Engineering of Natural α/β Hydrolases

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The family of α/β hydrolases is one of the largest known protein families, including a wide range of members such as epoxide hydrolases, dehalogenases, hydroxynitrile lyases, fungal lipases, amidases, dienelactone hydrolases, haloperoxidases, acetylcholine esterases, serine carboxypeptidases, serine carboxypeptidase-like acyltransferases and other enzymes with distinct functions. Although many natural enzymes have been screened as biocatalysts with excellent performance, most of them are still unable to meet the needs of industrial applications. Low catalytic activity, thermostability, and enantioselectivity under complex and harsh industrial process conditions are still the main limitations for the large-scale application of natural enzymes. With the development of protein engineering technology, functional improvements have been achieved for existing α/β hydrolases, specifically in key enzyme characteristics such as their enantioselectivity and stability, in order to tailor these enzymes for specific industrial applications.

chiral compound α/β hydrolase catalytic mechanism

1. Structure and Catalytic Mechanism of α/β Hydrolase

The family of α/β hydrolases have a characteristic conserved structure which consists of eight β-strands connected by α-helixes and folds into a mostly parallel central β-sheet surrounded by α-helixes [1]. The active sites of these enzymes usually contain a catalytic triad composed of three conserved residues in loops, including a nucleophile (serine, aspartate, or cysteine), a histidine, and a catalytic acid (aspartate or glutamate). Many α/β hydrolases also contain lid/cap domains located on top of the active site, and these domains are not conserved and vary considerably between different enzymes (**Figure 1**) [2][3]. The lid/cap domain also affects the properties of these enzymes. For example, in haloalkane dehalogenase, the conformational change of the cap domain is considered to be a key step to allow water to enter and solvate the halide ion [4]. In prolyl oligopeptidase, it is suggested that the lid domain helps to exclude bulk solvents [5]. Some studies revealed that the mutations in the lid/cap domains influence the substrate specificity of the enzymes [1][6].

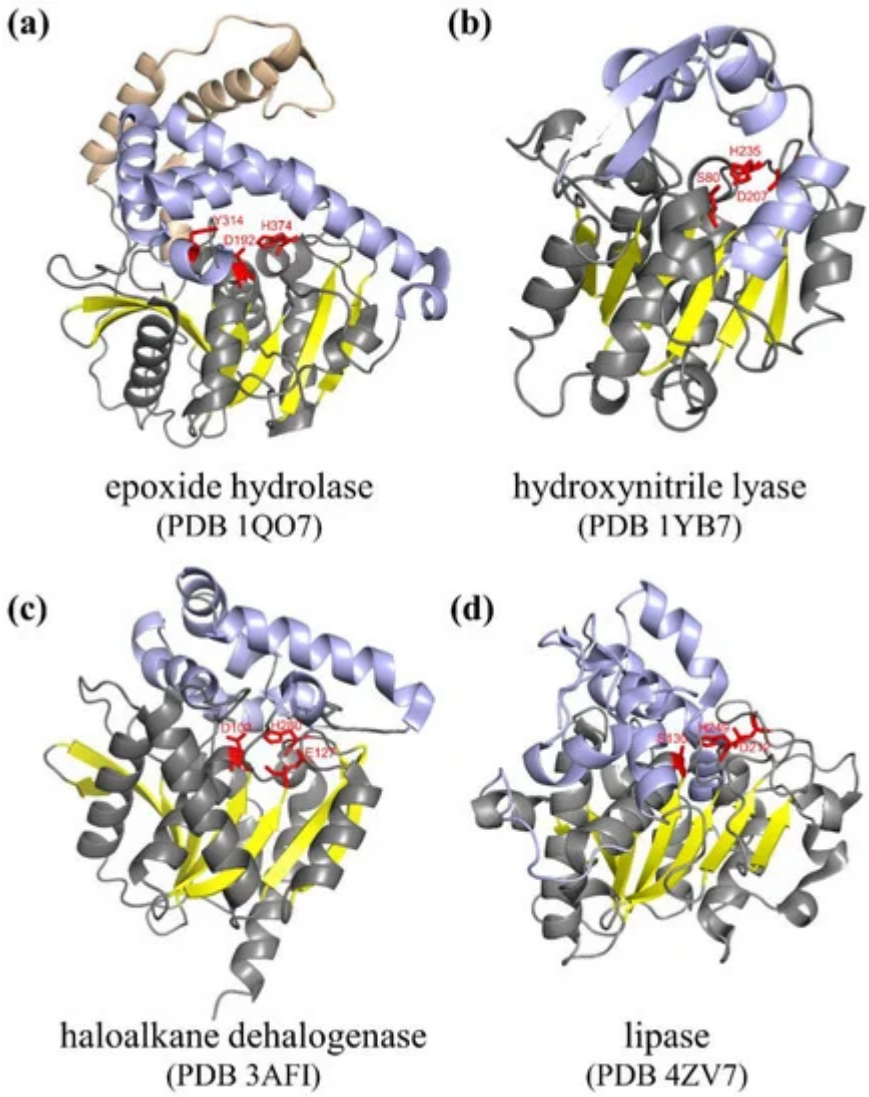

Figure 1. Structures of α/β hydrolases. (**a**) *Aspergillus niger* epoxide hydrolase [7]. (**b**) *Hevea brasiliensis* hydroxynitrile lyase [8]. (**c**) haloalkane dehalogenase DbjA [9]. (**d**) *Candida antarctica* lipase B [10]. The central β-sheet, flank helices, the cap domain, and the N-terminal meander are shown in yellow, gray, slateblue, and brown, respectively. The active site residues are shown as red sticks.

Members of α/β hydrolases catalyze a broad variety of chemical reactions with substrates containing diverse functional groups, but all of these reactions utilize the common mechanism of nucleophilic catalysis because of their highly similar three-dimensional structures and active site residues. The mechanism of substrate selectivity, regioselectivity, and stereoselectivity of α/β hydrolases are diverse and case-by-case depending on the structures of these enzymes. The catalytic mechanism was illustrated by *Aspergillus niger* epoxide hydrolase (*An*EH) [7]. The substrate-binding cavity is between the α/β-hydrolase fold domain and the lid domain, and the substrate is located at the enzyme active center by the hydrogen bonds between the oxirane ring and the hydroxyl groups of Tyr251 and Tyr314 on the cap domain. The catalytic triad is constituted of two aspartates and one histidine on the loops of the α/β-hydrolase fold domain. The reaction is catalyzed through a two-step mechanism. In the first step, a nucleophilic attack of an aspartic residue toward the oxirane ring forms a covalent ester intermediate with the ring opened by the classical push-pull mechanism. In the second step, the covalent intermediate is hydrolyzed to

release the final product by a water molecule in which the proton is extracted by the His374/Asp348 charge relay system.

2. Engineering of Natural α/β Hydrolases

Although many natural enzymes have been screened as biocatalysts with excellent performance, most of them are still unable to meet the needs of industrial applications. Low catalytic activity, thermostability, and enantioselectivity under complex and harsh industrial process conditions are still the main limitations for the large-scale application of natural enzymes. With the development of protein engineering technology, functional improvements have been achieved for existing α/β hydrolases, specifically in key enzyme characteristics such as their enantioselectivity and stability, in order to tailor these enzymes for specific industrial applications (**Table 1**).

Table 1. Native and engineered α/β hydrolases for chiral chemical production.

Type	Enzyme	Source	Mutation Site	Reaction/Effects	Reference
Epoxide hydrolase	Alp1U	*Streptomyces ambofaciens*	W187F/Y247F	Regioselective nucleophilic attack at C-2 of fluostatin C	[11]
	Alp1U	*Streptomyces ambofaciens*	Y247F	Highly regioselective attack at C-3 of fluostatin C	[11]
	*Am*EH	*Agromyces mediolanus* ZJB120203	-	Hydrolysis of (*R*)-ECH to enantiopure (*S*)-ECH	[12]
	*An*EH	*Aspergillus niger*	-	Hydrolysis of epoxides to the more water-soluble and usually less toxic diols	[7]
	*An*EH	*Aspergillus niger*	A217L	Improvement in enantioselectivity	[13]
	*An*EH	*Aspergillus niger*	A217V	Increase of the activity to allyl glycidyl ether	[13]
	*An*EH	*Aspergillus niger*	A217C	Increase of the activity towards	[13]

Type	Enzyme	Source	Mutation Site	Reaction/Effects	Reference
				allyl glycidyl ether and styrene oxide	
	*Ar*EH	*Agrobacterium radiobacter* AD1	T247K/I108L/D131S	Improvement of activity, enantioselectivity, and thermostability	[14]
	*Au*EH2	*Aspergillus usamii* E001	-	Resolution of racemic styrene oxide	[15]
	*Au*EH2	*Aspergillus usamii*	A214C/A250I	12.6-fold enhanced enantiomeric ratio toward *rac*-styrene oxide	[16]
	*Au*EH2	*Aspergillus usamii*	R322V/L344C	High enantioselectivity towards *rac*-ortho-trifluoromethyl styrene oxide	[17]
	EchA	*Agrobacterium radiobacter* AD1	I219F	Enhanced enantioselectivity for styrene oxide	[18]
	EchA	*Agrobacterium radiobacter* AD1	L190F	Enhanced activity for styrene oxide	[18]
	*Gm*EH3	*Glycine max*	-	Enantioconvergent hydrolysis of *rac*-epoxides with high enantiopurity and yield	[19]
	SgcF	*Streptomyces griseus* IFO 13 350	W236Y/Q237M	20-fold increased activity toward (*S*)-styrene oxide to yield an (*S*)-diol	[20]
	Sibe-EH	metagenomes	-	Desymmetrization of *cis*-2,3-epoxybutane producing the (2*R*,3*R*)-diol	[21]

Type	Enzyme	Source	Mutation Site	Reaction/Effects	Reference
	CH65-EH	metagenomes	-	EH activity toward a broad range of substrates and with high thermostability	[21]
	*Vr*EH1	*Vigna radiata*	-	Enantioconvergent hydrolysis of *p*-nitrostyrene oxide	[22][23]
	*Vr*EH2	*Vigna radiata*	-	Enantioconvergent hydrolysis of *p*-nitrostyrene oxide	[23]
	*Vr*EH3	*Vigna radiata*	-	High and complementary regioselectivity toward styrene oxides and high enantioselectivity toward *o*-cresyl glycidyl ether	[24]
Esterase	PFE	*Pseudomonas fluorescens*	replacement of a loop (A120-V139) with the corresponding element (P132-Y152) of the epoxide hydrolase EchA	Conversion of an esterase into an epoxide hydrolase towards *p*-nitrostyrene oxide	[25]
	*Rh*Est1	*Rhodococcus* sp. ECU1013	circular permutation mutants with G20/T19, S22/N21, and G24&A23 as new termini, respectively	Improved thermostability	[26]
	SABP2	*Nicotiana tabacum*	Q221M	Higher stability (6.6-fold half-life)	[27]
	SABP2	*Nicotiana tabacum*	G12T/M239K	Switching from an esterase to a hydroxynitrile lyase	[28]
Haloalkane dehalogenase	DbjA	*Bradyrhizobium japonicum* USDA110	-	Excellent enantioselectivity for α-bromoesters and high enantioselectivity for two β-bromoalkanes	[9]
	DbjA	*Bradyrhizobium japonicum*	H280F	Realized the transhalogenation	[29]

variants with random mutations in the protein-encoding gene is created through sequential error-prone PCR (ep-PCR) or in vitro DNA recombination, and then screening methods are used to isolate functionally improved mutant proteins for further study or the next round of improvement. Directed evolution does not rely on the knowledge of the enzyme structure and catalytic mechanism, but large libraries generated by directed evolution need to be screened for the best mutants with desired properties, which is considered to be time-consuming and laborious. The development of new efficient and convenient screening methods is the key to obtaining novel biocatalysts by directed evolution [37][38]. The second approach is the rational design which generates beneficial mutants based on protein sequences, structural information, and the relationship between enzyme structure and function. Advanced computational methods are often adopted to introduce specific mutagenesis in conserved functional domains, catalytic active sites, and certain amino acid positions for yielding remarkable desired properties (**Figure 2**). The combination of directed evolution and rational design strategies, named semi-rational design, with the benefits of both strategies has also been developed for improving substrate specificity, enantioselectivity, and stability.

Type	Enzyme	Source	Mutation Site	Reaction/Effects	Reference
		USDA110		reaction	
	DbeA	*Bradyrhizobium elkanii* USDA94	surface loop-helix transplantation from haloalkane dehalogenase DbjA	Lower stability but increased activity with various halogenated substrates and altered its enantioselectivity	[30]
	DhaA	*Rhodococcus rhodochrous*	E20S/F80R/C128F/T148L/A155P/A172I/C176F/D198W/V219W/C262L/D266F	Increased thermostability (ΔT_m 24.6 °C)	[31]
	DhaA	*Rhodococcus rhodochrous*	two mutants containing 13 and 17 mutation sites, respectively	Enantioselective production of (*R*)- and (*S*)-2,3-dichloropropan-1-ol, respectively	[32]
	LinB	*Sphingomonas paucimobilis* UT26	E15T/A53L/A81K/F169V/A197P/D255A/A247F	Increased thermostability ($\Delta T_{m,app}$ 23 °C)	[33]
Hydroxynitrile lyase	*Hb*HNL [8]	*Hevea brasiliensis*	L121Y	Improved activity on an unnatural substrate mandelonitrile	[34]
	*Hb*HNL	*Hevea brasiliensis*	T11G/E79H/K236G	Lower hydroxynitrile lyase activity and higher esterase-specific activity	[35]
Lipase	CALB	*Candida antarctica*	[39][40] -	Kinetic resolution of racemic alcohols and amines or desymmetrization of diols and diacetates	[10]
	CALB	*Candida antarctica*	a circular permutated variant of CALB with 283 /282 as the new termini [35]	Higher catalytic activity (2.6- to 9-fold) for trans and interesterification of the different substrates	[36]

ed stars: *asiliensis*

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attempted to convert the esterase of *Pseudomonas* into epoxide hydrolase by replacing the residues of active site loops in the esterase with the residues in the epoxide hydrolases. After residues in multiple sites were carefully chosen and replaced, the mutant enzyme showed an epoxide hydrolase activity with enantioselectivity for R-type enantiomers, but it was widely inhibited by substrates and had a limited turnover number [25]. These examples provide evidence for specificity engineering by utilizing the catalytic promiscuity of enzymes, although their performance, compared with natural enzymes, still needs further improvement.

Beier et al. reported a study on haloalkane dehalogenase which provides a new way to expand the application of α/β hydrolases in chiral preparations [29]. By mutating the catalytic residue histidine and blocking the hydrolysis process, the variant DbjA H280F could catalyze a transhalogenation reaction and realize the replacement of halogen substitutes. This strategy was further demonstrated in several different haloalkane dehalogenases [29].

4. Catalytic Activity Enhancement of α/β Hydrolases

High catalytic activities are required for enzymes in commercial applications. It is possible to increase the activities of natural enzymes through rational design and directed evolution approaches. Kotik et al. significantly improved the activity of *Aspergillus niger* M200 epoxide hydrolase through single site mutagenesis of Ala217 at the substrate entrance. For example, A217C enhanced the activity of the enzyme by three times towards allyl glycidyl ether and styrene oxide, while A217V increased the activity of the enzyme to allyl glycidyl ether by six times [13]. A completely different approach was used by Yu et al. to improve the activity of *Candida antarctica* lipase B. A mutant cp283 obtained through circular permutation showed higher catalytic activity, compared with the wild-type enzyme in the transesterification reaction with different substrates using 1-butanol and ethyl acetate as acyl receptors [36]. Langermann et al. replaced the active site residue in hydroxynitrile lyase *Hb*HNL with the corresponding site in esterase SABP2, thereby improving the catalytic activity of *Hb*HNL on an unnatural substrate mandelonitrile [34]. More recently, Marek et al. transplanted a nine-residue-long extension of L9 loop and α4 helix from DbjA into the corresponding site of another haloalkane dehalogenase DbeA. The mutation not only altered its enantio-preference with several linear β-bromoalkanes but also enhanced the catalytic activity of DbeA towards various halogenated substrates [30].

5. Regio- and Stereo-Selectivity Engineering of α/β Hydrolases

Since the resolution process of racemates is costly and often difficult, enzymatic catalytic synthesis of enantiopure compounds has gradually become a useful alternative to various chemical preparation routes. While enantio-convergent bioconversions by natural enzymes are usually not able to meet industrial requirements, it is necessary to improve or alter the enantioselectivity of biocatalysts by protein engineering.

Substrate binding sites are frequently engineered for the improvement of regio- and stereo-selectivity because they contain the catalytic residues and determine the location of substrates during the catalysis. By mutating the residues of the atypical oxirane oxygen hole (Trp186/Trp187/Tyr247) around the active site in epoxide hydrolase Alp1U, Zhang et al. obtained mutants Y247F and W187F/Y247F with high regioselectivity, respectively, towards C-2 and C-3 of fluostatin C [11]. Recently, Wen et al. reported the improvement of the enantio-specificity of *Au*EH2 towards racemic *ortho*-trifluoromethyl styrene oxide by tuning the substrate binding pocket [17]. With a similar method, Hu et al. performed the molecular docking of *Au*EH2 with (*R*)-styrene oxide by AutoDock Vina and screened suitable sites for site-directed saturation mutagenesis and combinatorial mutagenesis. A mutant A214C/A250I exhibited a 12.6-times increase of the enantiomeric ratio towards the substate *rac*-styrene oxide [16]. Accompanied by the deep understanding of the relationship between the structure and activity of enzymes, more successful studies have been reported to enhance the enantioselectivity by the design and engineering of active site residues [14][18][20][41].

In addition to the active site, the entrance tunnel of the enzyme has also been targeted to modulate the selectivity. For example, Kotik et al. reported that a single amino acid mutation at the entrance site of the *An*EH tunnel can notably increase its enantioselectivity [13].

6. Stability Enhancement of α/β Hydrolases

Enzymes with high thermostabilities are required in many industrial production processes because the elevated temperature has positive effects on many bioconversion processes such as improving the solubility of substrates, increasing the catalytic rate, and reducing microbial contaminants [42]. Through a combination of computational design and experimental screening, Floor et al. established a method named FRESCO, which based on the calculation of folding energies for all possible substitutions and the integration of conformational sampling in disulfide-bond designs, utilized the molecular dynamics as a fast-screening tool. Using this method, a multisite mutant of a large monomeric protein, haloalkane dehalogenase LinB, was obtained with drastically improved thermostability (a 23 °C increase in apparent melting temperature and an over 200-fold longer half-life at 60 °C) and retained moderate enantioselectivity [33]. Similarly, Bednar et al. established another method, FireProt, which is a computational strategy aimed at the prediction of highly stable multipoint mutations and significantly increased the thermostability of haloalkane dehalogenase DhaA [31]. Utilizing the circular permutation strategy to analyze the three characteristic regions of typical esterase *Rh*Est1, Li et al. obtained three mutants CP-20, CP-22, and CP-24 with ameliorated thermostability [26]. A systematic study by Jones et al. used and compared five protein engineering strategies (random mutagenesis, two computational methods Rosetta and FoldX, consensus mutation, and homoproline mutation) to enhance the stability of an α/β-hydrolase fold enzyme, salicylic acid binding protein 2. The results showed that all five methods could obtain mutants with enhanced stability, but consensus mutation and homoproline mutation seemed to be the best choices.

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