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Epoxide Hydrolases: Applications in Pharmacological and Synthetic Industry

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Abstract: In the last two decades the exploitation of enzyme and microbes by synthetic as well as pharmaceutical industry has increased substantially. Epoxide hydrolase (EH) is an important enzyme widely used in kinetic resolution and synthesis of vicinal diol. This a highly attractive biocatalyst used in the formation of a single enantiomeric diol from a racemic oxirane. The microbial epoxide hydrolase hydrolyses substrates of various structural types. EHs are cofactor-independent enzymes that are easy to use for organic synthesis. Moreover, these enzymes are ubiquitous and not restricted to the mammalian world only. These are found in bacteria, yeast, fungi, plants and insects. There is a wide range of applications of EHs in pharmaceuticals as well as in clinical industry. EHs may enable the preparation of enantiopure epoxides in a very simple way starting from cheap and easily available racemic epoxides. This review covers the structure, mechanism of action and catalytic potential of EHs in pharmacological and synthetic industry.

Key words: Epoxide hydrolases, recemic epoxides, enantiopure epoxides, synthetic industry.

Introduction

The use of enzymes to catalyze the synthetic reactions is superior to use of conventional methods of chemical modification due to high catalytic efficiency and inherent selectivity, which result in high yield of relatively pure products. Epoxide hydrolases (EC 3.3.2.10) of microbial origin is a class of highly versatile biocatalyst for the asymmetric hydrolysis of proteins that catalyze the hydration of chemically reactive epoxides to their corresponding dihydrodiol products. Simple epoxides are hydrated to their corresponding vicinal dihydrodiols and arene oxides to *trans*-dihydrodiols. In the human body the epoxides appeared following the degradation of aromatic compounds. Thus this enzyme plays a vital role in the metabolism and removal of these tricyclic ring structured compounds. Epoxide hydrolases (EHs) are very important from medical point of view as their deficiency in the body leads to 'Dress syndrome'. It has been reported that mainly in the patients who used to take antiepileptic drug - phenytoin are more prone to accumulation of epoxides and development of Dress syndrome. In recemic mixtures these enzymes selectively hydrolyze the epoxides and result in the formation of vicinal diols which in non-hydrolysed epoxides remain in non-recemic form. Some of the interesting features of EHs make them valuable biocatalyst from industrial as well as pharmaceutical point of view [1-3]. These are ubiquitous, co-factor independent and highly enantio-selective enzymes, found in bacteria, yeast, fungi, plants and insects. Moreover, they are easy to use in synthetic reactions in organic media.

In plants, EHs play an important biological roles in physiological responses, development and defense mechanism(s) by regulation of epoxides and lipid substrates in cells [4]. There are seven distinct epoxide hydrolases reported including the mammalian soluble epoxide hydrolase, the hepoxilin hydrolase, leukotriene A4 hydrolase, the

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microsomal epoxide hydrolase and the insect juvenile hormone epoxide hydrolase. Mammalian EHs are important in detoxification and metabolism regulation in body. In mammalian species, there are at least five epoxide hydrolase forms, microsomal cholesterol 5,6-oxide hydrolase, hepoxilin A3 hydrolase, leukotriene A4 hydrolase, soluble and microsomal epoxide hydrolase which are different chemically and immunologically [5]. As far as the medical implications of EHs are concerned, these enzymes play vital role in vascular and cardiac homeostasis by degrading/metabolizing the arachidonic acid and epoxyeicosatrienoic acids [6]. Epoxyeicosatrienoic acids maintain the vascular functioning by decreasing the inflammation and platelet aggregation. Many enzymes of mammalian origin have been investigated for detoxification of accumulated compounds in cells [7,8] but their use at large scale is not so much prominent [9,10]. In the last 4-5 years, the production of EHs and its role in medical and pharmaceutical industry has increased sharply. Thus in the present review the key features of microbial epoxide hydrolases and their substrate selectivity relationship, particularly in view of preparative-scale applications and medical implications if any, are summarized.

History and importance of EHs

Biocatalysis in organic and aqueous media being recognized as eco-friendly as well as (avoids production of potentially toxic waste) in industrial processes has gained much attention [11-14]. Synthesis of L(+)-tartaric acid at industrial scale is the first application of an EH catalyzed reaction which is widely used in the food industry, pharmaceutical industry, chemical analysis and textile industry [15]. During the last two decades, greater emphasis has been given to mammalian EHs due to their immense medical importance. But now a day, bacterial EHs have emerged as resourceful biocatalysts of commercial value for preparation of enantiopure pharmaceuticals, medicines, pesticides, fine chemicals, anti-obesity drugs, anticancer agents, N-methyl-D-aspartate receptor antagonists with neuroprotective and nematocidic properties [16-19]. The ubiquitous EHs from different sources have been reported over a period of time (Table 1). The major bacterial genera that produce EHs include *Rhizobium, Pseudomonas* [20], *Nocardia* [21], *Corynebacterium* [22] and *Rhodococcus* [23].

Table 1. Prominent sources of epoxide hydrolases

S. No.	Source(s)	Organism (s)	Reference (s)
1	Bacteria	*Rhodococcus erythropolis* DCL14	24
		Rhodococcus erythropolis DCL14	25
		Agrobacterium radiobacter	26
		Rhizobium, Pseudomonas, *Nocardia Corynebacterium* and *Rhodococcus*	27-30
2	Actinomycetes	*Rhodococcus* and *Nocardia* sp.	31
3	Fungus	*Aspergillus niger*, *Rhodotorula glutinis* and *Rhodosporidium toruloides*	32-33
		Fungus	33
		Aspergillus niger LCP 521	34
4	Mammalian	Teleost fish:rainbow trout (*Salmo gairdneri*)	35
		Golden medaka (*Oryzias latipes*),	36
		Fathead minnow (*Pimphalespromelas*),	37
		Caenorhabditis elegans	38
5	Yeast	*Rhodotorula*, *Rhodosporidium* and *Trichosporon*	39
6	Plants	Cress and potato	40
		Soybean (*Glycine max*)	41

table 1. (continued).

S. No.	Source(s)	Organism (s)	Reference (s)
		Mouse eared cress (*Arabidopsis thaliana*)	42
		Common tobacco (*Nicotianatabacum*)	43
		Oilseed rape (*Brassica napus)*	44
		Pineapple (*Ananas comosus)*	45
		Castor bean (*Ricinus communis*)	46

To determine activity of EHs, various analytical methods such as liquid chromatography [47-49] gas chromatography [50-52] and colorimetric methods such as UV/VIS spectro- and fluoro-photometry were used [53-55]. Some other assay methods based upon fluorophotometry were also used for the determination of EHs activity [56]. Furthermore, any epoxide which can be metabolized to a vicinal diol with at least one hydrogen substituent at the hydroxyl group can be quantified by analytical assay. Finally, the suggested assay for EHs activity toward any epoxide should be sensitive, reproducible and should be feasible in crude cell extracts.

Basic structure of epoxide hydrolase

The enzyme EHs belongs to α/β-hydrolase super-family which also includes other hydrolases like lipases, esterases and haloperoxidases [57]. The first X-ray crystal structure of fungal EH [53] was reported from *Aspergillus niger* LCP 521. It was reported that EH from *Aspergillus niger* showed a catalytic triad Asp-His-Asp with third residue identified as glutamic acid and two tyrosine residues exert an activating and stabilizing effect on the oxirane oxygen [58,59]. Each dimer of EH contains two active sites and residue of each active site was drawn from single subunit. The shape of the substrate-binding site, with the catalytic nucleophile waiting for the substrate at the end of a relatively narrow hydro-phobic tunnel, offers a perfect explanation for the observed inability of this class of EHs to hydrolyze the bulky *trans*-substituted epoxides. In addition, two tyrosine residues are observed in the active site that can bind the ring oxygen of the epoxide and assist catalysis by subsequent proton donation. The structure of EH showed variations based on α/β-hydrolase fold with a core domain structure which consists of an eight-slandered β-sheet surrounded by α-helices covering the relatively conserved core domain which share less structural conservation between isoenzymes. While in case of mammalian epoxide hydrolases, an additional N-terminal domain is present which swaps between two subunits of native dimeric structure [60]. A typical structure of EHs from *Mycobacterium tuberculosis* was resolved to a resolution of 2.5 Å using single wavelength anamalous dispersion from a selenomethionine substituted protein [61].

Mechanism of action of EHs

The mechanism of hydrolysis of epoxides to the corresponding diols by EHs has been described [62-64]. It is known that epoxides can be opened by direct attack of the nucleophile on the epoxide ring or *via* an intermediate in which there is a covalent link between the enzyme and the substrate (covalent catalysis). Epoxide hydrolases catalyze the hydrolytic reaction by the addition of a water molecule to an epoxide resulting in the formation of the corresponding vicinal diol. No co-factor/ prosthetic or metal ion are required for catalytic activity of EHs but the mechanism of action resembles to that of serine hydrolases like lipases. The mechanism of reaction catalyzed by EH involved two steps mechanism which was extensively studied by various researchers [65-67] and the same is depicted diagrammatically (Figure 1).

1. First step involves the formation of hydroxyl-alkyl-enzyme intermediate at carboxyl of nucleophile.
2. Second step involves the hydrolysis of the ester intermediate by an activated water molecule resulted in the formation of *vic*-diols.

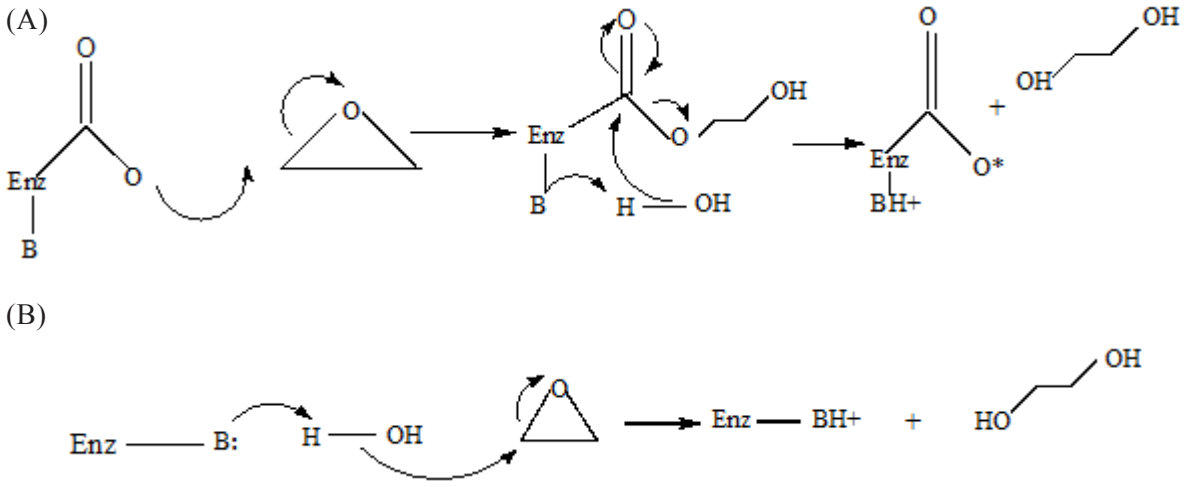

Figure 1. Basic mechanism of epoxide hydrolase catalysed reaction

However, after X-ray crystallographic study of EH, some other mechanisms of biocatalysis of epoxides were proposed depending upon the substrate chosen but the basic scheme remained same. The bio-catalytic mechanism of EH-catalysed reaction from *Agrobacterium radiobacter* was also described as two-steps process [68]. The *Aspergillus niger* enzyme has a similar structure and mechanism [69]. In another two-step mechanism [70], the first step involves the opening of epoxide ring in which two tyrosine molecules form hydrogen bonds with the oxygen of substrate and nucleophilic aspartate attaches epoxide carbons. This step also forms enzyme-substrate intermediate. Similarly, in the second step, catalytic histidine together with a second acidic residue (Asp or Glu) function as a charge-relay pair; this is responsible for activation of water molecule that subsequently hydrolysis the epoxide ring.

A kinetic mechanism of EHs catalyzed reaction was also proposed for various epoxide-containing substrates [71,72]. This mechanism did not take into account the regio-specificity of the enzymes. So it does not clear that which of the carbon atoms of the epoxide ring is to be attacked by the nucleophile, which in turn has importance in the synthesis of fine chemicals with predetermined stereochemistry of the final products of the reaction. It is interesting to note that there is only one oxygen atom from a water molecule got incorporated into product and regio-selectivity of EH determine the configuration of carbon atom whether is to be retained or inverted by attack of nucleophile. Thus overall opening of epoxide ring occurs in a *trans*-specific manner. This is in contrast to the kinetic resolution of esters (*e.g.* by esterases, proteases and lipases), where the absolute configuration at the stereogenic centre always remains the same throughout the reaction [73]. Another study showed that a *Radiobacter* EH involved the displacement of oxygen and produced a covalent intermediate and the hydrolysis of this intermediate by water is facilitated by a histidine residue. This leads to the regeneration of the enzyme and gives the product as a diol. It was proposed that the proton transfer to the oxygen of the substrate is concerned with the attack of the aspartate which leads to ring opening [74].

Purification strategies of EHs

A number of microbial EHs have been purified and characterized using conventional techniques of with respect to their enantio-selectivities [75-80]. Purification and stability of an enzyme are two major significant parameters that decide the catalytic potential of enzyme preparations. The presence of extra/ contaminated proteins in the enzyme preparation leads to decrease the stability of a biocatalyst. Such as presence of some proteases decreases leads to the hydrolysis of purified protein with time and results in decrease in residual activity. As far as the case of EHs has been concerned a few of these purified EHs show sufficient stability and enantio-selectivity in order

to be valuable biocatalysts in biotransformation reactions. Among fungal sources *Aspergillus niger* has proved to be a potential source of EH. A novel enantio-selective epoxide hydrolase was purified from *Aspergillus niger* M200 using ammonium sulphate precipitation, ionic exchange, hydrophobic interaction and size-exclusion chromatography to 186-fold with a yield of 15 %. The apparent molecular mass of the enzyme was determined to be 77 kDa under native conditions and 40 kDa under denaturing conditions, implying a dimeric structure of the native enzyme [81]. In another study, a highly enatio-selective EH from *Nocardia* sp. was purified to homogeneity by using a combination of four chromatographic steps that included hydrophobic interaction chromatography on Phenyl Sepharose CL-4B, anion exchange chromatography on SOURCE 30Q, followed by a second hydrophobic interaction chromatography on Phenyl Sepharose HP and gel-filtration on Superdex 75 HR to remove the unwanted proteins [82]. Previously, an EH was also successfully purified from *Rhodococcus erythropolis* strain DCL14 was originally isolated from a fresh-water sediment sample [83,84].

Catalytic potential in pharmacological and synthetic industry

Synthetic applications of EHs

EHs from microbial sources have gained increasing interest due to their ample availability through cultivation and potential applicability for the regio- and enantio-selective biotransformation of epoxides on a large scale [85]. EHs have been used for synthesis of many valuable enantiomers such as resolution of methyl-isobutylstyrene oxide thus opening the way to the synthesis of (S)-Ibuprofen, the biologically most active enantiomer of this major non-steroidal anti-inflammatory drug [86]. Most of the diol obtained from reactions catalysed by epoxy hydrolase could be cyclized back to the racemic epoxide, thus allowing recycling of this product. It was very important yield-limiting (and industrially expensive) inconvenient step to combine a chemical and an enzymatic step in a one-pot synthetic process. Such an approach was illustrated for the synthesis of Nifenalol [87]. This strategy has been again exploited for the synthesis of (R)-mevalonolactone [88]. A novel epoxide hydrolases from *Yarrowia lipolytica* has been shown to hydrolyze a variety of functionalized epoxides with good to excellent stereo-selectivity and at high volumetric productivities. Individual biotransformation products have been converted into optically active (R)-(tetrahydrofuran-2-yl) methanol,(S)-N-benzyl-3-hydroxypyrrolidine, (S)-3 hydroxytetra-hydrothiophene, (S) -N- benzyl -3-acetoxy-piperidine, (S)-3-hydroxytetrahydrofuran and (R)-(S)-N-benzyl-pyrrolidin-2-yl (phenyl)-methanol. New biotransformation system in order to provide products from less well-studied non-aromatic substrates was of commercial interest. These products on further transformation would provide noteworthy hydroxylated heterocyclic compounds. These experiments mainly concentrated on obtaining the epoxides at high optical purity, and hence the bio-transformations were run to relatively high conversion (62-73 %) in methanol [89].

Medical applications

Enantiopure epoxides and vicinal diols obtained as a product of epoxy hydrolase mediated reaction (s) are valuable chiral building blocks for the synthesis of optically active pharmaceuticals. These chiral building blocks can be prepared from available racemic epoxides using enantioselective or enantioconvergent EHs. Some examples of enantiopure epoxides that are valuable for the synthesis of pharmacologically active compounds are *p*-nitrostyrene oxide-chlorostyrene oxide, *m*-chlorostyrene oxide and *trans* and *cis*-1-phenyl-propene oxide. Soluble epoxy hydrolase is involved in the mechanism of arachidonic, linoleic and other fatty acids epoxides - the endogenous chemical mediators that play important roles in blood pressure regulation and inflammation [90]. Most of the mammalian soluble epoxide hydrolases (sEH) can be utilized as new target for drug development and chemical inhi-bition of this enzyme in animal models was shown to treat hypertension and vascular inxammation as well as related syndromes. Beneficial effects of several potent sEH inhibitors have been reported in cardiac hypertrophy [91-93].

Indeed sEH enzyme belongs to a relatively unexplored pathway of inflammatory lipid

mediators by cytochrome P450 enzymes, transforming arachidonic and linoleic acids to various biologically active compounds, including epoxyeicosatrienoic acids (EETs) or hydroxy-eicosatrienoic acids (HETEs) and epoxyoctadecenoic acids (EpOMEs), respectively. EETs and EpOMEs are further metabolized by sEH to their corresponding diols, dihydroxyeicosatrienoic acids (DHETs) and dihydroxyoctadecenoic acids (DHOMEs), respectively [94,95]. EETs have vaso-dilatory properties similar to that of endothelium-derived hyperpolarizing factor (EDHF) [96]. In addition, EETs produce an anti-inflammatory effect, at least in part, by inhibiting the activation of nuclear factor (NF)-κB-mediated gene transcription [97,98]. Enantiopure epoxides and vicinal diols are versatile synthetic intermediates for the preparation of enantiopure bioactive compounds. In particular enantiopure epichlorohydrin (ECH) is a valuable epoxide intermediate for producing optically active pharmaceuticals. Epoxide hydrolase from *Novosphingobium aromaticivorans* could preferentially hydrolyze (R)-styrene oxide. In this study, purified EH could be also effective in chiral resolution of racemic ECH. Particularly, soluble epoxide hydrolases catalyze the hydrolysis of epoxides in acyclic systems. In man this enzyme is the product of a single copy gene (EPXH-2) present on chromosome 8. Emerging roles of human sEH as endogenous substrates epoxygenated fatty acids, in inflammation and, hypertension make them valuable. One of the consequences of inhibiting sEH in rodent inflammation models is a profound decrease in the production of pro-inflammatory and pro-analgesic lipid metabolites including prostaglandins. This lead to hypothesize that sEH inhibitors may have antinociceptive properties [99]. Microsomal epoxide hydrolase (mEH) has a wide role as a bifunctional protein in carcinogen metabolism and is also able to mediate the sodium-dependent uptake of bile acids into hepatocytes. Studies have identified a subject with extremely elevated serum-bile salt levels in the absence of observable hepatocellular injury, suggesting a defect in bile acid uptake. In this individual, mEH protein and mEH mRNA levels were reduced by approximately 95 % and 85 %, respectively, whereas the expression and amino acid sequence of another bile acid transport protein (NTCP) was unaffected. As reported that mEH is greatly reduced in a patient with hypercholanemia, suggesting that mEH participates in sodium-dependent bile acid uptake in human liver where its absence may contribute to the etiology of this disease [100]. The beneficial effects of several potent sEH inhibitors in cardiac hypertrophy have been reported. One of the common causes of cardiac failure is myocardial infarction leading to myocardial cell loss. sEH catalyzes the conversion of epoxy-eicosatrienoic acids (EETs) to form the corresponding dihydroxyeicosatrienoic acids (DHETs). EETs are products of cytochrome P_{450} epoxy-genases that have vaso-dilatory properties and inhibit the activation of nuclear factor (NF)-κB-mediated gene transcription. EH motivates the potential to uncover a new class of therapeutic agents for cardiovascular diseases [101]. The study on peptidyl-urea based inhibitors of soluble epoxide hydrolases reveals the true potential of this noble enzyme [102]. Multiple roles of EH asvasodialiting agent(s) and anti-inflammatory agent(s) are also revealed by their endogenous sEH substrates including epoxyeicosatrienoic acids (EETs) [103,104, 105,106]. Hydrolysis of the epoxides by sEH diminished this activity [107]. The inhibition of sEH led to the accumulation of EETs and other lipid epoxides in the organism [108]. Furthermore, sEH inhibition in rodent models can successfully treat hypertension [109, 110] and inflammatory diseases [111] as well as protect against strenal damage caused by hypertension. Role for the sEH in blood pressure regulation [112] has been suggested by that the epoxy fatty acids are potent vasodilators [113]. This hypothesis was confirmed with sEH-null mice, for which the male systolic blood pressure was reduced to female levels [114] which confirms the value of epoxy hydrolase in pharmaceuticals [115].

EHs are key enzymes in providing defense against the hazardous properties of xenobiotic compounds [116] because of their property of converting epoxides to water soluble and less toxic diols. Epoxides are frequent intermediates in the metabolism of lipophilic xenobiotics. Because of their high ring tension and polarized C-O bonds,

they are often electro-philically reactive, forming adducts with cellular DNA that produce mutations leading to cancer. Epoxide hydrolase is one of the mammalian drug-metabolizing enzymes that metabolize a wide range of endogenous and xenobiotic compounds [117]. These enzymes either activate or the detoxify chemicals, for example, steroid hormones, therapeutic and recreational drugs, environmental and dietary chemicals, carcinogens and other toxic agents can be greatly influenced by the activities of these enzymes [118,119].

Industrial applications

EHs from microbial sources are highly versatile biocatalysts that can be practically used for the production of enantiopure epoxides and vicinal diols. Most recently, the biotechnological application of microbial epoxide hydrolases in two-phase or flow-through membrane reactors [120] and the development of continuous processes [121] have been evaluated. Such applications open the way to large-scale transformations and these microbial EHs can also be used successfully on preparative scale. Besides kinetic resolution these furnishes, the corresponding vicinal diol and remaining non-hydrolyzed epoxide in non-racemic form, enantio-convergent processes are possible which are highly attractive as they lead to the formation of a single enantiomeric diol from a racemic oxirane. During the last decade, microbial epoxide hydrolases that enantio-selectively hydrolyze epoxides have emerged as new biotechnological tools to produce enantiopure epoxides and vic-diols [122,123].

Epoxide hydrolase produced from various sources such as bacteria, yeasts, and filamentous fungi are enantio-selective hydrolases with a high potential of being useful biocatalysts in many biotransformation reactions. In most cases, the microorganisms adapted to harsh environmental conditions have been screened for the presence of enantio-selective epoxide-hydrolyzing activities. Petroleum-polluted soils can be treated by hydrocarbon-degrading microorganisms, which are ubiquitous in nature. This decontamination technology is called bio-remediation; one-half of the microbial samples were taken from petroleum contaminated bioremediation sites. Bio-filtration is used to remove volatile organic and odorous contaminants from exhaust gasses of industrial processes by microorganisms inherent in the bio-filter medium. Usefulness of these enzymes for organic synthesis some has been described in an interesting case is the preparation of (S)-2-pyridyloxirane using the *Aspergillus niger* EH. This product could not be obtained in a satisfactorily enantiopure form by using conventional chemistry approaches. This bio-catalysed resolution could be achieved using plain water instead of a buffer solution, a practical aspect that represents an important advantage as far as downstream processing for a potential industrial application is concerned. Application to other regioisomeric pyridyloxiranes was similarly successful. Obviously, the recent efforts devoted to the study of EHs make these newly discovered biocatalysts highly appealing for organic synthesis, and doubtlessly this will be true in the future for the fine chemical industry. The industrial applications of epoxide hydrolase have been explored due to some interesting point. Firstly, the first X-ray structure of three different EHs were described and led, in particular, to a better insight into the enzymatic mechanism. Secondly, new EHs were found that exhibited novel substrate and/ or enantioselectivity, complementary to the EHs known previously. Thirdly, it was shown that some of these enzymes can be used under interesting experimental conditions, including high concentration, bi-phasic systems and even by using plain water instead of buffers. Fourthly, several examples have illustrated the possible use of these 'new tools' for the preparation of epoxides in enantiopure form at a several-gram scale. Fifthly, some of these were implied as 'key synthons' in the synthetic strategy aimed at synthesizing biologically active products. Last but not least, in near future some of these enzymes that were recently cloned and over-expressed and two of them (from *A. radiobacter* and *A. niger*) may soon become commercially available [124].

Polycyclic aromatic hydrocarbons, a major class of environmental and dietary carcinogens, are metabolised by the CYP1 family of cytochromes P_{450} to form epoxides, which are detoxified through the action of EH. The toxicity and carcinogenicity

of these chemicals, both naturally occurring and anthropogenic, are due to their reactive intermediates that interact covalently with vital cellular macromolecules leading to mutations and toxicity. EH converts the epoxides to trans-dihydrodiols that are further oxidized by the CYP1 family to dihydrodiol-epoxides, the ultimate carcinogens [125,126]. Indeed, epoxides have been implicated in the toxicity/ carcinogenicity of many other structurally diverse chemicals including mycotoxins such as aflatoxin B1 [127] and halogenated aliphatic compounds such as vinyl chloride [128]; epoxides are valuable intermediates in the production of high added-value chemicals like pharmaceuticals due to high reactivity of their epoxide ring. Racemic mixtures of epoxides can be used to prepare enantiopure epoxides by kinetic resolution and a range of chemicals [129] and biocatalysts [130]. Epoxides readily react with halides, carbon, nitrogen, oxygen or sulfur nucleophiles that are useful in many industries. Several interesting concepts that have been developed over the past years for other biocatalysts might also be applicable for the enzymatic conversion of epoxides [131]. It is desirable to expand the current biosynthetic processes for epoxide conversion by finding new enzymes or by applying the above-mentioned concepts to broaden the scope and performance of existing epoxide-converting biocatalysts [132,133]. It was of commercial interest to subject less well-studied non-aromatic substrates to the new biotransformation system with the help of epoxide hydrolase in order to provide products which, on further transformation, would provide noteworthy hydroxylated heterocyclic compounds. For example pyrrolidinol is a valuable chiral intermediate for the synthesis of a wide range of pharmacologically active products and intermediates..

Epoxides and vicinal diols are chiral products essentially required for the synthesis of pharmacologically important heterocycles [134]. First clinical trials on sEH inhibition in man have begun and sEH inhibition is a novel pharmacological cardiovascular protective strategy with special regard to *in vivo* investigations. Pharmacological sEH inhibition influences several diseases. Epoxyeicosatrienoic acids (EETs) build a family consisting of four arachidonic acid derived regioisomers that are generated by P_{450} epoxygenases. In the past years, the growing interest in influencing EET level arose since EETs possess numerous beneficial effects in the cardiovascular system, for example, vasodilatation, anti-inflammation and elicit renal and myocardial protection. Because EETs are primarily metabolized by the sEH and potent inhibitors of this enzyme are currently available, pharmacological sEH inhibition seems to be a feasible approach to elevate EET level *in vivo* [135]. Because these enzymes are attractive biocatalysts, so development of methods for the application of epoxide hydrolases is of interest. They have broad substrate range, are cofactor independent and can react in general with a homologous range of epoxides. Much attention is given to the microbial enzymes because of their relatively easy availability on a large scale. For resolution of many interesting epoxides there are now suitable biocatalysts available with EH-activity. The versatility of EHs has recently been extended by the exceptional enantio-selective hydrolysis of unbranched aliphatic 1, 2-epoxides by *R. glutinis* and related yeast species. Future investigations with purified forms of the recently discovered novel epoxide hydrolases will reveal the actual scope and limitations of these versatile enzymes as novel biocatalysts [136].

Molecular aspects

Since only a few structures of EHs have been solved till now, the systematic analysis that could provide some "general" architecture allowing predictions of substrate specificity for new sequences was not possible. However, some attempts of clustering EHs by their sequence alignments were done and published [137]. The constant increase of accessible genome information from microbes and plants in combination with today's efficient gene cloning and expression systems is expected to greatly enhance the rate of discovery and isolation of new useful EH biocatalysts. Mutagenesis strategies aiming to improve on the catalytic properties of an enzyme leaves catalytic residues untouched due to the obvious risk of being detrimental to enzyme

activity. There are cases, however, where manipulation of kinetic rates by mutagenesis of catalytic groups has affected enantio-specificity to generate mutant enzymes of higher discrimination. In *S. tuberosum* StEH1, mutagenesis of non-substrate binding residues, shifted enantio-specificity 30-fold. The effect could be traced back to lowered affinity for the R, enantiomer combined with an increased hydrolysis rate of the alkylenzyme in one mutant [138].

Mutagenesis of the charge-relay residue Glu378 into Asp in a fish EH combined with two other active-site residues (Phe193 Tyr and Trp 200 Leu), improved on the catalytic efficiency with styrene oxide [139,140]. For manipulation of catalytic properties in epoxide hydrolases in a specific direction, structural alterations in non-catalytic residues have been performed. The degree of structural changes has varied from single [141] to multiple-site mutagenesis. Several methods for generation of libraries of enzyme mutants combined with activity screens [142] have enabled isolation of novel enzymes with improved enantio-specificities as compared to the parental proteins. Randomly distributed mutations in the *A. radiobacter* AD1 isoenzyme by error prone PCR generated a gene library encoding variant proteins. Expressed proteins displaying epoxide-hydrolase activity with *p*-nitrophenyl glycidyl ether, a set of mutants was isolated. The corresponding genes for these mutant enzymes that also exhibited improved enantio-selectivity for the substrate were combined in gene shuffling experiments [143].

Enzyme variants with up to 13-fold improvement in enantio-specificity could subsequently be isolated from the shuffled library [144]. The relatively modest improvement of the *A. niger* enzyme, applying random site-random substitution mutagenesis, induced the development of new methods for targeting and choosing residue replacements in the mutated enzymes [145,146]. The groups of 2-3 active-site residues were subjected to saturation mutagenesis using either the full codon set or a subset was used [147]. This approach generates relatively small protein libraries there by facilitating screening for functional mutants [148]. Metagenomic DNA is a rich source of genes encoding novel EHs. Two genes encoding functional EHs were retrieved from total DNA isolated from bio-filter-derived biomass, using PCR with EH specific degenerate primers followed by genome-walking PCR [149]. Many types of cultivable microorganisms, including bacteria, fungi, and yeast, exhibit EH activities. However, it is thought that many more EH-encoding genes exist in unknown or uncultivable microorganisms. Their genomes are collectively referred to as the metagenome and this metagenome is accessible to extract total DNA from a particular sample. EH-specific probes with metagenomic DNA can also be used to identify putative EH genes [150].

A PCR-based approach to identify EH genes in metagenomic DNA has been reported [151]. The use of PCR primers based on conserved regions plus metagenomic DNA as a template has been used to identify genes encoding novel EH [152]. The complete limonene-1, 2-epoxide hydrolase gene (*limA*) was isolated from a genomic library of *R. erythropolis* DCL14 using a combination of PCR and colony hybridization. The *limA* gene encoded a 149-residue polypeptide with a deduced molecular mass of 16.5 kDa. It was functionally expressed in *Escherichia coli* to improve EH activity [153]. sEH is a multifunctional protein encoded by the EPHX2 gene. The biological functions and enzyme kinetics of sEH have been extensively investigated, however, little is known about its transcriptional regulation and mechanisms of tissue specific expression. Here, a luciferase gene based reporter assay was used to identify the minimal promoter and *cis* regulatory elements of EPHX2 [151].

In the transcriptional regulation of the human sEH gene EPHX2 two EH-encoding genes were retrieved directly from metagenomic DNA showed that the two enzymes, termed Kau2 and Kau8 were functional EHs with promising bio-catalytic characteristics for the hydrolytic kinetic resolution of several aromatic epoxides. These generally showed opposite enantioselectivity and in several cases had a high potential for enantio-convergence. Kau2 was successfully used in preparative scale kinetic resolution reactions with epoxide [10] at high substrate concentrations as well as in an enantio-convergent process, allowing quantitative preparative access to enantiopure diol

(1R, 2R)-11a from epoxide. Currently, the synthetic potential of these new EHs in greater depth has been explored. In particular, additional non-terminal epoxides need to be tested. Metagenomic DNA contains many more EHs with industrial value that await discovery. The use of PCR with degenerate consensus primers and genome walking may help us to explore this untapped resource [153].

Conclusion

This review provides an overview to emphasize the pharmaceutical and industrial uses of epoxide hydrolase and to explore its catalytic potential. The preparation of optically active epoxides and optically active vicinal diols using EH technology has attracted great attention. However, to date EH technology has suffered from limitations in substrate concentration (and hence volumetric productivity) often rendering this approach inferior to alternative chemical technology tools. Obviously, the recent efforts devoted to the study of EHs make these highly valuabled, these newly discovered biocatalysts more appealing for organic synthesis.

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