Alteration of lipase properties by protein engineering methods

Uwe T. BORNSCHEUER

Department of Biotechnology and Enzyme Catalysis, Institute of Biochemistry, Greifswald University, Felix-Hausdorff-Str. 4, 17487 Greifswald, Germany
Tel.: +49 3834 86 4367,
Fax: +49 3834 86 80066
<uwe.bornscheuer@uni-greifswald.de>

Abstract: *Lipases are very important biocatalysts in lipid modification and a broad number of processes have already been established on large scale in industry. A current trend is to tailor-design enzymes for a given application and protein engineering methods are commonly used for this. In this dossier, basic principles of rational protein design and directed evolution are described together with recent examples for the successful application of these tools for the alteration of the substrate specificity, stereoselectivity, and stability of lipases.*

Key words: *lipase, esterase, protein design, protein engineering, directed evolution, stereoselectivity, stability*

Introduction

Lipases (EC 3.1.1.3, triacylglycerol hydrolases) are the most widely used biocatalysts for the modification of fats and oils [1-3], but they are also employed in organic synthesis [4, 5]. They do not require cofactors, many of them are commercially available and they exhibit high activity and stability, even in non-aqueous systems such as organic solvents. Thus, a plethora of publications dealing with lipases have appeared in the last decades. The majority of examples from academic research and in industrial applications rely on commercially available (usually immobilized) lipases and use just a few types, which all originate from microorganisms (*i.e.* lipase from *Rhizomucor miehei* (Lipozyme RM IM), *Thermomyces lanuginosa* (Lipozyme TL IM), *Candida antarctica* type B (Novozyme 435, CAL-B) or *Burkholderia cepacia* (Amano PS)).

In lipid modification, lipases have been often used for the tailoring of natural lipids to meet nutritional properties, especially for humans. The most prominent example is the synthesis of cocoa-butter equivalent [6]. Cocoa butter is predominantly 1,3-disaturated-2-oleylglyceride, where palmitic, stearic and oleic acids account for more than 95% of the total fatty acids. Cocoa butter is crystalline and melts between 25 and 35°C providing the desirable 'mouth feel'. Unilever [7] and Fuji Oil [8] filed the first patents for the lipase-catalyzed synthesis of cocoa butter equivalent from palm oil and stearic acid. Reactions are usually performed as transesterification or acidolysis of cheap oils using tristearin or stearic acid as acyl donors and a 1,3-specific lipase. Structured triglycerides (sTAG) with a defined distribution of different fatty acids along the glycerol backbone are another area where lipases have been used, as these sTAG are important compounds for human nutrition. sTAG containing medium chain fatty acids at the *sn*1- and *sn*3-position and a long (preferentially polyunsaturated) fatty acid at the *sn*2-position are used to treat patient with pancreatic insufficiency and for rapid energy supply (*i.e.* for sports). Another important example is Betapol™ used in infant nutrition, which contains oleic acid at the *sn*1- and *sn*3- and palmitic acid at the *sn*2-position. Currently, Betapol™ is manufactured by interesterification of tripalmitin with oleic acid using Lipozyme RM IM. Also, a two-step lipase-catalyzed process was developed *(figure 1)*, in which tripalmitin is first subjected to alcoholysis with ethanol using a lipase from *Rhizopus delemar* immobilized on a polypropylene carrier (EP-100) yielding 95% monopalmitin with a purity >90% after crystallization. Subsequent enzymatic esterification with oleic acid in hexane proceeded quantitatively within a few hours and the final OPO (yield 70%) contained up to 96% palmitic acid in the *sn*2-position [9].

Other more recent examples for successfully industrialized processes include lipase-catalyzed production of zero-*trans* margarines (ADM/Novozymes) and diglyceride-based cooking and frying oils (Kao Corp./ADM) [10]. The zero-*trans* and reduced *trans* oils and fats are produced on industrial scale by transesterification using Novozyme TL IM in combination with a cost-effective immobilization technology.

In addition, lipases have been used on industrial case to produce simple esters, *e.g.* for cosmetic applications. Prominent examples are cetyl ricinoleate and myristyl myristate [11, 12]. Although both esters have been chemically synthesized for a long time, enzyme technology allows higher yields and substantially purer products. The higher costs for the biocatalyst are compensated by energy savings (ambient temperature instead of 160-180°C) and product purification (*i.e.* a bleaching and deodorization step can be omitted).

However, it often occurs that an enzyme does not meet the requirements for a certain application and its properties have therefore to be optimized. This usually includes the chemo-, regio- and stereoselectivity of the biocatalyst, but also process-related aspects such as long-term stability at high temperatures or pH-values and activity in the presence of large substrate concentrations need to be improved. Beside rather classical strategies such as immobilization, additives or process engineering, molecular biology techniques nowadays represent the most important methodologies to tailor-design an enzyme for a given application. Two different (but increasingly complementary) strategies are the methods of choice: rational protein design or directed (molecular) evolution *(figure 2)*, which both profited from important developments in research since the mid 90s leading to a set of new methodologies.

This article focuses on these protein engineering methods with special emphasis on their use to alter the properties of lipases for lipid modification.

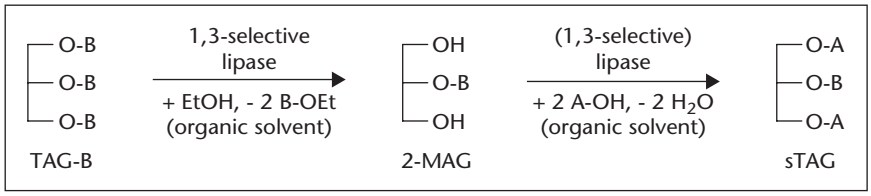

Figure 1. *Principle of the lipase-catalyzed two-step synthesis to obtain sTAG in high purity. A and B denote different fatty acids. MAG: monoacylglyceride, TAG: triacylglyceride.*

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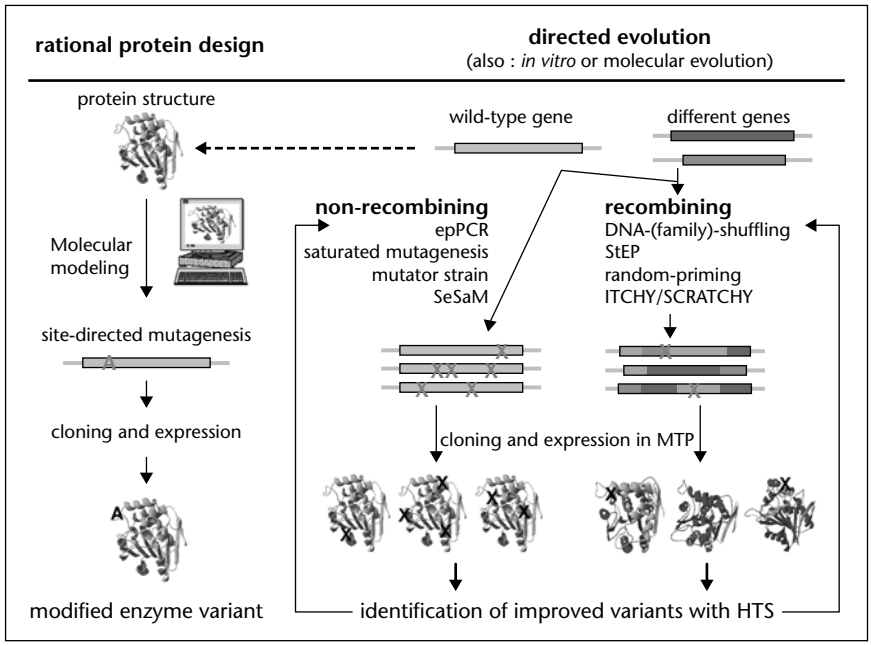

Figure 2. *Rational design starts from a protein structure (or a homology model), from which key amino acid residues are identified. These are then introduced on the gene level and the resulting mutant is produced and verified for desired properties. Directed evolution starts from one or several (homologous) genes, which are subjected to a range of random mutagenesis methods. From the resulting libraries of mutants or chimeras, desired variants then need to be identified by high-throughput screening or selection methods.*

Background of Directed Evolution and Rational Protein Design

Directed evolution

In principle, directed evolution is comprised of two steps: first, the random generation of mutant libraries and second, the identification of desired variants within these libraries using a suitable screening or selection system. Two different strategies for the generation of mutant libraries have been described *(figure 2)*, an asexual (non-recombining) evolution, in which a parent gene is subjected to random mutagenesis to yield variants with point mutations, and the sexual (recombining) evolution, in which several parental genes are randomly fragmented, shuffled and reconstructed to create a pool of recombined chimera.

In the last 15 years many methods have been developed and are covered in a number of books [13-15] and reviews [16-18]. The most widely used non-recombining method is error-prone polymerase chain reaction (epPCR) [19]. Here, non-optimal reaction conditions are used to create a mutant library [19, 20]. For example, increasing the Mg^{2+} concentration, adding Mn^{2+} and usage of unbalanced dNTP concentrations can substantially increase the error rate of the commonly used polymerase from *Thermus aquaticus* (*Taq*) from 0.001 to ~1%. The first recombining method was developed by Stemmer (named DNA- or gene shuffling) [21] and consists of a DNAse catalyzed degradation followed by a subsequent recombination of the fragments without primers (self-priming PCR) and finally a PCR with primers.

All random mutagenesis methods generate hughes mutant libraries (usually in the range of 10^4-10^8 variants) and rapid and highly reliable high-throughput screening or selection systems are therefore necessary to identify desired mutants within short experimental time. Overviews of recently described assays can be found in a book [22] and a number of reviews [23-27].

For libraries expressed in microorganisms, high-throughput screening can be sometimes directly performed on colonies growing in a solid culture like an agar-plate. Assays on agar-plated colonies typically enable the screening of $>10^4$ variants in a matter of days, but they are often limited in sensitivity: soluble products diffuse away from the colony and hence only very active variants are detected or false positives occur. Assays based on insoluble products have higher sensitivity, but their scope is rather limited. Solid-phase screening relies on product solubilization following an enzymatic reaction that gives rise to a zone of clearance, a fluorescent product, a pH-shift visualized by a pH-indicator or a strongly absorbing (chromogenic) product like X-gal or α-naphthyl acetate and Fast Blue/Fast Red as an example for lipase activity detection [28]. Lipolytic activity can still be screened in a high-throughput format, on-plate, with triolein- or tributyrin-agar through halo formation. Alternatively, a high-throughput assay in solid phase was recently developed by Babiak and Reymond using esters of coumarin [29].

However, many assays cannot be applied in a solid-phase format. Thus, individual clones must be grown and assayed in microtiter plates (MTP). These assays are significantly more time-consuming than solid-phase assays. However, by using robot automation and colony picking technology, throughput can be substantially increased. In addition, MTP-based assays have the major advantage that screening provides significantly more information compared to a selection approach as the activity can be directly and quantitatively measured and even allows to determine the kinetics. For lipases, these assays are usually based on commerically available *p*-nitrophenyl esters of varying fatty acid chain length, but also the use of resorufin or umbelliferyl esters has been described. Hydrolysis of these substrates releases a chromophore/fluorophore that can be quantitatively measured time-resolved at high sensitivity. However, these artificial substrates differ from the true lipase substrate and hence can lead to false positive hits. Furthermore, the substrates are often unstable at extreme pH or temperature and this autohydrolysis can limit their use in certain screening efforts. Alternative compounds were suggested by Reymond *et al.* coupled with a periodate treatment and β-elimination to release the chromophore/fluorophore [30-32]. However, these substrates have to be chemically synthesized and only end-point measurements are possible. For the screening of lipases active in the synthesis (*i.e.* esterification or transesterification) reaction, a fluorometric method was described as well [33]. This method is based on the transesterification between an alcohol and a vinyl ester of a carboxylic acid. Acetaldehyde generated from the vinyl alcohol by keto-enol tautomerization is reacted with a (non-fluorescent) hydrazine (NBD-H) to produce the corresponding highly fluorescent hydrazone, which is then quantified by fluorimetric measurement *(figure 3)*.

Alternatively, lipase synthesis activity can be indirectly measured in an organic solvent using a modified *p*-nitrophenyl ester assay [34]. Transesterification activity of immobilized esterases was determined by sampling p-nitrophenol released and subsequent spectrophotometric quantification in an aqueous

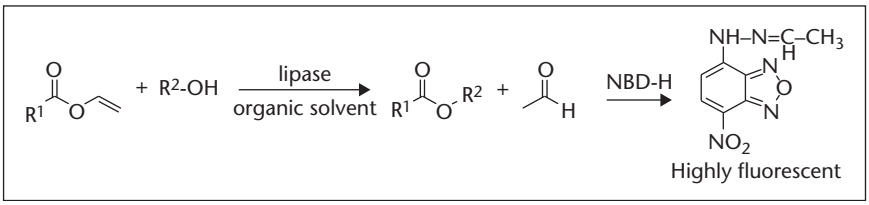

Figure 3. *High-throughput assay for the determination of transesterification activity of lipases.*

system. A similar method was reported very recently for the determination of the synthesis activity of a lipase [35].

A totally different strategy to find the best variant in a mutant library is bacterial surface display. Kolmar *et al.* showed that *E. coli* bacteria that display esterases or lipases on their cell surface together with horseradish peroxidase (HRP) are capable of hydrolyzing carboxylic acid esters of biotin tyramide. The tyramide radicals generated by the coupled lipase-peroxidase reaction were short-lived and therefore became covalently attached to reactive tyrosine residues that are located in close vicinity on the surface of a bacterial cell that displayed hydrolase activity. Differences in cellular esterase activity were found to correlate well with the amount of biotin tyramide deposited on the cell surface. This selective biotin tyramide labeling of cells that had lipase activity allowed their isolation by magnetic cell sorting [36].

Rational design

Engineering a protein by rational design requires the availability of the enzymes tertiary structure or at least a homology model of sufficient quality. Furthermore, detailed information about the structure-function relationship and usually the reaction mechanism is required to allow for the prediction of amino acid residues to be mutated. In the past decades, the number of protein structures deposited in the Brookhaven protein database (pdb) and protein sequence information in various databases substantially facilitated the rational design of proteins. Furthermore, a plethora of modeling software has been developed, which makes this methodology easier to use and also enhances the success rate of modeling predictions. Usually, the information derived from computer modeling identifies certain amino acids (hot spots) which should be altered to lead to a change in the enzymes properties such as broadened or restricted substrate range or altered selectivity. Site-directed mutagenesis (SDM) is then performed at these positions using for example, the QuikChange™ Site-Directed Mutagenesis method from Stratagene. In many cases, it might be more advantageous to directly perform a saturation mutagenesis at the selected position(s), which will introduce all 19 proteinogenic amino acids and hence increase the chance to find desired variants. A combination of rational protein design with directed evolution (CASTing) has been described by the Reetz group [37].

Examples

The chain-length selectivity of lipases was altered by rational protein design and SDM, as shown by Joerger and Haas for the *Rhizopus oryzae* (formerly *Rhizopus delemar*) lipase (RDL) [38, 39]. Based on the crystal structure, they applied molecular modeling to identify the molecular determinants of acyl chain length specificity of this enzyme and were able to change this property significantly. In another example – while trying to isolate new enzyme variants of the extracellular lipase from *Thermomyces lanuginosa* with enhanced activity in the presence of detergent – Danielsen *et al.* randomized nine amino acids in two regions flanking the flexible α-helical lid. A S83T mutation was found in six of the seven most active variants, which in the homologous RDL had been proven to determine the chain-length preference [40]. More recently, an esterase was subjected to directed evolution and a mutant was identified having a lipase-like chain-length specificity [41]. Moreover, the variant also was shown to have distinct *sn*2-specificity, a unique feature, which makes this enzyme very attractive for the synthesis of structured triglycerides as outlined in the introduction. A similar change in chain-length specificity was recently achieved by random mutagenesis of a distinct region of an esterase from *Pseudomonas fluorescens*. The best variants had a 10-fold higher catalytic activity towards p-nitrophenyl dodecanoate than the wild-type enzyme [42].

Fujii *et al.* reported the enhancement of amidase activity of a *Pseudomonas aeruginosa* lipase after one single round of random mutagenesis. Mutant libraries were screened for hydrolytic activity against oleylnaphthylamide *vs.* the hydrolysis of the corresponding carboxylic acid ester. Three mutational sites were identified to enhance amidase activity, and the double mutant F207S/A213D was found to have the highest amidase activity, 2-fold that of the wild-type. These mutations were located near the calcium binding site, far from the active site [43]. For organic synthesis, the stereoselectivity of lipases is one of the most important features and directed evolution was also applied to alter this characteristics. Thus, Reetz *et al.* enhanced the selectivity of a lipase from *Pseudomonas aeruginosa* for the kinetic resolution of 2-methyldecanoic acid *p*-nitrophenyl ester from E=1.1 to the practically useful value of E=51 [44, 45].

CAL-B is probably the most useful lipase and it could be show that its thermostability could be improved by directed evolution as variants were found after two rounds of epPCR, that are 20-fold more stable at 70°C than the wild type. Positions 221 and 281 were found to be critical to prevent irreversible inactivation and protein aggregation and the variants were also found to be more active against p-nitrophenyl butyrate and 6,8-difluoro-4-methylumbelliferyl octanoate [46].

Stability of lipases is very important in large scale applications to make processes more cost-efficient. In order to enhance the stability of a lipase from *Rhizopus oryzae* (ROL) towards lipid oxidation, products such as aldehydes, six lysine and all histidine residues (except for the catalytic His) out of 22 amino acid residues (15 Lys, 7 His, *figure 4*) prone to react with aldehydes were chosen. These selected positions were then subjected to saturation mutagenesis using the gene encoding the prolipase. In order to quickly and reliably identify stability mutants within the resulting libraries, active variants were pre-screened by an activity staining method on agar plates. Active mutants were expressed in *E. coli* in a 96-well MTP format and a stability test using octanal as model deactivating agent was performed. The most stable histidine mutant (H201S) conferred a stability increase of 60%, which was further enhanced to 100% by combination with a lysine mutant (H201S/K168I). This increase in stability was also confirmed for other aldehydes. Interestingly, the mutations did not affect specific activity, as this was still similar to the wild type enzyme [47].

Further examples for the successful application of protein engineering of lipases and esterases are summarized in *table 1*.

Conclusion

Protein engineering methods have emerged as very powerful tools to alter the properties of enzymes for biocatalysis. Especially directed evolution became a mature technology within just a few years and the diverse set of molecular biology tools to create well-balanced mutant libraries as well as suitable high-througput screening methods allowed to create biocatalysts including lipases with substantially altered

Table 1. *Selected examples of lipases (and esterases) improved by protein engineering methods.*

Enzyme (origin)	Target	Mutagenesis method	Assay	Improved property	References
P. aeruginosa lipase	Enantioselectivity	epPCR and others	MTP assay with chiral *p*-nitrophenyl esters	Increased enantioselectivity	[44, 45]
P. aeruginosa lipase	Improve amidase activity	epPCR Saturation mutagenesis	Activity staining on agar plate	2-fold higher amidase activity	[43]
P. aeruginosa lipase	Substrate specificity	CASTing	MTP assay with *p*-nitrophenyl esters	Expanded substrate acceptance for different carboxylic acid esters	[37]
R. oryzae lipase	Substrate specificity	Saturation mutagenesis	Agar-plates containing rhodamine B	Altered chain-length preference	[38, 39]
R. oryzae lipase	Enhanced stability	Saturation mutagenesis	MTP assay	Increased stability towards aldehydes	[47, 48]
R. arrhizus lipase	Thermostability	epPCR Shuffling	Agar-plates containing rhodamine B	Improved thermostability and higher temperature optimum	[49]
C. antartica lipase B	Thermostability	epPCR Saturation mutagenesis	MTP with *p*-nitrophenyl esters and heating	>20-fold improvement in half-life at 70°C	[46]
B. gladioli esterase	Stability in organic solvents	epPCR	pH indicator	100-fold improvement of activity in 35% DMF	[50]
B. subtilis lipase A	Enantioselectivity towards 1,2-*O*-isopropylidene-glycerol	Saturation mutagenesis near the active site	Phage display	Inverted enantioselectivity	[51]
Esterase from deep sea	Broadened substrate range	epPCR	MTP with *p*-nitrophenyl esters	Lipase-like chain-length selectivity, also *sn*2-specific	[41]
P. fluorescens esterase	Broadened substrate range	Randomized saturation mutagenesis	MTP with *p*-nitrophenyl esters	Lipase-like chain-length selectivity	[42]

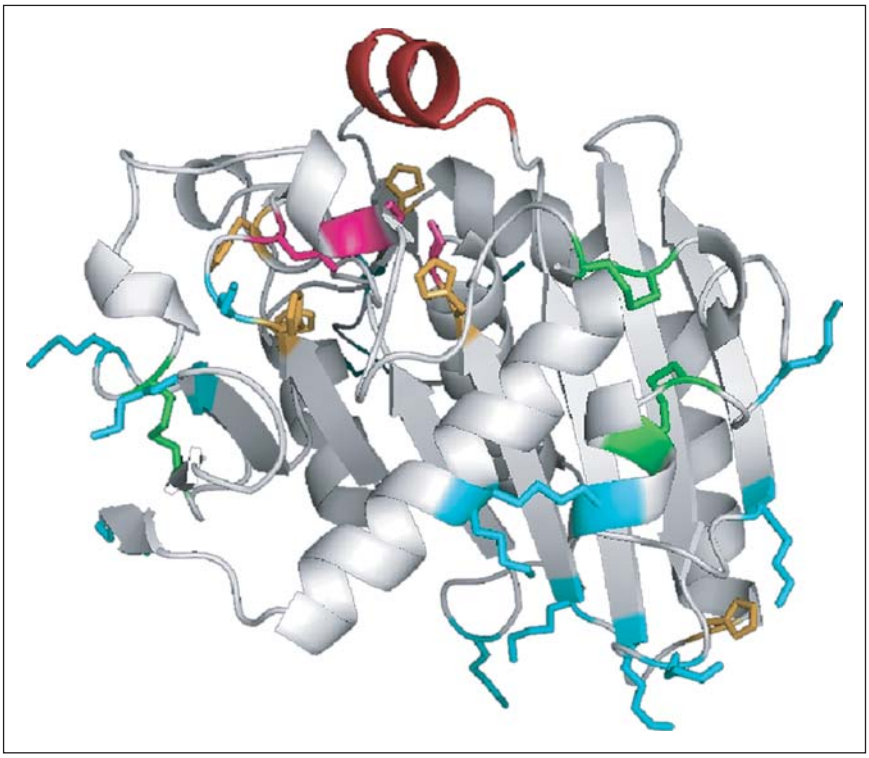

Figure 4. *Visualization of the target residues in the lipase from Rhizopus oryzae (ROL) for saturation mutagenesis to increase the stability of ROL towards aldehydes. Red: lid, pink: active site, orange: His residues, light blue: lysine residues, green: cysteine residues.*

properties. Furthermore, rational protein design was shown to be a useful method to change lipase specificities and with an increasing number of protein structures available, this approach will certainly become even more important in the future.

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