al. 1998).

The amino acid sequence did not show similarity to other EHs or to any other sequence in protein databases (Barbirato et al. 1998). Furthermore, the imidazole-modifying compounds 2-bromo-4'-nitroacetophenone and diethylpyrocarbonate did not affect limonene-1,2-epoxide hydrolase activity which suggests that, unlike in α/β hydrolase folded EHs, no catalytic histidine is involved in this enzyme (van der Werf et al. 1998).

Eph1 in biocatalysis

EPH1 was expressed in *E. coli* and resting cells showed a high specific activity of 200 nmol min^{-1} (mg protein)$^{-1}$ towards 1,2-epoxyhexane. This activity is 1.7 times higher as compared to *R. glutinis* cells (120 nmol min^{-1} (mg $protein^{-1}$), Kronenburg et al. 1999). Previously, bacterial EH encoding genes have been expressed successfully in *E. coli*. The *ech*A gene of *A. radiobacter* AD1 was expressed under control of a T7 promoter in a soluble and active form up to 40% of the total cellular protein content of *E. coli* BL21(DE3) (Rink et al. 1997). The *lim*A gene of *R. erythropolis* DCL14 was expressed by the endogenous promoter in *E. coli* DH5α. However, a 30 times lower specific enzyme activity was observed as compared to *R. erythropolis* DCL14 (Barbirato et al. 1998).

The improved EH activity of the recombinant *E. coli* as compared to *R. glutinis* and the obtained sequence information of *EPH1* opens the way to conduct protein engineering experiments in order to furnish a *R. glutinis* mutant Eph1 with desired properties. The over-expression of such a mutant EH could result in large amounts of an efficient epoxide biocatalyst.

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Chapter 5

Construction and Characterization of a Genetically Engineered *Escherichia coli* Strain for the Epoxide Hydrolase-Catalyzed Kinetic Resolution of Epoxides

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ABSTRACT

The *Rhodotorula glutinis* epoxide hydrolase, Eph1, was produced in the heterologous host *Escherichia coli* BL21(DE3) in order to develop a highly effective epoxide hydrolysis system. A 138-fold increase in Eph1 activity was found in cell extracts of the recombinant *E. coli* when compared to cell extracts of *Rhodotorula glutinis*, despite the formation of Eph1 inclusion bodies. Optimization of cultivation conditions and co-expression of molecular chaperones resulted in a further increase in activity and a reduction of the inclusion bodies formation. Compared to *Rhodotorula glutinis* cells and cell extracts, a total increase in Eph1 activity of over 200 times was found for both fermenter-cultured *E. coli* cells and crude enzyme preparations of fermenter-cultured *E. coli* cells, without loss of enantioselectivity. The improved conditions for recombinant Eph1 production were used to demonstrate the Eph1-catalyzed kinetic resolution of a new Eph1 substrate, 1-oxa-spiro[2.5]octane-2-carbonitrile.

INTRODUCTION

Enantiopure epoxides and their corresponding enantiopure diols are important chiral building blocks in the preparation of more complex enantiopure biologically active compounds or as end products with biological activity. Therefore, a great interest exists in the development of methods for the synthesis of enantiopure epoxides and diols. An interesting approach is to use a biocatalytic process that involves the epoxide hydrolase (EH) catalyzed racemic resolution of epoxides. These enzymes are enantioselective in the cofactor-independent hydrolysis of structurally divergent epoxides (1, 26).

EHs catalyze the hydrolysis of epoxides to the corresponding diols. These enzymes are found in both prokaryotic and eukaryotic organisms where they have different functions. For example, the intensively studied microsomal EH of mammals is a liver enzyme involved in the detoxification of noxious epoxides (16, 18). The limonene-1,2-EH from the bacterium *Rhodococcus erythropolis* DCL14, on the other hand, is involved in the limonene degradation pathway of this organism (21). Also several basidiomycetous yeast species possessing EH activity have been described (2) but a biological function of yeast EH has not been revealed yet.

The EH of the red-colored yeast *Rhodotorula glutinis* is an enzyme that can accept a broad range of substrates, which are hydrolyzed with moderate to high enantioselectivities (24, 25). These enzyme characteristics are very interesting for the development of a commercial process for the production of enantiopure epoxides and diols. However, to enable the application of a *R. glutinis* EH for preparative purposes an increase in EH activity is desired. Attempts to improve the enzyme's production

level in *R. glutinis* by searching for conditions that would induce EH gene expression have failed so far (C. A. G. M. Weijers, personal communication).

Recently the EH-encoding gene, *EPH1*, from *R. glutinis* was isolated. *EPH1* function was demonstrated by its expression in *Escherichia coli* (23). The isolation of *EPH1* allows the development of Eph1 overproducing strains by genetic engineering. In this paper we report the construction and analysis of an *R. glutinis* Eph1 overproduction system.

MATERIALS AND METHODS

Strains, plasmids and DNA manipulations

Escherichia coli strains BL21(DE3) and BL21(DE3)pLysS, and plasmid pET28a(+) were purchased from Novagen Inc. (Madison, WI, USA). The plasmids pTf16, pG-Tf2 and pG-KJE8 were a gift from Professor Takashi Yura (14). *E. coli* strain XL1-BlueMRF' (Stratagene, La Jolla, CA, USA) was used for general cloning purposes. If not stated otherwise standard molecular cloning techniques were used (17). The *R. glutinis* epoxide hydrolase-encoding region (1.2 kb) was released from plasmid pKKRgEph1 (23) with endonucleases *Eco*RI and *Hind*III. This fragment was cloned in frame to a (N-terminal) His-tag encoding sequence in the corresponding sites of plasmid pET28a(+) resulting in plasmid pEph1.

The preparation of electro-competent *E. coli* cells and the transformation of these cells by electroporation was preformed as described previously (22). After transformation of *E. coli* BL21(DE3)(pEph1) cells with pTf16, pG-Tf2 or pG-KJE8 positive colonies were selected from Luria-Bertani (LB) agar plates containing both kanamycin (50 μg/mL) and chloramphenicol (20 μg/mL).

Expression of *EPH1*

A single *E. coli* BL21(DE3)(pEph1) colony was taken from a LB agar plate, supplemented with kanamycin (50 μg/mL), and used to inoculate 50 mL LB medium in a 250 mL Erlenmeyer flask. This inoculum was incubated at 37°C and 200 revolutions per minute (rpm) until the optical density at 600 nm (OD_{600}) reached 0.5-1. Two mL of this pre-culture were used to inoculate a 50 mL culture. This culture was grown at 37°C and 200 rpm until OD_{600} reached 0.5-1 and subsequently *EPH1* gene expression was induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Diagnostic Chemicals Limited, UK). After an additional 3 hours of incubation the cells were harvested by centrifugation at 4°C and 20,000 × *g* for 5 minutes and washed with 50 mM potassium phosphate (KPi) buffer (pH 7). The cells were resuspended in 5 mL KPi buffer. A cell extract was prepared by sonication (5 minutes, duty cycle 30%, output control 3) using a Branson sonifier 250. To separate the cell extract in a soluble (S-) and insoluble (P-) fraction the disrupted cells were centrifuged for 10 minutes at 4°C

and 20,000 × *g*. The pellet, containing cell debris and insoluble proteins, was resuspended in an equal volume of KPi buffer.

Fermentation conditions

Five hundred mL of LB medium, supplemented with 50 μg/mL kanamycin, was inoculated with *E. coli* BL21(DE3)(pEph1). This pre-culture were incubated for 16 hours at 200 rpm and 37°C and used to inoculate a 15 L fermenter (Applikon, Schiedam, The Netherlands), which was filled with 10 L of LB medium supplemented with 50 μg/mL kanamycin, to an OD_{600} of 0.2. The fermenter content was kept at pH 7, stirred by two impellers (250 rpm), cooled (21°C) and aerated (750 mL/min). At an OD_{600} of 0.65 IPTG was added to a final concentration of 100 μM to induce gene expression. After 20 hours the cells were harvested by centrifugation at 10,000 × *g* for 5 minutes and 4°C and a cell extract was prepared as described above.

Eph1 assay

Suspensions of whole cells, cell extract, S- or P-fractions in KPi buffer were pre-incubated in sealed tubes for 5 minutes at 35°C and 250 rpm in a waterbath. The reaction was started by addition of 10 mM 1,2-epoxyhexane and incubated at 35°C and 250 rpm. To monitor epoxide degradation headspace samples were taken over a period of time and were analyzed using GC analysis on a Chrompack CP9000 gas chromatograph equipped with a β-cyclodextrin 120 chiral column (Supelco, Zwijndrecht, The Netherlands). Protein contents were determined using the Bio-Rad *DC* Protein Assay (Bio-Rad Laboratories BV, Veenendaal, The Netherlands). Specific enzyme activities were expressed as units per mg of protein U $(mg\ protein)^{-1}$. One unit is equivalent to 1 μmol of epoxide converted per minute.

1-Oxa-spiro[2.5]octane-2-carbonitrile analysis

Hydrolysis of 1-oxa-spiro[2.5]octane-2-carbonitrile was analyzed by the same gas chromatography method as was described for styrene oxide hydrolysis analysis by *R. glutinis* (24). However, the oven temperature of the gas chromatograph was set at 120°C instead of 85°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed according to Laemmli (12). In brief, the protein composition of samples was analyzed by separation on SDS-PAGE (12.5%) gels using a Hoeffer Mighty Small system (Pharmacia, Roosendaal, The Netherlands). After electrophoresis the separated proteins were stained with Bio-Safe Coomassie Stain (Bio-Rad). The broad range Precision Protein Standard (Bio-Rad) was used to estimate protein size. The Quantity one 4.2.1. software package (Bio-Rad) was used to prepare digital images of the gels.

RESULTS

Expression of recombinant Eph1

The T7 polymerase-based *E. coli* expression system (pET-System™, Novagen, Madison, WI, USA) was chosen because this system can result in the production of high levels of heterologous proteins. The *R. glutinis* Eph1 encoding DNA sequence was cloned into pET28a(+), yielding plasmid pEph1, in such a manner that upon expression a fusion-protein consisting of Eph1 with an additional N-terminal histidine-tag would be produced. This tag was added to facilitate purification of recombinant Eph1 by metal affinity chromatography. Plasmid pEph1 was introduced into *E. coli* BL21(DE3) by electrotransformation resulting in strain BL21(DE3)(pEph1). *EPH1* gene expression was induced for 3 hours at 37°C in LB medium containing 1 mM IPTG. Analysis of whole cells of *E. coli* BL21(DE3)(pEph1) using the Eph1 assay revealed epoxide hydrolase activity towards model substrate 1,2-epoxyhexane, whereas no epoxide hydrolase activity could be detected in strain BL21(DE3) carrying plasmid pET28a(+).

SDS-PAGE analysis of whole cell lysates of *E. coli* BL21(DE3)(pEph1) and *E. coli* BL21(DE3)(pET28a(+)) showed a protein band of ± 50 kDa only in the lane carrying *E. coli* BL21(DE3)(pEph1) material indicating the presence of recombinant Eph1 (data not shown). The molecular weight of this band corresponds very well with the predicted molecular weight of recombinant Eph1 based on the amino acid sequence. Subsequently, the cells were disrupted and the soluble proteins (S-fraction) were separated from the insoluble proteins (P-fraction) by centrifugation and both fractions were analyzed for EH activity. The S-fraction showed an activity of 1.5 U (mg protein)$^{-1}$ whereas no EH activity could be detected in the P-fraction. In the SDS-PAGE separated S-fraction of these cells no 50 kDa protein band, corresponding to the Eph1 protein, was distinguishable from the other proteins. Apparently, the amount of soluble Eph1 was below the detection limit. Although no Eph1 activity was measured in the P-fraction the majority of recombinant Eph1 was present in the P-fraction (Fig. 1). It is likely that most of the Eph1 protein is deposited in an inactive form in inclusion bodies.

Manipulation of the *EPH1* expression level

The high level expression of heterologous genes in *E. coli* often results in the deposition of the recombinant protein in inclusion bodies. In particular, overproduced proteins from eukaryotic sources undergo this problem (9). Because of the high amount of Eph1 in the inclusion bodies we searched for conditions that would result in more Eph1 in an active form. One approach was to decrease *EPH1* expression (production rate) and thereby allowing proper folding of Eph1 by the cell's protein folding machinery. Three parameters were tested: the temperature of cultivation, the expression of *EPH1* in *E. coli* strain BL21(DE3)pLysS and the concentration of inducer IPTG.

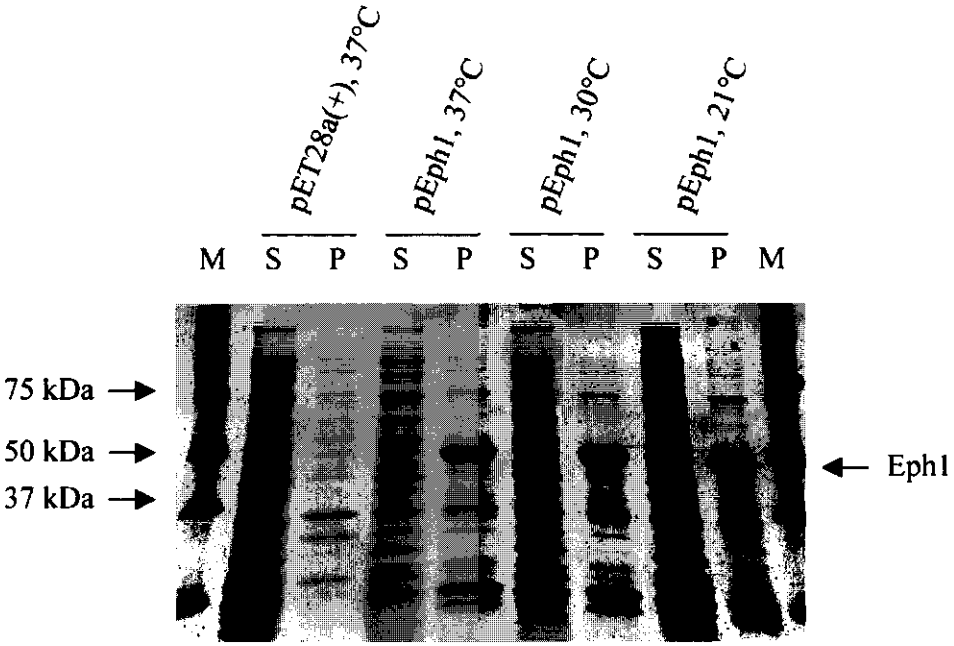

Figure 1. Coomassie stained protein gel loaded with *E. coli* BL21(DE3)(pEph1), which was cultured at different temperatures. M, protein precision marker. The molecular weights of three marker proteins covering the area of interest are indicated on the left of the gel. S, soluble fraction of a cell extract. P, insoluble fraction of a cell extract. The arrow at the right indicates the position of the Eph1 band.

Temperature effect

It has been shown that expression of heterologous genes in *E. coli* at temperatures lower than 37°C can reduce the formation of inclusion bodies (13, 19). Therefore, recombinant *EPH1* gene expression was, in addition to 37°C, also tested at 30°C and 21°C. The specific activity in the S-fractions of *E. coli* BL21(DE3)(pEph1) could be increased 10 times, from 1.5 to 15 U (mg protein)$^{-1}$ by lowering the temperature from 37°C to 21°C. Furthermore, SDS-PAGE analysis of the S-fractions showed an increasing level of a 50 kDa protein, whereas no significant changes were observed in the composition of the total protein content in cells cultivated at lower temperatures. However, the Eph1 bands present in the P-fractions were still of the same intensity (Fig. 1).

***EPH1* expression in *E. coli* BL21(DE3)(pLysS)(pEph1)**

An alternative approach to prevent inclusion body formation was to lower the expression level of the T7 promoter. Therefore, plasmid pEph1 was introduced into *E. coli* strain BL21(DE3)(pLysS) and the resulting strain was denominated BL21(DE3)(pLysS)(pEph1). This strain contains plasmid pLysS, which provides the cell with a small amount of T7 lysozyme upon expression. T7 lysozyme inhibits T7 RNA polymerase directed gene expression by binding to T7 RNA polymerase (15).

Expression of *EPH1* was performed at 21°C with 1 mM IPTG. The specific Eph1 activity in the S-fraction was 1 U (mg protein)$^{-1}$. SDS-PAGE analysis revealed the absence of Eph1 aggregates in the P-fraction but also of soluble Eph1 in the S-fraction (data not shown). Apparently, the overall level of Eph1 produced was very low.

IPTG concentration effect

Initially, in our experiments 1 mM IPTG was used to induce *EPH1* gene expression. However, a lower IPTG concentration might also result in a decreased level of *EPH1* expression. A lower *EPH1* expression level could give the cell more time to properly fold Eph1 molecules resulting in less inclusion body formation and an increased level of soluble Eph1. Therefore, expression of *EPH1* was carried out at 21°C with different concentrations of IPTG. The results showed that between 100 μM and 1,000 μM IPTG there is very little difference in specific enzyme activity. Protein analysis by SDS-PAGE showed no difference in the amount and distribution of Eph1 between the S- and P-fractions. At an IPTG concentration lower than 100 μM an overall decrease in enzyme activity was observed.

Co-expression of molecular chaperones

The purpose of the experiments described above was to lower *EPH1* gene expression and therefore to give the cell's folding machinery more time to fold newly synthesized Eph1 correctly. Another approach to obtain an elevated level of soluble Eph1 was based on the co-expression of molecular chaperones. The production level of nascent, partially (mis)folded recombinant Eph1 intermediates is likely to be very high in the *E. coli* BL21(DE3)(pEph1) expression system. A lack of sufficient molecular chaperones to correctly fold the recombinant Eph1 might be a reason for the accumulation of inactive Eph1 molecules within inclusion bodies. To overcome this problem Eph1 was overproduced together with different combinations of the endogenous chaperones DnaK-DnaJ-GrpE, GroEL-GroES and trigger factor (TF) (14).

The folding chaperones DnaK-DnaJ-GrpE are e.g. involved in (i) preventing newly synthesized proteins to aggregate and (ii) in assisting off-pathway folding intermediates of a protein to reenter the proper folding pathway (4). The ribosome-bound trigger factor is the major protein associated with nascent polypeptides and it shows prolyl isomerase activity *in vitro* (5). The TF cooperates with GroEL in the degradation of abnormal proteins (7) and with DnaK in protein folding (3).

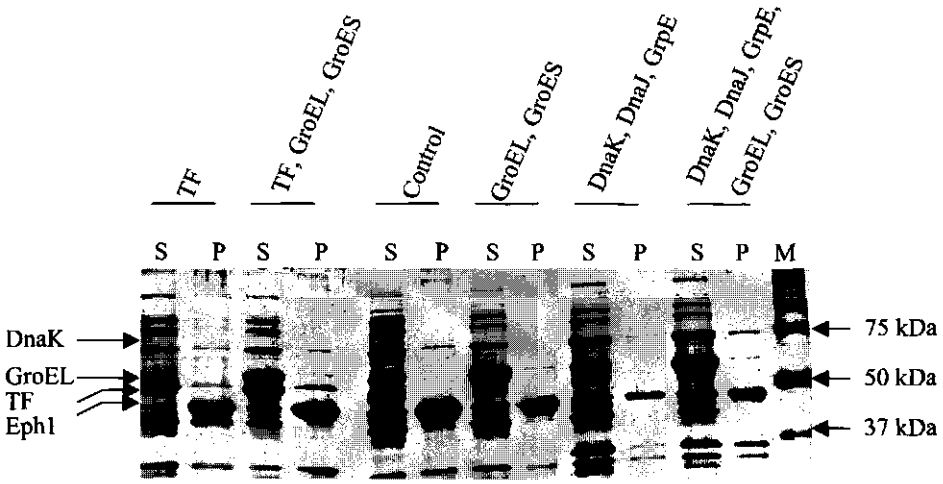

Figure 2. Coomassie stained protein gel of cell material corresponding to *E. coli* BL21(DE3)(pEph1) with or without (control) co-produced molecular chaperones at 37°C. The molecular chaperones are indicated above the lanes. Each sample was divided into a soluble (S) and an insoluble (P) fraction. M, protein precision marker. Arrows at the right of the gel indicate the molecular weights of three marker proteins covering the area of interest. The strong bands present in the P-fractions correspond to Eph1. Arrows at the left of the gel indicate the position of chaperones DnaK, GroEL and TF as well as Eph1.

The expression of the molecular chaperones was induced at the start of the culture by the addition of arabinose or tetracycline as described by Nishihara *et al.* (14). In these induced chaperones cultures *EPH1* expression was induced at $OD_{600} = 0.5$ by the addition of 100 μM IPTG.

The overproduction of molecular chaperones at 37°C was clearly visible by the high amounts of the chaperone proteins in the S-fraction of cells analyzed by SDS-PAGE (Fig. 2). It should be noticed that none of the molecular chaperones were observed as inclusion bodies in the corresponding P-fractions. On the other hand, the P-fraction of *E. coli* BL21(DE3)(pEph1) cells still contained the major portion of Eph1. Co-expression of the genes encoding DnaK-DnaJ-GrpE seemed to have some effect on

the formation of Eph1 aggregates. In these cases the level of Eph1 aggregates was reduced. However, all lanes containing the S-fractions of the various combinations did not reveal a significant increase in the amount of soluble Eph1. Moreover, in all combinations of expression of *EPH1* in the presence of overproduced molecular chaperones the Eph1 activity was reduced compared to the control (Table 1).

Table 1. The effect of co-produced molecular chaperones on Eph1 activity of the soluble fraction of *E. coli* BL21(DE3)(pEph1) cell extracts.

Chaperones	Relative activity (%)*	
	37°C	21°C
None (control)	100	100
TF	7	94
TF, GroEL, GroES	60	41
GroEL, GroES	55	70
DnaK, DnaJ, GrpE	24	90
GroEL, GroES, DnaK, DnaJ, GrpE	40	35

*. The Eph1 activity of the control (no chaperones) was set at 100%. Activities of samples containing chaperones were compared to the control and given as a percentage thereof.

As presented earlier, a shift in growth temperature from 37°C to 21°C resulted in a 10-fold increase in soluble Eph1 and Eph1 activity. Therefore, the experiment using the overproduced chaperones was also conducted at 21°C. Under these conditions co-expression of the molecular chaperones had a significant effect on the formation of inclusion bodies (Fig. 3). The coomassie stained protein gel showed that co-expression of TF with Eph1 resulted in a slight decrease in the amount of Eph1 inclusion bodies, whereas inclusion bodies were nearly absent when Eph1 was produced together with TF and GroEL-GroES. This was also observed when *EPH1* was expressed together with the chaperone team DnaK-DnaJ-GrpE and / or GroEL-GroES. However, the reduction in inclusion body formation did not result in an equivalent increase of soluble Eph1. Nevertheless, Eph1 activity in presence of TF or DnaK-DnaJ-GrpE increased nearly to that of the control when the induction temperature was reduced to 21°C. High amounts of molecular chaperone proteins were clearly present in the lanes of a protein gel containing the S-fractions (Fig. 3). As a result of this, lower Eph1 enzyme activities in presence of the molecular chaperones were found as compared to the control (Table 1).

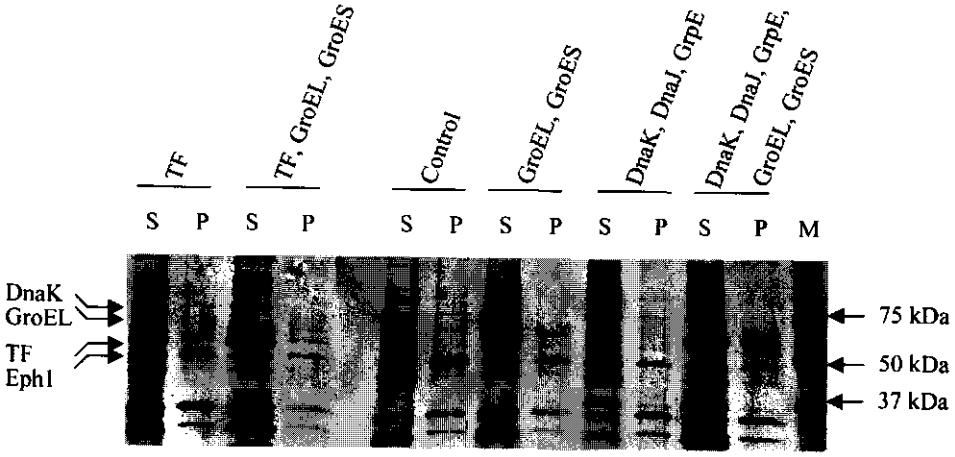

Figure 3. Coomassie stained protein gel of cell material corresponding to *E. coli* BL21(DE3)(pEph1) with or without (control) co-produced molecular chaperones at 21°C. The molecular chaperones are indicated above the lanes. Each sample was divided into a soluble (S) and an insoluble (P) fraction. M, protein precision marker. Arrows at the right of the gel indicate the molecular weights of three marker proteins covering the area of interest. The strong band present in the P-fraction of the control corresponds to Eph1. Arrows at the left of the gel indicate the positions of chaperones DnaK, GroEL and TF as well as Eph1.

Increased cultivation volume

The initial *EPH1* expression experiments described above were conducted at a 50 mL scale in a 250 mL Erlenmeyer. The calculated total activity of the S-fraction obtained from cells cultivated at 21°C was 195 units for 1,2-epoxyhexane. To increase the absolute amount of active enzyme by using a larger cultivation volume, we also tested *EPH1* expression in a 450 mL culture in a 1 L Erlenmeyer and a 10 L culture in a 15 L fermenter. The 450 mL culture was conducted in a similar way to that of the 50 mL cultures incubated at 21°C and induced with 100 μM of IPTG. The S-fraction of these cells showed the enantioselective hydrolysis of 1,2-epoxyhexane with an activity of 15 U (mg protein)$^{-1}$, which is the same as obtained from 50 mL of cells. The calculated total activity from this batch was $2.6 \cdot 10^3$ units. Protein analysis showed that

most of the enzyme was present in the P-fraction in which no Eph1 activity could be detected.

The fermenter culture was induced at an OD_{600} of 0.65. After 20 hours of induction the OD_{600} had reached the value of 5. Both whole cells and the S-fraction of the cell extract of these cells were applied in the Eph1 assay. The enantioselective 1,2-epoxyhexane hydrolysis observed for the S-fraction was comparable to that of *R. glutinis* cell extracts (11). The activity of whole cells was 14 U (mg dw)$^{-1}$, while the activity of the S-fraction was 23 U (mg protein)$^{-1}$. The calculated total activities of these batches of biocatalysts were $2.8 \cdot 10^5$ and $1.7 \cdot 10^5$ U respectively. Like in the other situations, the P-fraction of these fermenter-cultured cells contained most of the recombinant Eph1.

The *EPH1* over-expression system described in this paper was developed for the efficient hydrolysis of Eph1 substrates, especially those for which *R. glutinis* exhibits low EH activity. An example of such a substrate is 1-oxa-spiro[2.5]octane-2-carbonitrile an interesting chiral building block and new *R. glutinis* Eph1 substrate (Fig. 4).

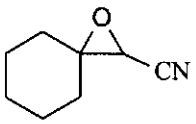

Figure 4. Structure of 1-oxa-spiro[2.5]octane-2-carbonitrile.

R. glutinis showed an activity of $6 \cdot 10^{-3}$ U (mg dw)$^{-1}$ in the hydrolysis of 20 mM 1-oxa-spiro[2.5]octane-2-carbonitrile. However, the activities of the fermenter-cultured *E. coli* BL21(DE3)(pEph1) cells and the S-fraction thereof were $6 \cdot 10^{-2}$ U (mg dw)$^{-1}$ and 3 U (mg protein)$^{-1}$ respectively. Importantly, in all three cases, the yield of the remaining enantiopure epoxide was more than 49%, while the maximal yield is 50%.

DISCUSSION

Previously, the *R. glutinis* Eph1 encoding gene was expressed in *E. coli* using the prokaryotic expression vector pKK223-3 to demonstrate the function of the encoded enzyme (23). Compared to *R. glutinis* cells, a two-fold increase in enzymatic activity for the hydrolysis of 1,2-epoxyhexane was found with this expression system. The results reported here show a strong improvement of enzyme activity using the pET-system. The enzyme activity for the hydrolysis of 1,2-epoxyhexane found in the *E. coli* BL21(DE3)(pEph1) S-fractions of 15 U (mg protein)$^{-1}$ corresponds to a 138-fold

increase as compared to *R. glutinis* (11). Furthermore, fermenter-cultured cells showed that the activity had increased another 1.5 times to 23 U (mg protein)$^{-1}$, probably as a result of better culture conditions. Therefore, a total increase in activity of over 200 times was found for the *E. coli* BL21(DE3)(pEph1) S-fraction when compared to *R. glutinis* cell extract. A similar improvement was observed with whole *E. coli*BL21(DE3)(pEph1) cells versus *R. glutinis* cells as biocatalyst (25).

Like other heterologous proteins produced in *E. coli* (13, 19) the majority of Eph1 produced in *E. coli* BL21(DE3)(pEph1) is aggregated into inclusion bodies. It is likely that the rapid accumulation of Eph1 folding intermediates causes the formation of these aggregates. Apparently, the cell's protein folding machinery is unable to keep up with this production rate probably due to the physical properties of Eph1 and an insufficient level of molecular chaperones.

The overproduction of molecular chaperones in *E. coli* clearly shows that it is possible for a cell to have a high concentration of one or a few soluble proteins without the formation of aggregates of the same proteins. Therefore, we tried to balance the Eph1 production rate and the Eph1 folding rate in order to gain more soluble Eph1. One approach was the decrease of *EPH1* expression by lowering the cultivation temperature. A 10-fold increase in specific activity was found when Eph1 expression was performed at 21°C instead of 37°C. However, the level of inclusion bodies was not affected. Other attempts to lower *EPH1* expression by decreasing the concentration of the inducer IPTG and the use of *E. coli* strain BL21(DE3)(pLysS) were not successful.

The other approach was to increase the cell's protein folding capacity by the overproduction of molecular chaperones. In general, the overproduced chaperones did not increase the amount of soluble active Eph1 as was observed by protein gel electrophoresis and Eph1 activity measurements.

Mouse endostatin, human oxygen-regulated protein ORP150 and human lysozyme are proteins that are prone to aggregation when they were expressed in *E. coli*. Nishihara *et al.* (14) demonstrated that expression of these proteins in *E. coli* together with TF, GroEL-GroES and / or DnaK-DnaJ-GrpE could successfully prevent the formation of aggregates. However, although overproduced TF did prevent mouse endostatin to aggregate the total yield of mouse endostatin dropped by about 50%. Additionally, expression of GroEL-GroES together with mouse endostatin hardly increased the amount in the soluble fraction and reduced the total yield.

A similar situation was encountered in case of Eph1. The chaperone teams DnaK-DnaJ-GrpE and GroEL-GroES in particular showed a strong reduction in the level of Eph1 inclusion bodies formation, without an equivalent increase in soluble Eph1. A proteolytic degradation event might explain this because molecular chaperones are involved both in protein folding as well as in protein degradation. Straus *et al.* (20) have shown that *E. coli* heat shock gene mutants (*dnaK, dnaJ, grpE* and *groEL*) were defective in proteolysis and thereby indicated a role of these proteins in this process. Moreover, they also showed that overproduction of heat shock proteins led to a more

rapid peptide degradation rate. Additionaly, chaperones GroEL, TF, and DnaJ are required for the degradation of abnormal proteins in *E. coli* (6,8).

Regarding this information misfolded Eph1 might also be degraded in the presence of overproduced molecular chaperones instead of being folded correctly. However, the molecular mechanisms behind the effects of the overproduced molecular chaperones on both active and aggregated inactive Eph1 remain unclear.

The recombinant Eph1 carries an N-terminal histidine tag. The tag was added to facilitate the purification of recombinant Eph1 by means of metal affinity chromatography. However, initial experiments to purify native recombinant Eph1 using Ni(II)-loaded columns were not successful. Zou *et al* (27) reported the X-ray structure of the *Aspergillus niger* EH. The N-terminal meander of this soluble dimeric enzyme is involved in the formation of the homodimer. The *R. glutinis* EH was also found to exist as a homodimer (10). The primary stuctures of *A. niger* EH and *R. glutinis* Eph1 are quite similar (29% identity, data not shown). Therefore, it might be possible that recombinant Eph1 forms a homodimer involving the N-terminus. The histidine tags might therefore be inaccessible to the Ni(II)-loaded column. The histidine tags and the dimerization might also affect the formation of inclusion bodies in *E. coli*.

Three cultivation volumes (50 mL, 450 mL and 10 L) were tested for Eph1 yield. These cultivation set-ups were different from another and therefore one cannot speak of a real scale-up process. Nevertheless, the activity of 15 U (mg protein)$^{-1}$ of the S-fraction of shake flask cultured cells was further increased to 23 U (mg protein)$^{-1}$ by cultivation of *E. coli* BL21(DE3)(pEph1) in a fermenter. Comparing the data of the 10 L and 50 mL fermentation showed that the calculated total activity of the S-fraction was increased 867-fold, whereas the culture volume was increased by a factor of 200 only. A reasonable explanation for this observation is a higher cell density, caused by e.g. better culture aeration, of the fermenter culture.

These results indicate that a powerful tool for enantioselective epoxide hydrolysis was developed. The usefulness of this system was demonstrated by the enantioselective hydrolysis of the new *R. glutinis* EH substrate 1-oxa-spiro[2.5]octane-2-carbonitrile (Fig. 4). *R. glutinis* hydrolyzed 1-oxa-spiro[2.5]octane-2-carbonitrile in an enantioselective manner resulting in a high yield of enantiopure epoxide. Because of the excellent enantioselectivity and the high activity, *E. coli* BL21(DE3)(pEph1) is a promising candidate to be used in the enantioselective epoxide hydrolysis on a preparative scale.

ACKNOWLEDGMENTS

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Chapter 6

Cloning, characterization and heterologous expression of epoxide hydrolase-encoding genes from yeasts in *Escherichia coli*: identification of a new highly enantioselective epoxide hydrolase

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Submitted for publication

ABSTRACT

Epoxide hydrolase-encoding cDNA sequences were isolated from the carotenoid producing yeast species *Rhodosporidium toruloides* CBS 349, *Rhodosporidium toruloides* CBS 14 and *Rhodotorula araucariae* CBS 6031 in order to evaluate the molecular data and potential application of this type of enzymes. The deduced amino acid sequences were similar to those of the previously cloned epoxide hydrolases from *Rhodotorula glutinis* CBS 8761, *Xanthophyllomyces dendrorhous* CBS 6938 and *Aspergillus niger* LCP 521, which all correspond to the group of the microsomal epoxide hydrolases. The homology between the EH of yeasts and *A. niger* epoxide hydrolase, which belongs to the α/β hydrolase fold family of enzymes, suggests that they belong to the same family. The epoxide hydrolase encoding cDNAs of the *Rhodosporidium* and *Rhodotorula* species were expressed in *Escherichia coli*. The recombinant strains were able to hydrolyze *trans*-1-phenyl-1,2-epoxypropane in an enantioselective manner. The recombinant counterpart of *Rhodosporidium toruloides* CBS 14 was found to be a highly active and enantioselective biocatalyst for this substrate, despite the low performance of *Rhodosporidium toruloides* CBS 14 itself.

INTRODUCTION

Epoxide hydrolases (EHs) catalyze the hydrolysis of epoxides to the corresponding diols. Presently, six different types of EHs are known. Soluble EH (sEH) and microsomal EH (mEH) exhibit broad substrate specificity and are involved in processes such as the detoxification of epoxides derived from xenobiotic or endogenous compounds [1], catabolism [2] and plant cutin biosynthesis [3]. The other four type of EHs, cholesterol 5,6-EH, leukotriene A_4 (LTA_4) hydrolase, hepoxilin A_3 hydrolase and limonene-1,2-epoxide hydrolase are rather restricted to their specific substrates i.e. cholesterol 5,6-epoxide, LTA_4, hepoxilin A_3 and limonene-1,2-epoxide respectively [4,5].

Several sEH and mEH encoding genes from mammalian, insect, plant and bacterial sources have been cloned [6-10]. Despite the low level of amino acid sequence similarity between sEH and mEH, it was suggested that both type EHs belong to the same class of enzymes known as the α/β hydrolase fold family [11], which are structurally and mechanistically related and hydrolyze their substrates via an enzyme-ester-intermediate [12]. Several biochemical and molecular studies [13-15] including the determination of EH x-ray structures [16-18] confirmed this hypothesis.

The EH activities of the fungi *Aspergillus niger* and *Beauveria bassiana* as well as the red yeast *Rhodotorula glutinis* CBS 8761 were studied in more detail especially related to the efficiency and enantioselectivity by which these enzymes hydrolyze different types of epoxides [19,20]. These fungal enzymes have been shown to be useful in the production of enantiopure epoxides and diols, which are both valuable

intermediates in the synthesis of fine chemicals. Therefore, EHs are attractive epoxide biocatalysts, which might be applied in a production scheme [21]. For example, *A. niger* EH has been used to perform the enzymatic part of the chemo-enzymatic synthesis of several valuable target-compounds such as the β-adrenergic blocker (*R*)-Nifénalol [22].

The yeast *R. glutinis* CBS 8761 exhibits high activities and enantioselectivities toward several epoxides [19]. Furthermore, research has been carried out to improve or manipulate the properties of partly purified *R. glutinis* EH by addition of detergents or by immobilized imprinting [23,24]. Moreover, the knowledge of EHs from yeasts was expanded to the molecular level with the isolation and characterization of the mEH related EH-encoding genes (*EPH1*) from *Xanthophyllomyces dendrorhous* CBS 6938 and *R. glutinis* CBS 8761 [25,26].

Additional yeast strains have been reported to hydrolyze 1,2-epoxyalkanes. Some of these strains show good activity and / or enantioselectivity, while others don't [27].

We intend to further explore the molecular data and potential industrial use of yeast EHs. Therefore, the EH-encoding cDNA sequences from *Rhodosporidium toruloides* CBS 349, *Rhodosporidium toruloides* CBS 14 and *Rhodotorula araucariae* CBS 6031 were isolated and the derived amino acid sequences analyzed. In addition, the cDNA sequences were expressed in *E. coli* and the hydrolysis of *trans*-1-phenyl-1,2-epoxypropane by both the yeast strains and the corresponding recombinant *E. coli* was analyzed.

MATERIALS AND METHODS

Strains and plasmids

Escherichia coli XL1-BlueMRF' (Stratagene, La Jolla, CA, USA) and *Escherichia coli* BL21(DE3) (Novagen Inc., Madison, WI, USA) were used for cloning and expression experiments respectively. Plasmid pGEM-T Easy (Promega, Leiden, The Netherlands) was used for cloning of Supertaq (SphearoQ, Leiden, The Netherlands) amplified PCR products. Plasmid pET28a(+) (Novagen Inc., Madison, WI, USA) was used for expression of EH-encoding cDNA sequences in *E. coli* BL21(DE3). Plasmid pKKRgEph1 was described previously [26]. *Rhodotorula glutinis* CBS 8761, *Rhodosporidium toruloides* CBS 349, *Rhodosporidium toruloides* CBS 14 and *Rhodotorula araucariae* CBS 6031 were obtained from the Centraal Bureau voor Schimmelcultures (Utrecht, The Netherlands). The latter three yeast strains are also referred to as UOFS Y-0471 (CBS 349), UOFS Y-0473 (CBS 6031) and UOFS Y-0472 (CBS 14) as reported by Botes *et al.* [27]. *R. glutinis* strain CBS 8761 is also known as *R. glutinis* ATCC 201718 and *R. glutinis* CIMW 147.

DNA manipulations

Standard molecular biological techniques were used unless stated otherwise [28]. The nucleotide sequences of custom primers, which were used in this study, are given in Table 1. Isolation of yeast chromosomal DNA and total RNA was performed as described previously [26]. Messenger RNA was purified from total RNA using the Qiagen Oligotex mRNA purification kit (Westburg BV, Leusden, The Netherlands). The 5′/3′ RACE (rapid amplification of cDNA ends) kit (Roche Diagnostics Nederland BV, Almere, The Netherlands) was used to generate first strand cDNA sequences by reverse transcription (RT) of mRNA. The *Not*I primer adapter was obtained from Promega Benelux BV, Leiden, The Netherlands. The PCR technique was applied to amplify partial or full-length EH-encoding DNA sequences using Supertaq DNA polymerase (SphaeroQ, Leiden, The Netherlands). The PCR cycle profile using degenerate primers and yeast chromosomal DNA as template was as follows: 8'at 94°C, subsequently 30 cycles of 1'at 94°C, 1'at 45°C, 2'at 72°C and ending by 5'at 72°C. The PCR cycle profile using specific primers and RT products or pKKRgEph1 as template was as follows: 5'at 94°, subsequently 30 cycles of 30''at 94°C, 30''or 1'at 50°C, 55°C or 60°C, 1'or 2'at 72°C, and ending by 5'at 72°C. PCR products were cloned into pGEM T Easy and transferred to *E. coli* XL1-Blue MRF' by means of electroporation as described previously [25].

Table 1 Custom primers used in this study.

Primer	Sequence (5′ → 3′)
PEHATGNCOI	TTTTCCATGGATGGCGACACACACATTCGC[1]
PEHTAG	AGCTCTGCAGCTACTTCTCCCACATGACGCC[2]
PSMPGY	CCNWSIATGCCNGGNTAY
PANEH4A	TCVAGWGCWGCRAARTGDCCDCC
P60315N	GGACCCCCAGTCACCGCCC
P60315	CTTGCAGTGTCTTCCATGCCC
P60313N	GTTGTACACCACGCTCTCGC
P60313	CCTCTTTCCTCCCCTACTCGC
P6031ATG	ATATACCATGGGCGAGCACAGCTTCGAGGCCCCG[1]
P6031TGA	ATATAGCGGCCGCTCACGACGACAGCATGGCCTTGACTGCC[3]

Nucleotide codes: N: A+T+C+G, W: A+T, S: C+G, I: deoxyinosine, Y: C+T, V: A+C+G, R: A+G, D: A+T+G. Restriction endonuclease sites are underlined. [1], *Nco*I. [2], *Eco*RI. [3], *Not*I.

Plasmid DNA was isolated from ampicillin-resistant colonies and analyzed by DNA restriction analysis. Both DNA strands of all cloned PCR products were sequenced by the Laboratory of Molecular Biology (Wageningen University, The Netherlands) using AmpliTaq FS DNA polymerase fluorescent dye terminator reactions as recommended by the supplier (Perkin-Elmer Nederland, Nieuwerkerk a/d IJssel, The Netherlands). The DNASTAR software package was used to analyze nucleotide and deduced amino acid sequences.

The Eph1-encoding cDNA sequences from *R. toruloides* CBS 349, *R. toruloides* CBS 14 and *R. araucariae* have been deposited in the GenBank database under accession numbers AF416991, AF416992 and AF416993 respectively.

Induction of heterologous EPH1 expression

Expression of recombinant *EPH1* cDNA was performed according to the pET system suppliers instructions (Novagen) with some minor modifications. In brief, *EPH1*-expressing *E. coli* BL21(DE3) strains were cultivated in 100 mL of Luria-Bertani (LB) medium supplemented with 50 μg ml^{-1} kanamycin at 200 revolutions per minute (rpm) and at 21°C until the optical density at 600 nm (OD_{600}) reached approximately 1. One hundred μM of isopropyl-β-D-thiogalactopyranoside (IPTG, Diagnostic Chemicals Limited, UK) were added to induce *EPH1* gene expression. After 4 hours of induction the cells were harvested by centrifugation (4°C, 8000 × *g*, 5 minutes), washed with potassium phosphate (KPi) buffer (50 mM, pH 7) and stored at -20°C in 10 ml of KPi-buffer supplemented with 10% glycerol.

Cultivation and preparation of yeast cells

R. glutinis, *R. toruloides* CBS 349, *R. toruloides* CBS 14 and *R. araucariae* were cultivated in 5 liter Erlenmeyer flasks containing 500 ml of a mineral medium supplemented with 1% $^{w}/_{v}$ glucose and 0.2% $^{w}/_{v}$ yeast extract at 30°C on a shaking platform at 60 revolutions per minute (rpm). When the color of the yeast culture turned orange to red, i.e. 48-72 hours of incubation, the cells were harvested by centrifugation (4°C, 8000 × *g*, 5 minutes), washed with KPi-buffer and stored at -20°C in a minimum volume of KPi-buffer supplemented with 10% glycerol.

Enzyme assay

Trans-1-phenyl-1,2-epoxypropane hydrolysis was measured as described previously [29]. In brief, cells were mixed with KPi-buffer to give a final volume of 20 ml in a 100 ml screw-capped bottle. After 5 minutes of pre-incubation at 35°C, the reaction was started by addition of 10 mM of *trans*-1-phenyl-1,2-epoxypropane. The reaction mixture was incubated at 35°C and shaken at 200 rpm. One ml samples were taken over a period of time. These samples were centrifuged for 1 minute at 12,000 × *g*. Epoxide was extracted from 500 μl of supernatant using 1 ml ethylacetate and analyzed by chiral gas-liquid chromatography as described before [29]. Specific enzyme activities were expressed as units per mg of dry cell mass (dry weight, dw): U · $(mg\ dw)^{-1}$. One unit is equivalent to 1 μmol of epoxide converted per minute.

RESULTS

Isolation of the epoxide hydrolase-encoding cDNA sequences

Initially, chromosomal DNA was isolated from *R. glutinis*, *R. toruloides* CBS 349, *R. toruloides* CBS 14 and *R. araucariae*. These DNA samples were digested with several endonucleases and subjected to Southern blot analysis using the *R. glutinis EPH1* cDNA as a probe in order to investigate the presence of similar DNA sequences in these yeast species. After hybridization at 60°C and washing (50°C, 2 × 15 minutes) with 2×SSC / 0,1% SDS a film was exposed to the blot. The hybridization patterns for *R. toruloides* CBS 349, *R. toruloides* CBS 14 and *R. glutinis* were identical. This indicates that the EH-encoding genes are situated on the same restriction fragment. This type of conservation suggested that nucleotide sequences of the *EPH1* genes from *R. toruloides* CBS 349 and *R. toruloides* CBS 14 might be very similar to that of *R. glutinis EPH1*.

Therefore, total RNA was isolated from *R. toruloides* CBS 349 and *R. toruloides* CBS 14 and applied in an RT-reaction using the *Not*I primer adapter to generate a first strand of cDNA. Because of the identical hybridization patterns mentioned above, the specific *R. glutinis EPH1* primers PEHATGNCOI and PEHTAG, corresponding to both ends of the *R. glutinis EPH1* open reading frame, were applied in the PCR using first strand cDNA of *R. toruloides* CBS 349 or *R. toruloides* CBS 14 as template. Each reaction resulted in the amplification of a DNA fragment of approximately 1.2 kbp, as was shown by agarose gel electrophoresis. Both PCR products were cloned, sequenced and compared the *R. glutinis EPH1* sequence. The nucleotide sequences of the *R. glutinis EPH1* cDNA and *R. toruloides* CBS 349 PCR product were identical. The nucleotide sequence of the *R. toruloides* CBS 14 PCR product was 96% identical to that of the *R. glutinis EPH1* cDNA.

Based on the Southern blot analysis it seemed that the *R. araucariae* EH-encoding DNA sequence is different from that of *R. glutinis*. Therefore, it was attempted to synthesize a specific EH fragment for *R. araucariae* using degenerate primes corresponding to conserved regions of known EHs. This approach was successful in the isolation of the *X. dendrorhous EPH1* gene [25]. Two degenerate primers named PSMPGY, corresponding to conserved domain SMPGY, and PANEH4A, corresponding to conserved domain GGHFAALE, were designed. A 1.2 kbp DNA fragment was amplified using these primers and chromosomal *R. araucariae* DNA as template. Nucleotide sequence analysis revealed a putative EH-encoding sequence, which was interrupted by intron sequences. The presence of the amino acid sequence, GGDWG, which corresponds to an α/β hydrolase fold EH specific motif [12] containing the catalytic nucleophilic residue of these enzymes supports this preliminary conclusion. This 1,2 kb *R. araucariae* EH probe was denominated pRaEH1.

Based on the pRaEH1 nucleotide sequence, the specific primers P60315 and P60313 were designed. These primers were used for the amplification of the 5'- and 3'-cDNA ends using the 5'/3' RACE kit. Primer P60315 was used for the first strand cDNA

synthesis in the 5' RACE. An initial PCR with the oligo (d)T anchor primer, targeted against the poly adenylated 5'-cDNA ends, and primer P60315 did not result in a PCR product. Therefore, a nested PCR was performed using primer P60315N and the oligo (d)T anchor primer and on a sample of the first PCR mixture as template. The result of this nested PCR was a specific product of approximately 570 bp. This fragment was cloned in pGEM T Easy, the ligation mixture was transferred to *E. coli* XL1-BlueMRF', yielding the plasmid pRaEH2. The amino acid sequence, deduced from the nucleotide sequence of pRAEH2, showed sequence similarity to the N-terminal amino acid sequence of *R. glutinis* Eph1. Moreover, an α/β hydrolase fold EH specific motif, HGWP corresponding to the putative oxyanion hole of the enzyme, was present. These results indicated that the 5'-end of the *R. araucariae EPH1* cDNA was cloned.

A similar approach was followed in the 3' RACE. The oligo (d)T anchor primer was used for the first strand cDNA synthesis in the 3' RACE. An initial PCR using the oligo (d)T anchor primer, targeted against the 3'poly $(A)^+$ cDNA end, and primer P60313 gave no PCR products. The nested PCR with primer P60313N and the oligo (d)T anchor primer, however, resulted in the amplification of a 400 bp specific PCR product. This DNA fragment was cloned in pGEM T Easy yielding pRaEH3. The nucleotide sequence of the insert in pRaEH3 was determined and the deduced amino acid sequence shared similarity to the C-terminal amino acid sequence of *R. glutinis* Eph1. Moreover, a conserved amino acid sequence, GGHFAA, found in mEHs and containing the catalytic histidine, was present. These results indicated that, in addition to the 5' end, also the 3'end of the *R. araucariae EPH1* cDNA was isolated.

Specific primers targeted to the *R. araucariae EPH1* ATG start-codon region (P6031ATG) and the TGA stop-codon region (P6031TGA) were designed to amplify the entire Eph1 encoding cDNA sequence. Initially, the RT reaction mixture was used as template in the PCR reaction. However, no DNA fragment could be synthesized. Therefore, messenger RNA (mRNA) was isolated from total RNA using the Oligotex kit (Qiagen). A subsequent RT-PCR reaction, with mRNA as template in the RT reaction and primers P6031ATG and P6031TGA in the PCR reaction, resulted in the amplification of a DNA fragment of approximately 1,2 kb. This product was cloned and sequenced. The deduced amino acid sequence indicated that the complete putative Eph1-encoding cDNA of *R. araucariae* was isolated.

Eph1 amino acid sequence alignment

The *EPH1* cDNA sequences of *R. toruloides* CBS 349, *R. toruloides* CBS 14 and *R. araucariae* encode polypeptides of 409, 409 and 410 amino acids respectively. The calculated molecular weights correspond to 46 kDa. The amino acid sequences of yeast Eph1 enzymes, including *X. dendrorhous* Eph1, as well as the *A. niger* EH (AnEH) were aligned and typical α/β hydrolase fold EH motifs were identified, including putative active site residues (Fig. 1). The level of amino acid identity between these enzymes is depicted in Table 2.

Table 2. EH amino acid-sequence similarity.

	*An*EH	*Xd*Eph1	*Rg*Eph1	*Rt*14Eph1	*Ra*Eph1
*An*EH	-	28.4	29.4	28.6	28.6
*Xd*Eph1		-	30.3	30.3	27.8
*Rg*Eph1			-	96.1	67.0
*Rt*14Eph1				-	67.5
*Ra*Eph1					-

The amino acid sequence similarity (%) between the EHs from *A. niger* (*An*EH), *X. dendrorhous* (*Xd*Eph1), *R. glutinis* (*Rg*Eph1), *R. toruloides* CBS 14 (*Rt*14Eph1) and *R. araucariae* (*Ra*Eph1) is given.

Construction of EPH1-expressing *E. coli* BL21(DE3) strains

With regard to this study, the amino acid sequences of the Eph1s from *R. toruloides* CBS 14 and *R. araucariae* were denominated *Rt*14Eph1 and *Ra*Eph1 respectively. Since the nucleotide and amino acid sequences of *R. glutinis* and *R. toruloides* CBS 349 Eph1 were identical, the corresponding enzyme was referred to as *Rg*Eph1.

The Eph1-encoding cDNA sequences were cloned into the *Nco*I and *Eco*RI or *Not*I endonuclease sites of the expression vector pET28a(+) and transferred to *E. coli* BL21(DE3) resulting in strains BL21(DE3)*Rg*Eph1, BL21(DE3)*Rt*14Eph1 and BL21(DE3)*Ra*Eph1.

Hydrolysis of *trans*-1-phenyl-1,2-epoxypropane

The hydrolysis of *trans*-1-phenyl-1,2-epoxypropane hydrolysis by the wild type yeast strains and the recombinant *E. coli* was evaluated (Table 3). In all cases, the enantioselective hydrolysis of *trans*-1-phenyl-1,2-epoxypropane was observed yielding (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane as residual epoxide.

Of the four yeast strains, *R. toruloides* CBS 349 showed the highest initial reaction rate, while the highest yield of (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane was observed for *R. glutinis*. Both, *R. toruloides* CBS 14 and *R. araucariae* exhibit low activity and enantioselectivity. The highest yields of (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane were obtained with the recombinant *E. coli* strains BL21(DE3)*Rg*Eph1 and BL21(DE3)*Rt*14Eph1. Compared to these two *E. coli* strains, the third strain BL21(DE3)*Ra*Eph1 gave a rather low initial reaction rate and corresponding yield of (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane.

In general, the *E. coli* strains exhibited much higher activities when compared to their yeast counterparts (Table 3). The best improvement was that observed for BL21(DE3)*Rt*14Eph1 versus *R. toruloides* CBS 14.

Table 3 Hydrolysis of *trans*-1-phenyl-1,2-epoxypropane.

Organism	Strain	EH activity (mU · mg dw^{-1})	Yield (%)
R. glutinis	CBS 8761	6.5	40
R. toruloides	CBS 349	3.2	42
R. toruloides	CBS 14	0.2	10
R. araucariae	CBS 6031	0.3	21
E. coli	BL21(DE3)*Rg*Eph1	810	46
E. coli	BL21(DE3)*Rt*14Eph1	970	48
E. coli	BL21(DE3)*Ra*Eph1	52	40

Hydrolysis of 10 mM racemic *trans*-1-phenyl-1,2-epoxypropane in 20 mL reaction mixture with wild type yeast cells or recombinant *E. coli* cells, which express the corresponding yeast *EPH1* genes. The yield of the residual substrate (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane is given. Theoretically, when (1*S*,2*S*)-*trans*-1-phenyl-1,2-epoxypropane is completely converted a maximal yield of 50% enantiopure (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane remains, which represents a 100% enantiomeric excess (e.e.). Reactions were stopped when the e.e. of (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane was higher than 98%.

DISCUSSION

To explore the biological diversity and potential industrial use of yeast EHs the Eph1-encoding cDNA sequences from *R. toruloides* CBS 349, *R. toruloides* CBS 14 and *R. araucariae* were isolated, characterized and expressed in *E. coli*.

The Eph1-encoding cDNA sequence of *R. toruloides* CBS 349 was 100% identical to that of the *R. glutinis* sequence. This is striking since the Eph1 activities and enantioselectivities exhibited by these yeasts toward the 1,2-epoxyalkanes [27] and *trans*-1-phenyl-1,2-epoxypropane are different. Because *R. glutinis* is the anamorphic state of *R. toruloides*, these yeast species have a highly similar genetic background, which could explain the identical *EPH1* genes. However, at the Eph1 protein level contradicting data have been reported. While the size of the purified *R. glutinis* Eph1 was 46 kDa large [33], the purified EH from *R. toruloides* CBS 349 was 54 kDa, although heavily glycosylated [34]. We found 39 proline and 5 cysteine residues in the *R. glutinis* Eph1 amino acid sequence, whereas these residues were absent in an acid hydrolysate of the purified *R. toruloides* CBS 349 Eph1. Unfortunately, no amino acid sequence information of the purified EH from *R. toruloides* CBS 349 EH has been reported, which could support these results. However, based on the Southern analysis we found no evidence for more than one Eph1-encoding gene in both *R. glutinis* and *R. toruloides* CBS 349. Moreover, a single *EPH1* gene is present in *X. dendrorhous* [25].

The *A. niger* EH is a well characterized enzyme, which is related to mEH [35]. The AnEH crystal structure is known and active site amino acid residues have been identified [16]. AnEH belongs to the α/β hydrolase fold family of enzymes [16] and hydrolyzes epoxides via a characteristic two-step mechanism involving an enzyme-

substrate-ester intermediate. Based on amino acid sequence similarity to AnEH, positions of putative active site residues were assigned in the primary structure of the cloned yeast Eph1s (Fig 1). Additionally, amino acid sequences corresponding to the oxyanion hole and nucleophile motifs of α/β hydrolase EHs were observed in the Eph1 sequences from yeasts. Therefore, the yeast Eph1 is related to mEH as AnEH is and it is conceivable that the examined yeast Eph1 enzymes belong the α/β hydrolase fold EHs and hydrolyze epoxides via an enzyme-substrate-ester intermediate.

	HGXP	sxnxss	Y
*An*EH	114-LHGWPG-119/	/189-QGGDIGSF-196/	/250-AYA-252
*Rg*Eph1	114-CHGWPG-119/	/187-QGGDWGS··-193/	/260-AYY-262
*Ra*Eph1	115-CHGWPG-120/	/188-QGGDWGS··-194/	/261-AYY-263
*Rt*14Eph1	114-CHGWPG-119/	/187-QGGDWGS··-193/	/260-AYY-262
*Xd*Eph1	104-SHGWPS-109/	/178-GAGDWGSW-185/	/262-GYQ-264

	Y	A	H
*An*EH	313-TYR-315/	/347-KDL-349/	/372-EGGHFAAL-379
*Rg*Eph1	330-PYS-332/	/347-GEI-349/	/382-EGGHFAAL-389
*Ra*Eph1	331-PYS-333/	/348-GEL-350/	/383-DGGHFAAL-390
*Rt*14Eph1	330-PYS-332/	/347-GEI-349/	/382-DGGHFAAL-389
*Xd*Eph1	332-PYK-334/	/358-AEI-360/	/382-RGGHFAAV-389

Fig. 1. Comparison of the amino acid sequences of EHs from *A. niger* (*An*EH) [34], *R. glutinis* / *R. toruloides* CBS 349 (*Rg*Eph1), *R. toruloides* CBS 14 (*Rt*14Eph1), *R. araucariae* (*Ra*Eph1) and *X. dendrorhous* (*Xd*Eph1) [25]. Depicted are, in the one letter code, sequence regions corresponding to the conserved oxyanion hole (HGXP) and nucleophile (sxnxss) motifs as well as active site residues. The nucleophile motif consists of small residues (s), any residues (x) and the catalytic nucleophilic residue (n). The active site residues of *An*EH have been determined [16,35]. The corresponding yeast residues are appointed based on the homology to *An*EH and are therefore marked as putative. All (putative) active site residues are in bold face. Tyrosine residues that are possibly involved in substrate binding and activation are indicated by Y. Furthermore, the catalytic acidic residue (A) and the catalytic histidine (H) are shown. Numbers indicate amino acid positions. The nucleotide sequences of the *R. toruloides* CBS 349, *R. toruloides* CBS 14 and *R. araucariae* Eph1-encoding cDNAs have been deposited in the GenBank database under accession numbers AF416991, AF416992 and AF416993 respectively.

Excellent activity and enantioselectivity were observed for *R. toruloides* CBS 14 Eph1 expressed in *E. coli* in the hydrolysis of *trans*-1-phenyl-1,2-epoxypropane, while the activity and enantioselectivity of *R. toruloides* CBS 14 itself were quite low (Table 3). Apparently, the *Rt*14Eph1 enzyme in *R. toruloides* CBS 14 is either produced at a very low level or the enzyme is minimally active due to e.g. unfavorable intracellular Eph1 conditions, which makes it difficult to determine its enantioselectivity. Therefore, by means of over-expression of the *EPH1* cDNA in *E. coli* sufficient Eph1 is produced,

which enables the determination of the actual enantioselectivity of the enzyme in question. Consequently, the *R. toruloides* CBS 14 Eph1 revealed to be highly enantioselective. These results illustrate the great value of the cloning and over-expression of genes in heterologous hosts as has now been shown for the EH-encoding genes from yeasts in *E. coli*. In this way, related but better yeast Eph1s can be found, which would otherwise not be recognized in screening programs due to the low enzyme activity of the corresponding host. Therefore, the isolation of Eph1-encoding genes from yeasts by (degenerate) polymerase chain reactions should be quite feasible. The expression system, which was used in this study, is very suitable for the evaluation of the potential of the corresponding Eph1 activities.

Under optimized conditions the maximal yield of (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane from the hydrolysis of 1-phenyl-1,2-epoxypropane by *R. glutinis* was 45% [29]. In this study, moderate yields (~40%) of (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane were obtained in the enantioselective hydrolysis of *trans*-1-phenyl-1,2-epoxypropane by *R. glutinis* and *R. toruloides* CBS 349. *R. toruloides* CBS 14 and *R. araucariae* exhibited yields of only 10 and 21% respectively. Additionally, low (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane yields were also reported for the mammalian mEH catalyzed *trans*-1-phenyl-1,2-epoxypropane hydrolysis [30]. More promising results (~30% yield) have been found by using *Beauvaria bassiana* (formally named *B. sulfurescens*) and recombinant human sEH [31,32]. Nevertheless, for now, the recombinant *E. coli* that produce *R. glutinis* or *R. toruloides* CBS 14 Eph1 are probably the best choice for the production of enantiopure (1*R*,2*R*)-*trans*-1-phenyl-1,2-epoxypropane because (i) the enantioselectivity of these cells is at least equal to, but probably better than, that of *R. glutinis* under optimized conditions, resulting in high yields of enantiopure epoxide (up to 48%) and (ii) the recombinant Eph1 is over-produced in *E. coli* resulting in cells with Eph1 activities, which are 2 to 3 orders of magnitude higher than that of the wild type yeast cells.

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INTRODUCTION

Enantiopure epoxides and diols are versatile chemical building blocks in the organic synthesis of fine chemicals such as pharmaceuticals and agrochemicals. Epoxide hydrolases (EHs) catalyze the co-factor independent hydrolysis of epoxides to the corresponding diols. Interestingly, in many cases mixtures of racemic epoxide can be hydrolyzed by EHs in an enantioselective manner yielding enantiopure epoxides and / or diols. Therefore, EHs might be used as a biocatalyst in the production of these respective enantiopure compounds.

EHs are omnipresent and have been characterized from various organisms. The EH activity from the basidiomycetous yeast *Rhodotorula glutinis* in particular exhibits interesting characteristics, such as a broad substrate range, a high activity and enantioselectivity. Because of these properties *R. glutinis* is a promising source of an EH biocatalyst, which could be applied in the enantioselective hydrolysis of epoxides.

At the start of this research project no information was available about the molecular biology of EHs from yeast species. This kind of information was highly desired since it would open the way for the development of an efficient yeast EH-based epoxide hydrolysis system. Therefore, the research described in this thesis focused on two items. First, the isolation and characterization of EH-encoding genes from 5 yeast species elucidated the identities of the yeasts EHs (chapters 2, 3 and 5). Second, the yeast EH-encoding genes were overexpressed in the heterologous host *E. coli* in order to develop efficient tools for the EH-catalyzed enantioselective hydrolysis of epoxides (chapters 4 and 5).

ISOLATION AND CHARACTERIZATION OF EH-ENCODING GENES FROM YEASTS

The first aim of the research described in this thesis was to clone and characterize EH-encoding genes from yeast species. The EH-encoding gene from *Xanthophyllomyces dendrorhous* CBS 6938 was the first one cloned from a yeast species and it was denominated *EPH1* (chapter 2). Based on this information, additional *EPH1* genes from *R. glutinis* CBS 8761, *R. toruloides* CBS 349, *R. toruloides* CBS 14, and *R. araucariae* CBS 6031 were isolated (chapters 3 and 5). These genes all encode polypeptides of approximately 410 amino acids large with calculated molecular masses of 46 kDa. Comparison of the Eph1 amino acid sequences to that of other EH sequences showed a clear similarity to the mEH type of the α/β hydrolase fold EHs, while homology to sEH was low (Figure 1).

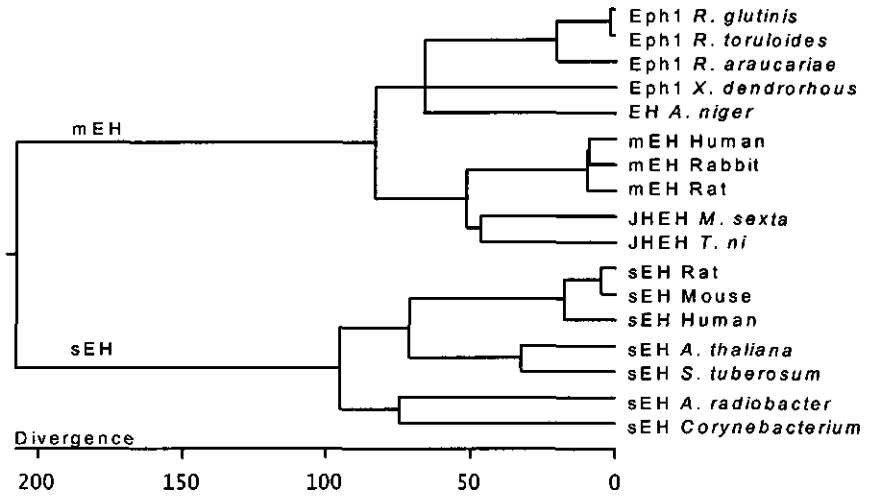

Figure 1. Phylogenetic tree of (putative) α/β hydrolase fold EHs. EH, epoxide hydrolase. Eph1, yeast EH. JHEH, juvenile hormone EH. mEH, microsomal EH. sEH, soluble EH. *R. glutinis*, *Rhodotorula glutinis*. *R. toruloides*, *Rhodosporidium toruloides* CBS 14. *R. araucariae*, *Rhodotorula araucariae*. *A. niger*, *Aspergillus niger*. *X. dendrorhous*, *Xanthophyllomyces dendrorhous*. *M. sexta*, *Manduca sexta*. *T. ni*, *Trichoplusia ni*. *S. tuberosum*, *Solanum tuberosum*. *A. thaliana*, *Arabidopsis thaliana*. *A. radiobacter*, *Agrobacterium radiobacter*.

Two typical α/β hydrolase fold motifs are present in the Eph1 sequence. The first one is the nucleophile motif s-x-n-x-s-s, where s is a small amino acid, x is any amino acid and n is the nucleophilic residue of the catalytic triad. The second motif, HGXP, corresponds to the oxyanion hole of the enzyme and consists of His (H), Gly (G), any (X) and Pro (P) amino acid residues. The Eph1 HGXP motif contains a Trp residue at the X position.

Based on the amino acid sequence homology of yeasts Eph1s to mEHs putative Eph1 active-site residues can be appointed in the primary enzyme structure as they align with the active site residues of e.g. rat mEH and *A. niger* EH (Figure 2). The first member of the putative Eph1 catalytic triad is an Asp residue, which fits perfectly to the nucleophile motif of α/β hydrolase fold enzymes. The so-called charge relay system is represented by the other two members of the catalytic triad, an acidic residue (Glu or Asp) and a His residue. Except for the *A. niger* EH, the acidic residue of the charge relay system corresponds to a Glu residue in all mEH sequences. The cloned yeasts Eph1s make no exception: a Glu residue represents the corresponding acidic residue of the putative Eph1 catalytic triad. The third putative member is a His residue, which aligns to the perfectly conserved catalytic His residues of other EHs. Therefore, the

catalytic triad of the putative Eph1 active site consists of Asp-Glu-His. Furthermore, putative active site Tyr residues are present in the yeast Eph1 sequence at positions corresponding to two *A. niger* EH active site Tyr residues, which are involved in epoxide positioning and activation.

Taken together, the cloned yeast Eph1s are related to mEH and they probably are α/β hydrolase fold enzymes, which hydrolyzes epoxides via an enzyme-substrate-ester intermediate analogous to mEH and sEH.

The mammalian mEH is linked to the microsomal membrane by means of an N-terminal membrane anchor sequence. The main difference between mammalian mEH and the fungal mEH related enzymes is the absence of this anchor sequence in the *A. niger* EH and the yeasts Eph1s. Consequently, both wild type and recombinant *A. niger* EH were found to be a soluble enzyme (Arand *et al.*, 1999a). In agreement with this, the active recombinant *R. glutinis* Eph1 is soluble in *E. coli*. However, the corresponding Eph1 activity in *R. glutinis* is membrane associated (Kronenburg *et al.*, 1999). Additionally, membrane associated Eph1 activity was demonstrated in other yeasts (Botes *et al.*, 1999; Visser *et al.*, 1999). To clarify this discrepancy, attempts to determine the *X. dendrorhous* Eph1 intracellular location were conducted. A gene encoding an Eph1-Enhanced Green Fluorescent Protein (EGFP) fusion protein was constructed and cloned into a *X. dendrorhous* transformation vector. The vector was targeted to the endogenous *EPH1* gene for insertion into the chromosome. Upon expression from the endogenous *EPH1* promoter the fusion protein would be produced and transported intracellularly. By fluorescence microscopy the position of the fusion protein would be visualized, which might indicate the cellular location of Eph1 in *X. dendrorhous*. Unfortunately, this experiment was not successful.

FUNCTION OF EPH1 FROM YEASTS

The mEHs of mammals are a group of important enzymes involved in the detoxification of mutagenic and cytotoxic epoxides. They exhibit broad substrate ranges, which is a prerequisite property for such an enzyme.

Similarly, it has been demonstrated that *R. glutinis* hydrolyzes a versatile group of epoxides. This could indicate that also the Eph1s from yeasts are involved, just like mammalian mEH, in the detoxification of harmful epoxides. However, no paper that demonstrates direct evidence for this putative role has been reported. Nevertheless, it is likely that the yeast is challenged by noxious epoxides, either directly or indirectly.

Directly, the yeast cell might encounter plant or fungal metabolites, which carry an epoxide moiety and exhibit anti-microbial properties. For example, the plant genus *Rutaceae* is capable of producing acridone alkaloides.

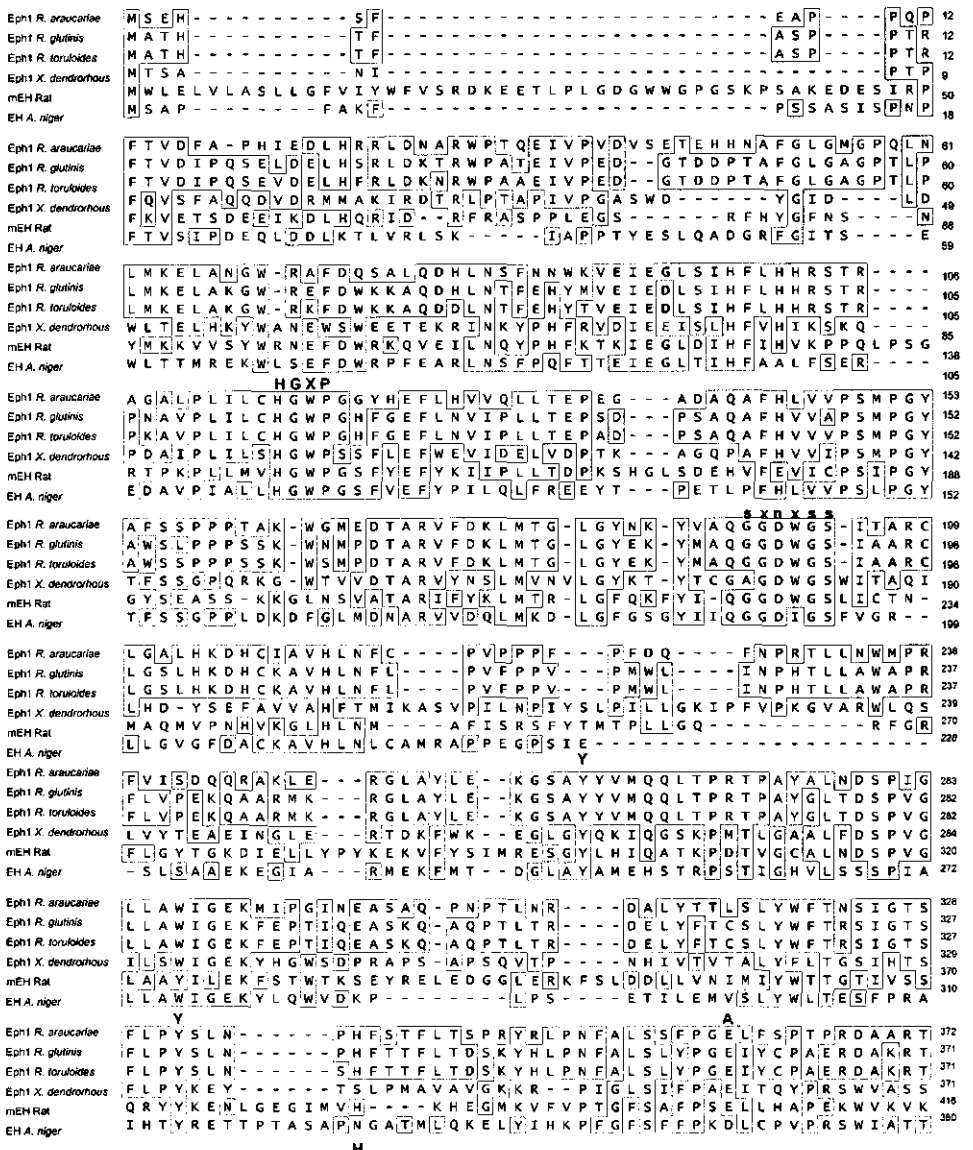

Figure 2. Amino acid sequence comparison of the mEH related EHs from *R. glutinis*, *R. aruacariae*, *R. toruloides* CBS 14, *X. dendrorhous*, *A. niger* and rat. Eph1, yeast EH. Amino acid residues which are similar to the consensus sequence are indicated in boxes. sxnxss, nucleophile motif (see text). HGXP, oxyanion motif. Y, (putative) active site Tyr residues. A, (putative) acidic residue of the charge relay system of the catalytic triad. H, (putative) catalytic histidine.

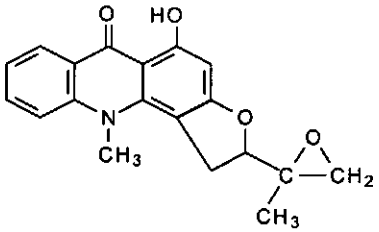

Figure 3. Chemical structure of rutacridone epoxide.

When suspension-cultured cells of *Ruta graveolens* L. are treated with an autoclaved culture homogenate of *Rhodotorula rubra* the plant responds with a rapid accumulation of acridone epoxides (Figure 3), which have antibiotic properties (Eilert *et al.*, 1984; Eilert and Wolters, 1989). EH activity has been demonstrated in *R. rubra* (Botes *et al.*, 1999). Additionally, a partial sequence similar to Eph1 was isolated from this yeast (GenBank accession number AY062024, Visser *et al.*, unpublished results). Therefore, *R. rubra* might use an EH to defend itself. However, it is not known whether the *R. rubra* Eph1 accepts acridone epoxides as substrates.

Trichothecenes comprise a group of mycotoxins, which are mainly produced by fungi of the genus *Fusarium*. Trichothecenes are sesquiterpenoid compounds, which all possess a double bond at C-9,10 and an epoxide ring at C-12,13. Presently, over 150 different members of the trichothecenes are known (Coulombe, 1993). These so-called 12,13-epoxytrichothecenes are cytotoxic to eukaryotes and inhibit protein synthesis. Hydrolysis of the 12,13-epoxide group results in a nontoxic compound (Ueno, 1985). Therefore, it is tempting to suggest that Eph1 could hydrolyze the particular epoxide ring, which then implies a detoxifying role of this enzyme in yeast. However, T-2 toxin (a 12,13-epoxytrichothecene, Figure 4) has been shown to be toxic to *R. rubra*, *R. glutinis* and a number of other yeast species tested (Burmeister and Hesseltine, 1970). Nevertheless, it might be possible that one or several trichothecenes are detoxified by yeast Eph1-catalyzed hydrolysis.

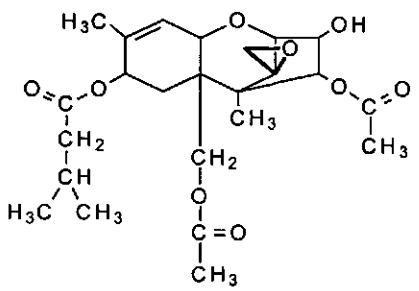

Figure 4. Chemical structure of T2-toxin. The bonds of the epoxide ring are indicated in bold.

The metabolism or detoxification of both biotic and xenobiotic compounds may be initiated by oxidative reactions conducted by the cytochrome P450 system resulting in the formation of an epoxide ring on the specific compound. Therefore, the yeast is also exposed to noxious epoxides in an indirect way. Conversion of these epoxides to the generally less toxic diol products is important to prevent cell damage. Considering its broad substrate range Eph1 might perform this conversion. Therefore, Eph1 might represent an important detoxifying step, analogous to mammalian liver mEH, in the degradation pathway of noxious compounds.

The importance of the *EPH1* gene for growth was analyzed for *X. dendrorhous*. A mutant strain, in which the *EPH1* gene was disrupted, grew normally in rich medium. Therefore, it seems that under these laboratory-conditions *X. dendrorhous* does not need Eph1 for growth.

TOWARDS THE APPLICATION OF RECOMBINANT EPH1 IN BIOCATALYSIS

As already stated above *R. glutinis* is a promising candidate to be applied in the enantioselective epoxide hydrolysis. However, for some substrates it shows a rather low activity. This implies that the cell density of the *R. glutinis* reaction mixture needs to be very high in order to convert a relatively low amount of epoxide. From a practical point of view it is desirable to have an Eph1 production host, which produces higher amounts of Eph1 than *R. glutinis* does. Therefore, the second aim of the research described in this thesis was to improve Eph1 production by over-expression of its corresponding gene in a suitable host.

The use of the prokaryotic T7 RNA polymerase based *E. coli* expression system for heterologous gene expression often leads to very high levels of recombinant gene products. Therefore, this system was chosen for the overproduction of recombinant *R. glutinis* Eph1 (chapter 4). Compared to *R. glutinis* the Eph1 activity (units·mg protein) of the recombinant *E. coli* (both whole cells as well as cell free extract) toward 1,2-epoxyhexane was improved over 200 times. Importantly, *R. glutinis* Eph1 was overproduced without loss of enantioselectivity. These results are an essential step forward towards the industrial application of this yeast enzyme.

In chapter 5 the same expression system was used for the analysis of the Eph1-encoding genes from three additional yeast species i.e. *R. toruloides* CBS 349, *R. toruloides* CBS 14 and *R. araucariae* CBS 6031. The low epoxide hydrolysis rates and concomitant enantioselectivity for *trans*-1-phenyl-1,2-epoxypropane and a range of terminal epoxyalkanes by *R. toruloides* CBS 14 is the reason why this yeast is not the first choice for the enantioselective hydrolysis of these epoxides. However, when the corresponding gene was over-expressed in the *E. coli* system, it revealed to encode an enzyme, which is actually highly enantioselective toward *trans*-1-phenyl-1,2-epoxypropane. These results indicate that a low enantioselectivity toward a certain

epoxide of a yeast species can be caused by a low EH expression level. Higher EH expression levels are therefore necessary to be able to determine the actual enantioselectivity. Therefore, if e.g. in a screening program for yeasts EHs activities, one or more yeast strains with low activity and enantioselectivity are found, it is recommendable to clone and express the genes in *E. coli* or other hosts and analyze the recombinant enzyme. Cloning of these genes should be facilitated using the available sequence information of the EHs from yeasts and *A. niger*. In this way, an interesting Eph1, like the one from *R. toruloides* CBS 14 might be obtained.

On one hand it is important to have a high yield Eph1 production system to guarantee sufficient Eph1 for biocatalysis purposes. However, on the other hand molecular data on epoxide hydrolases led to new insights in the way that these enzymes operate. This essential information can be used to further improve the efficiency of epoxide hydrolysis in another way, namely by adjusting enzyme characteristics by means of recombinant DNA technology as has already been shown for other EHs.

For example, an interesting mutant enzyme of rat mEH was found in the characterization of the active site residues of this enzyme (Arand *et al.*, 1999b). The replacement of Glu^{404} (the catalytic acidic residue of the charge relay system) by Asp strongly increased the V_{max} of the enzyme with styrene oxide (23-fold) and 9,10-epoxystearic acid (39-fold). Of all known mEHs, the *A. niger* EH is the only mEH-related enzyme that contains an Asp at the corresponding position in the native enzyme. Consequently, the fungal enzyme exhibits a remarkably high turnover number when compared to native mammalian mEHs. Both the *A. niger* EH as well as the mutant ($Glu^{404}Asp$) rat mEH are interesting tools for the preparation of optically pure epoxides.

EH crystal structures provide very valuable data such as a detailed spatial view on these enzymes. The shape of the catalytic cavity and the active site amino acid residues presumably determine the enantioselectivity of the EH. When the crystal structure is known computer-modeling experiments with epoxides of interest can be conducted. A similar approach was used to explain the enantioselectivity of the *A. niger* EH regarding its model-substrate 4-nitrostyrene oxide. The different direction of the nucleophilic attack by Asp^{192} on the epoxide ring of 4-nitrostyrene oxide, caused by the different arrangements of both enantiomers in the catalytic cavity, explains the enantiopreference of the *A. niger* EH for the (*R*) enantiomer (Zou *et al.*, 2000). Therefore, based on the shapes of the EH and the epoxide, it might be possible to predict whether a certain epoxide could be a substrate and what the enantiopreference of the EH will be. Furthermore, the structural data can be used to alter or improve EH properties by adding, removing or changing amino acid residues to result in a kind of "tailor-made" enzyme for the (altered) enantioselective hydrolysis of a target epoxide.

FUTURE PROSPECTS

The major drawback of using *R. glutinis* as a general biocatalyst in the enantioselective hydrolysis of epoxides was its low activity towards substrates such as 1-oxa-spiro[2.5]octane-2-carbonitrile. This problem was eliminated by the Eph1 overproduction system, which is described in chapter 4.

To make the *R. glutinis* Eph1 an even more attractive biocatalyst it needs to be further analyzed. Similar to the $Glu^{404}Asp$ mutation in rat mEH, which caused a strong increase in the substrate turnover number, the Glu residue of the putative catalytic Eph1 triad could be replaced by Asp. This mutant Eph1 should then be analyzed to determine whether or not the substrate turnover rate has increased. Given the very valuable information that is gained upon determination of the crystal structure of EHs from *A. niger* (Zou *et al.*, 2000), *A. radiobacter* (Nardini *et al.*, 1999) and mouse (Argiriardi *et al.*, 1999), the determination of the *R. glutinis* Eph1 crystal structure is in progress. Its structural data can initiate the rational design of Eph1 mutant enzymes with improved or altered characteristics.

Additionally, improved Eph1 enzymes might be obtained by directed evolution using techniques such as gene shuffling (Stemmer, 1994) or error prone PCR (Leung *et al.*, 1989). These techniques have been used successfully to alter enzyme properties such as the substrate specificity of β-galactosidase (Zhang *et al.* 1997) or the inversion of the enantioselectivity of hydantoinase (May *et al.*, 2000). However, the success of this approach is highly dependent on the availability of a good high throughput screening method. So far, the absence of such a method has hampered the design of new EHs by directed evolution. However, a styrene oxide based screening method with high throughput screening potential was reported recently (Zocher *et al.*, 1999).

These approaches will probably result in a very efficient biocatalyst. Nevertheless, a variety of molecules that contain an epoxide ring exist, each having its own distinctive properties and structure. Therefore, Eph1 has to be optimized with regard to the epoxide of interest.

Whether the industrial potential of Eph1 will be exploited depends on a lot of factors such as the type of the epoxide or diol of interest, the type of reaction medium, the type of reactor, the stability of the biocatalyst, product recovery and so on. The total of these factors determines whether or not a biocatalytic process is economically feasible (Schmid *et al*, 2001). An important point to consider is that many epoxides are insoluble in water and are toxic to whole cells, including cells of *E. coli*. Therefore, the recombinant *E. coli* strains, which overproduce yeast (*R. glutinis* or *R. toruloides*) Eph1 (chapters 4 and 5) cannot simply be used in an aqueous medium as whole-cell biocatalyst in the presence of high concentrations of epoxides. A solution to this problem could be to use a two-liquid-phase medium. In this system an aqueous phase contains the biocatalyst, while an apolar solvent phase contains the toxic substrate at a high concentration.

E. coli has been used successfully in the biocatalysis of alkanes, toluene and styrene derivatives in two-liquid-phase media, despite the low solvent tolerance of this bacterium (Schmid *et al.*, 2001). Both wild type and recombinant *E. coli* are able to withstand the presence of high amounts of apolar phases as long as the cells are growing (at growth rates even less than 0.1 h^{-1}) and the log *P* value of the solvent exceeds 4.0 (Wubbolts *et al.*, 1996). Moreover, organic solvents such as dodecane, hexadecane and bis(2-ethylhexyl)phthalate were used in two-phase biocatalytic processes for the production of (*S*)-styrene oxide from styrene by recombinant *E. coli* (Wubbolts *et al.*, 1996; Panke *et al.*, 2000). A maximal volumetric productivity of 2.2 g (*S*)-styrene oxide per liter of liquid volume per hour has been shown (Panke *et al.*, 2000).

Large-scale biocatalysis of epoxides by whole cells of *R. glutinis* has been reported by Choi *et al.* (1999, 2000). A cascade two-phase hollow-fiber membrane bioreactor was used to minimize the toxic effect of dodecane, which was used to dissolve the feed-epoxide 1,2-epoxyhexane and to extract residual enantiopure (*S*)-1,2-epoxyhexane. After 12 days of operation this system yielded 38 g of enantiopure (*S*)-1,2-epoxyhexane, which corresponds to a volumetric productivity of 3.8 $g\ l^{-1}\ h^{-1}$.

One approach to improve productivity is to increase the amount of enzyme activity by overproduction. Exactly this has been done for the *R. glutinis* Eph1 in *E. coli* and this biocatalyst was effective in the enantioselective hydrolysis of 1-oxa-spiro[2.5]octane-2-carbonitrile (chapter 4). The next step would be to investigate the use of the *R. glutinis* Eph1 overproducing *E. coli* strain in a two-phase biocatalytic process, analogous to that described in the previous paragraphs, for the enantioselective hydrolysis of epoxides. Given the strong improvement in Eph1 activity of the recombinant *E. coli* when compared to *R. glutinis* it is conceivable that a higher productivity can be achieved.

In addition to whole cells, free (a crude or purified enzyme preparation) recombinant Eph1 may also be used. This is particularly interesting if e.g. the epoxide of interest has difficulty to enter the cell by crossing the cell membrane. The functionality of a crude preparation of recombinant *R. glutinis* Eph1 was demonstrated in the preparative-scale Eph1-catalyzed kinetic resolution of 1-oxa-spiro[2.5]octane-2-carbonitrile (de Oliveira Villela Filho, 2001). This process was performed in an aqueous buffered reaction medium, which is in line with so-called "green-chemistry" processes. The objective these processes is to minimize or even exclude environmental pollution by e.g. organic solvents that are used in production processes by the chemical industries. Nevertheless, catalytic properties of an enzyme such as activity, specificity, selectivity and stability can be improved in pure organic solvents (Klibanov, 2001). However, Eph1 needs water to hydrolyze epoxides. The minimum moisture content of crude *R. glutinis* Eph1 to maintain activity in a dodecane-containing reaction medium is 10% (Weijers, 2001). The scope and limitations of the applicability of *R. glutinis* Eph1 in organic solvents can now be explored since it is no longer hampered by the limited availability of sufficient Eph1.

In some occasions, special requirements such as high solvent tolerance or rigid cell structures might prefer the use of another host than *E. coli*. Bacteria from the genus *Pseudomonas* posses several properties that enable these bacteria to deal with solvents with a log *P* value below 4 (Isken and de Bont, 1998). Therefore, when such a solvent would be necessary in an enantioselective Eph1-catalyzed epoxide hydrolysis process *Pseudomonas* would be a better Eph1 host than *E. coli*. When a larger and more rigid cell type is desired *R. glutinis* itself might be the desired host to use. Additionally, it harbors the natural Eph1 environment, which might be optimal for enzyme activity. However, the problem with these alternative hosts is that the tools to genetically modify these organisms are limited and not as efficient as is they are for *E. coli*. Therefore, it is difficult to obtain a stable transformant, which expresses recombinant Eph1 at a high level. Furthermore, *Pseudomonas* species are well known for their ability to degrade toxic compounds such as epoxides or toluene. Consequently, the epoxide or diol of interest might be metabolized.

Among all EHs investigated so far, data concerning the application of EHs as biocatalysts has been restricted to that of the microbial EHs. Various types of epoxides have been hydrolyzed in an enantioselective manner using microbial EHs. Highly substituted (2,2- and 2,3-disubstituted) epoxides are hydrolyzed with good enantioselectivity by bacterial EHs, whereas fungal EHs give good results in case of styrene oxide-type of substrates (Orru and Faber, 1999). *R. glutinis* shows a broad substrate specificity which is partly complementary to and overlapping with that of the other microbial EHs (Weijers, 1997). Moreover, Weijers (2001) reported that the asymmetric hydrolysis of *meso*-epoxides is restricted to *R. glutinis* EH. Furthermore, *R. glutinis* EH is the only suitable biocatalyst known to date for the kinetic resolution of monosubstituted aliphatic epoxides such as 1,2-epoxyhexane oxide and 1,2-epoxyhexene oxide. Finally, in general, the yeast (*R. glutinis*) EH hydrolyzes monosubstituted aliphatic epoxides, 2,3-disubstituted aliphatic and aryl epoxides, and trisubstituted alicyclic epoxides with significant enantioselectivities (Weijers, 2001).

This pool of information should help the organic chemist to select the most appropriate EH for the hydrolysis of the epoxide of interest. Moreover, due to the overlapping substrate specificities several EHs might be optional. Clearly, EHs from yeasts are a very good option, especially in case of *meso*-epoxides and monosubstituted aliphatic epoxides. Furthermore, the overproduction of recombinant yeasts Eph1s allows the enantioselective hydrolysis of substrates for which the yeasts themselves show low activities and enantioselectivities, such as styrene oxide-type of substrates.

Therefore, yeast species having EH activities are very interesting for the preparation of enantiopure epoxides. The collection of promising yeasts EHs can be enlarged by screening additional yeast strains for EH activity or by improving cloned Eph1s by recombinant DNA technology. In case of the former, the cloning and over-expression of Eph1-encoding genes will reveal the actual potential of yeasts Eph1s from yeasts that show low EH activity and enantioselectivity themselves as was shown in this thesis.

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SUMMARY

Epoxides are cyclic ethers that readily react with various nucleophilic compounds. Consequently, epoxides can be used in many chemical synthesis reactions. Two enantiomeric forms of an epoxide are possible if one of the carbon atoms is chiral. This means that the epoxide is actually a racemic mixture of its two enantiomers. Due to the universal presence of chirality in nature it is important to use the proper epoxide enantiomer in the synthesis of compounds such as pharmaceuticals and agrochemicals, which should affect biological processes. Therefore, enantiopure epoxides are valuable intermediates in the synthesis of biologically active compounds by the pharmaceutical and agrochemical industries.

Epoxide hydrolases (EHs) catalyze the hydrolysis of an epoxide into its corresponding diol. Moreover, EHs can hydrolyze racemic epoxide mixtures in an enantioselective manner. This results in the hydrolysis of one enantiomer, while the other remains unaffected and thus enantiopure. Therefore, EHs might be valuable tools to obtain enantiopure epoxides from racemic mixtures.

EHs from yeast species, *Rhodotorula glutinis* in particular, have been used to hydrolyze various epoxides with high activity and enantioselectivity. Consequently, the EHs from yeast species are promising biocatalysts that can be used in the production of enantiopure epoxides.

In chapter 1 the work that is presented in this thesis is introduced. The aims of the research project were to gain important fundamental knowledge on EHs from yeasts and to develop biotechnological processes based on the use of these enzymes for the production of enantiopure epoxides. To introduce EHs in general, a broad overview is given in chapter 2 dealing with the molecular biology, biochemistry and potential application of these enzymes.

In order to determine the relationship of these yeasts EHs to other known EHs, the EH-encoding genes and cDNA sequences from the yeast strains *Xanthophyllomyces dendrorhous* and *R. glutinis* were isolated (chapters 3 and 4). The genes were denominated *EPH1*. Whereas the *X. dendrorhous EPH1* open reading frame (ORF) of 1236 bp was interrupted by 8 introns, the 1230 bp-large *R. glutinis EPH1* ORF was interrupted by 9 introns. The genes encoded polypeptides of 411 and 409 amino acids respectively, with corresponding calculated molecular masses of 46 kDa. The deduced amino acid sequences were similar to that of mammalian microsomal epoxide hydrolases. These enzymes belong to the α/β hydrolase fold family of enzymes, which have similar enzymatic structures and mechanisms. The *EPH1* cDNA sequences were expressed in *Escherichia coli* to demonstrate their function. The epoxides, 1,2-epoxyhexane and 1-methyl-cyclohexene oxide, were hydrolyzed in an enantioselective manner. The inactivation of the *EPH1* gene of *X. dendrorhous* showed that it was not essential for growth in rich medium under laboratory conditions.

The epoxide hydrolase of *R. glutinis* was overproduced in the heterologous host *Escherichia coli* BL21(DE3) in order to develop a highly effective epoxide hydrolysis

system (chapter 5). A strong improvement in Eph1 activity was found in cell extracts of the recombinant *E. coli* when compared to cell extracts of *Rhodotorula glutinis*, despite the formation of inactive Eph1 inclusion bodies. Co-expression of genes encoding molecular chaperones (DnaK-DnaJ-GrpE, GroEL-GroES, and trigger factor) decreased the amount of Eph1 inclusion bodies. However, there was no equivalent increase in active soluble Eph1. An increase in the level of soluble Eph1 was demonstrated by lowering the cultivation temperature from 37°C to 21°C and by using a fermenter for cultivation. Compared to *R. glutinis* the total increase in Eph1 activity for the recombinant *E. coli* towards 1,2-epoxyhexane was over 200 times, without loss of enantioselectivity. The utility of this Eph1 overproduction system was demonstrated by the hydrolysis of 1-oxa-spiro[2.5]octane-2-carbonitrile, which is a new *R. glutinis* Eph1 substrate and a versatile building block in organic synthesis. Whereas the recombinant *E. coli*, expressing *R. glutinis EPH1*, could be used to hydrolyze 1-oxa-spiro[2.5]octane-2-carbonitrile with high Eph1 activity in an enantioselective manner. This was not possible using *R. glutinis* itself.

To explore the biological diversity and potential industrial use of EHs from yeasts the Eph1-encoding cDNA sequences were also isolated from the carotenoid producing yeast species *Rhodosporidium toruloides* CBS 349, *Rhodosporidium toruloides* CBS 14 and *Rhodotorula araucariae* CBS 6031. These cDNA sequences encoded polypeptides of 409, 409, and 410 amino acids large respectively with molecular masses of 46 kDa. The deduced amino acid sequences were similar to that of the epoxide hydrolases from *R. glutinis*, *X. dendrorhous* and *Aspergillus niger*, which all correspond to the microsomal epoxide hydrolase sequence. Consequently, these cloned Eph1s probably belong to the α/β hydrolase fold family of enzymes. The epoxide hydrolase encoding cDNAs of the *Rhodosporidium* and *Rhodotorula* species were expressed in *Escherichia coli* BL21(DE3). The recombinant strains were able to hydrolyze *trans*-1-phenyl-1,2-epoxypropane in an enantioselective manner. The recombinant counterpart of *Rhodosporidium toruloides* CBS 14 was found to be a highly active and enantioselective biocatalyst for this substrate, despite the low activity and enantioselectivity of *Rhodosporidium toruloides* CBS 14 itself.

SAMENVATTING

Epoxiden zijn cyclische ethers die gemakkelijk kunnen reageren met een verscheidenheid aan nucleofiele stoffen. Daarom kunnen epoxiden in veel chemische synthesereacties gebruikt worden. Er bestaan twee enantiomeren van een epoxide als een van de koolstofatomen van het epoxide chiraal is. Dit betekent dat zo'n epoxide eigenlijk een racemisch mengsel is van de beide enantiomeren. Chiraliteit is overal in de natuur aanwezig. Daarom is het belangrijk het juiste epoxide enantiomeer te gebruiken in de synthese van stoffen die een effect op een biologisch proces moeten uitoefenen, zoals medicijnen of landbouwchemicaliën. Enantiomeerzuivere epoxiden zijn om deze reden waardevolle bouwstenen voor de synthese van medicijnen en landbouwchemicaliën door de farmaceutische en agrochemische industrieën.

Epoxidehydrolases (EHs) katalyseren de hydrolyse van epoxiden naar diolen. Het is zelfs zo dat een groot aantal EHs enantioselectief zijn voor racemische epoxiden. Als gevolg hiervan zet, in het meest ideale geval, een dergelijk EH het ene epoxide enantiomeer om, terwijl het andere epoxide enantiomeer onaangetast blijft. Op deze manier kunnen EHs gebruikt worden om enantiomeerzuivere epoxiden te verkrijgen uit een racemisch epoxiden mengsel.

EHs uit gisten, *Rhodotorula glutinis* in het bijzonder, kunnen verschillende soorten epoxiden met hoge activiteit en enantioselectiviteit hydrolyseren. Deze eigenschappen zorgen ervoor dat EHs uit gisten veelbelovende biokatalysatoren kunnen zijn voor het verkrijgen van enantiomeerzuivere epoxiden.

Het werk zoals beschreven in dit proefschrift wordt ingeleid in hoofdstuk 1. De twee doelstellingen van het onderzoeksproject waren ten eerste het vergaren van belangrijke fundamentele kennis van EHs uit gisten en ten tweede het ontwikkelen van biotechnologische processen voor het produceren van enantiomeerzuivere epoxiden gebruikmakend van EHs uit gisten. In hoofdstuk 2 wordt ingegaan op de moleculaire biologie, biochemie and de potentiële toepassing van EHs om een overzicht van dit soort enzymen in het algemeen te geven.

De genen en cDNA moleculen die coderen voor de EHs uit de giststammen *Xanthophyllomyces dendrorhous* en *R. glutinis* werden geïsoleerd om de verwantschap van deze gist EHs met andere, reeds bekende, EHs te analyseren (hoofdstukken 3 en 4). Deze EH coderende genen kregen de naam *EPH1*. Het *X. dendrorhous EPH1* open leesraam van 1236 bp bleek te zijn onderbroken door 8 intronen, terwijl het *R. glutinis EPH1* open leesraam van 1230 bp door 9 intronen werd onderbroken. Deze genen coderen voor eiwitten (EHs) met respectievelijke ketenlengtes van 411 en 409 aminozuren en een berekende moleculaire massa van 46 kDa. De afgeleide aminozuurvolgorden vertoonden gelijkenis met die van de microsomale EHs uit zoogdieren. Deze enzymen behoren tot de familie van de zogenoemde "α/β hydrolase fold" eiwitten, welke gelijke enzymatische mechanismen en structuren hebben. Om de werking van de gevonden EHs te onderzoeken, werden de *EPH1* cDNA nucleotidenvolgorden tot expressie gebracht in *Escherichia coli*. De epoxiden 1,2-

epoxyhexaan en 1-methyl-cyclohexeen oxide werden op een enantioselectieve manier gehydrolyseerd. Het *EPH1* gen van *X. dendrorhous* werd uitgeschakeld via insertie mutagenese. Hieruit bleek dat dit gen voor de gist niet essentieel is voor groei op rijk medium onder laboratorium condities.

Ten behoeve van de ontwikkeling van een efficiënt epoxiden hydrolyse systeem werd het *R. glutinis* EH eiwit (Eph1) in de heterologe gastheer *Escherichia coli* BL21(DE3) overgeproduceerd (hoofdstuk 5). De recombinant *E. coli* gaf een sterke verbetering in Eph1 activiteit voor 1,2-epoxyhexaan hydrolyse ten opzichte van *R. glutinis*, ondanks de vorming van inactieve Eph1 aggregaten. De gelijktijdige expressie van *EPH1* en genen die coderen voor moleculaire chaperonne eiwitten (DnaK-DnaJ-GrpE, GroEL-GroES en "trigger factor") resulteerde in een afname van Eph1 aggregaten. Helaas resulteerde dit niet in een evenredige toename van epoxide hydrolase activiteit. Door echter de *E. coli* cultuur te kweken in een fermentor en door de temperatuur van de cultuur van 37°C terug te brengen tot 21°C werd wel een toename bewerkstelligd in de hoeveelheid actief en oplosbaar Eph1. Dit resulteerde in een meer dan 200-maal hogere Eph1 activiteit ten opzichte van *R. glutinis*, waarbij ook de enantioselectiviteit behouden bleef. Het nut van dit Eph1 overproducerende systeem werd aangetoond met de hydrolyse van 1-oxa-spiro[2.5]octaan-2-carbonitril. Dit is een nieuw *R. glutinis* Eph1 substraat en een veelzijdige bouwsteen in de organische synthese. Met dit systeem kon 1-oxa-spiro[2.5]octaan-2-carbonitril met een hoge activiteit op een enantioselectieve manier worden gehydrolyseerd, terwijl dit met *R. glutinis* zelf niet mogelijk was.

Om de biologische diversiteit en potentiële industriële toepassing van EHs van gisten te onderzoeken werden ook de Eph1 coderende cDNA moleculen van de carotenoïden producerende gistsoorten *Rhodosporidium toruloides* CBS 349, *Rhodosporidium toruloides* CBS 14 en *Rhodotorula araucariae* CBS 6031 geïsoleerd. Deze moleculen coderen voor eiwitten met een respectievelijke ketenlengte van 409, 409 en 410 aminozuren en een berekende moleculaire massa van 46 kDa. De afgeleide aminozuurvolgorden vertoonden gelijkenis met de EHs van *R. glutinis*, *X. dendrorhous* en *Aspergillus niger*, welke behoren tot de familie van eiwitten met de zogenaamde "α/β-hydrolase fold". De *EPH1* cDNA moleculen van de *Rhodosporidium* en *Rhodotorula* soorten werden tot expressie gebracht in *Escherichia coli*. Deze recombinant *E. coli* stammen waren in staat om *trans*-1-fenyl-1,2-epoxypropaan op een enantioselectieve manier te hydrolyseren. *Rhodosporidium toruloides* CBS 14 vertoonde een lage EH activiteit en enantioselectiviteit. Toen het bijbehorende *EPH1* gen in *E. coli* tot overexpressie werd gebracht bleek het verantwoordelijke Eph1 echter zeer actief en enantioselectief voor dit substraat te zijn.

NAWOORD

Een proefschrift samenstellen doe je niet alleen. Daarom wil ik graag een aantal mensen noemen zonder wiens hulp dit proefschrift niet tot stand was gekomen.

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CURRICULUM VITAE

Johannes Heinrich (Hans) Visser werd op 20 december 1970 te Arnhem geboren en groeide op in Lobith, een dorpje in de Liemers. In 1988 werd het HAVO diploma behaald aan de St. Andreas Scholengemeenschap te Zevenaar. In hetzelfde jaar werd begonnen aan de studie chemische laboratoriumopleiding, afstudeerrichting biotechnologie, aan de Hogeschool Gelderland te Arnhem en Nijmegen. Deze opleiding werd in 1992 afgesloten na een stageperiode bij het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede. Aansluitend werd begonnen met de studie Biologie aan de Katholieke Universiteit Nijmegen. In september 1995 werd deze studie afgerond met als hoofdvak moleculaire biologie en als bijvak moleculaire plantenfysiologie.

Als assistent in opleiding en toegevoegd onderzoeker heeft de auteur vanaf februari 1996 promotie onderzoek verricht bij de sectie Industriële Microbiologie van Wageningen Universiteit. De resultaten van dat onderzoek staan beschreven in dit proefschrift.

Vanaf februari 2002 is de auteur aangesteld als post-doctoraal onderzoeker binnen het EG-project "Production of fungal carotenoids for healthy nutrition" bij het Laboratorium voor Microbiologie van Wageningen Universiteit.

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