

Methods to increase enantioselectivity of lipases and esterases

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Lipases and esterases are frequently used in the synthesis of optically pure compounds; however, natural enzymes do not always show sufficiently high enantioselectivity. Variation of the structure of the substrates, modification of the reaction system or protein engineering (e.g. the expression of pure enzymes, rational design or directed evolution) are strategies that can be employed to improve the distinction between two enantiomers or enantiotopic groups.

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Current Opinion in Biotechnology 2002, 13:543–547

0958-1669/02/\$ — see front matter
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Published online 31 October 2002

Abbreviations

BsubpNBE	<i>Bacillus subtilis</i> p-nitrobenzylesterase
CAL-B	<i>Candida antarctica</i> lipase
CRL	<i>Candida rugosa</i> lipase
DMSO	dimethyl sulfoxide
IL	ionic liquid
PCL	<i>Pseudomonas cepacia</i> lipase
PLE	pig liver esterase

Introduction

The application of enzymes, especially in organic synthesis, is now well documented in the literature [1–3]. In the past decade, a considerable number of processes have been commercialized in industry [4,5]. Hydrolytic enzymes such as lipases and esterases are frequently used because they accept a broad range of substrates and often exhibit high enantioselectivity [6,7]. Researchers can now choose from a large number (>50) of commercial lipases and esterases. The availability of even more enzymes is to be expected because of the strong efforts to access biodiversity using novel methods for the cloning and expression of enzymes [8] and owing to advances in protein engineering and directed (molecular) evolution techniques [9].

In spite of this, enzymes do not always show satisfying performance in terms of activity, stability and most importantly enantioselectivity. These properties can be optimized by alteration of the substrates, the reaction system (medium engineering) or by protein engineering techniques. The molecular basis for enantioselectivity (usually expressed as an E-value, the ratio of the activity towards each enantiomer [10]) of a given enzyme is still not well understood. Especially in the absence of structural data, predictions as to how enantioselectivity will change are almost impossible. It is even more difficult to predict how enantioselectivity will change when the amino acid

sequence of the enzyme remains the same, but there are changes in the substrate structure, the carrier for immobilization and the solvent system.

In this article, recent achievements focusing on methods to change the enantioselectivity of lipases or esterases are reviewed. Readers are also referred to a recent article covering this aspect in a much broader context [11•].

Protein engineering approaches

Methods for protein engineering can be subdivided into three major categories: functional expression of (iso-)enzymes, rational (computer-guided) protein design, and directed evolution. Our understanding of the basis of enantioselectivity on a molecular level is improving and, consequently, protein engineering is proving to be a useful approach for increasing enantioselectivity. A number of successful examples for increased enantioselectivity are discussed below.

Functional expression of (iso-)enzymes

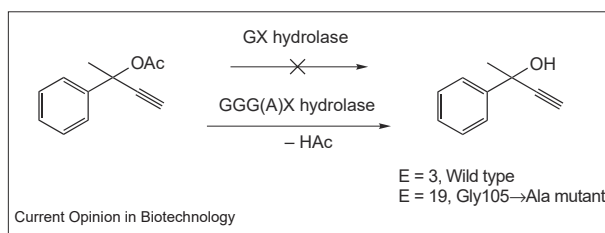
Wild-type enzymes are often produced by the host organism as a mixture of isoenzymes. These usually differ in their amino acid sequence, but sometimes only in their glycosylation pattern.

For practical reasons and cost-effectiveness, most enzymes are sold as crude products without any attempts to purify a distinct biocatalyst. As a consequence, it can happen that the activity and stereoselectivity of these ill-defined preparations reflects only a weighed average of the properties of the various isoenzymes and other enzymatically active ‘impurities’. Moreover, enantioselectivity can change from batch to batch and also change with reaction time due to differences in the operational stability of the isoenzymes.

Most prominent examples are pig liver esterase (PLE) and *Candida rugosa* lipase (CRL). PLE is isolated from pig liver by extraction and consists of several isoenzymes with the so-called α -, β - and γ -subunits as the most dominant ones. In the literature, a constant debate exists as to whether these isoenzymes differ [12] or not [13] in their enantioselectivity. Recently, the first functional overexpression of active PLE (the γ -isoenzyme) in the yeast *Pichia pastoris* was reported [14]. This allows the production of recombinant PLE (rPLE) at stable product quality without interfering influences of other isoenzymes and hydrolases. More importantly, rPLE shows substantially higher E-values in the resolution of acetates of secondary alcohols. For instance, the resolution of (R,S)-1-phenyl-2-butyl acetate proceeded with E <4 using commercial PLE, but with E >100 using the recombinant enzyme, which contains only a single isoenzyme [15•].

A similar situation was reported for CRL. One of the first examples was the observation that cross-linked enzyme

Figure 1



A single amino acid motif determines the activity of lipases and esterases towards tertiary alcohols. Furthermore, the enantioselectivity of a GGGX esterase from *Bacillus subtilis* was improved sixfold by site-directed mutagenesis [28**].

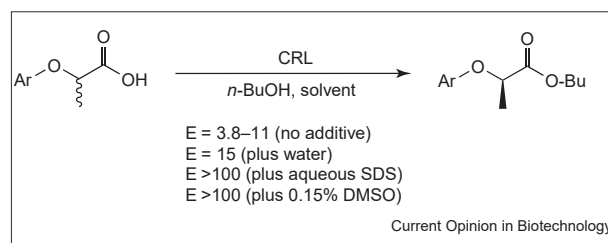
crystals of CRL showed much higher enantioselectivity than the commercial preparation in the resolution of ketoprofen. This increase in enantioselectivity was attributed to the selective enrichment of one isoenzyme during the crystallization process [16]. This is supported by a study using purified fractions of lipase OF, which also originates from *Candida rugosa* [17]. More recently this was confirmed for the resolution of D,L-menthol benzoate using the isoenzyme LIP1 of CRL obtained by heterologous expression in the yeast *Pichia pastoris* [18]. Commercially available preparations of this lipase showed only minor selectivity ($E = 15$), whereas recombinant CRL gave excellent enantiomeric purity ($E > 100$).

Rational computer-guided protein design

Crystallographers have already solved many lipase structures and have paved the way for the use of computer modeling to rationalize the substrate specificity and enantioselectivity of these enzymes [19*]. Various approaches to understand the origin of enantioselectivity of lipases and to provide guidelines for improving activity and selectivity by rational design are described [20–22]. In an impressive example, the enantioselectivity of the *Candida antarctica* B lipase-catalyzed resolution of 1-chloro-2-octanol was improved from $E = 14$ to 28 by a single amino acid exchange (Ser47→Ala), as predicted by molecular modeling [23]. In another approach [24**], a transition-state stabilizing threonine near the active site was replaced by valine using site-directed mutagenesis resulting in a loss of lipase activity. Interestingly, this could be restored by using 2-hydroxypropanoate as a substrate, which provides the ‘missing’ hydroxyl group. This also resulted in an improved enantioselectivity (from $E = 1.6$ in the wild type to 22 in the Thr40→Val mutant). Other recent examples include the development of models to understand the stereoselectivity of lipase from *Pseudomonas cepacia* towards secondary alcohols [25] and γ - and δ -lactones [26].

According to Pleiss and co-workers [27] lipases differ in a certain motif located in the active site, and this motif is responsible for the stabilization of the tetrahedral intermediate during catalysis. These are either named GX or

Figure 2



The effect of additives on lipase-catalyzed resolutions. Ar, aryl.

GGGX motif, are located in the oxyanion-binding pocket, and so far have only been described in a phenomenological manner. In a recent work, it could be shown that this motif also controls the activity of lipases towards tertiary alcohols [28**] — an important class of compounds that so far have only been accepted by a few hydrolases (e.g. CRL or lipase A from *Candida antarctica* [CAL-A]). Indeed, it could be shown that not only CRL, which is known to contain the GGGX motif, acts on acetates of tertiary alcohols, but also a considerable number of other GGGX-motif hydrolases (e.g. three acetylcholine esterases, PLE, and a *p*-nitrobenzylesterase from *Bacillus subtilis* [BsubpNBE]) efficiently hydrolyzed four model acetates albeit at moderate enantioselectivity ($E = 1.5\text{--}11$) [28**]. On the basis of a thorough computer modeling, mutants were predicted for BsubpNBE that show at least sixfold improved enantioselectivity ($E = 6$ in the wild type versus $E = 19$ in the Gly105→Ala mutant) in the resolution of (*R,S*)-2-phenyl-3-buten-2-yl acetate (Figure 1) (E Henke, UT Bornscheuer, J Pleiss, unpublished data). GX-motif hydrolases were all inactive towards the tertiary alcohols used in these studies.

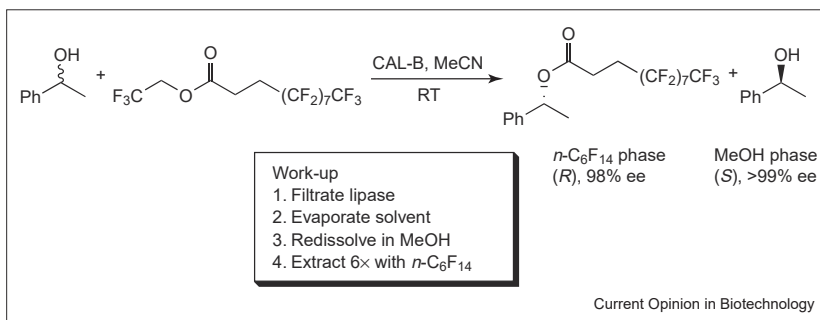
Directed evolution

Directed evolution represents an efficient alternative to rational protein design when the structure and mechanism of the targeted enzymes are not available. In short, this method consists of the generation of large mutant libraries using various methods for random mutagenesis and recombination followed by advanced high-throughput screening or selection methods [29] to identify desired variants. In the past few years, a considerable number of reviews [9,30,31] and a book [32] have addressed in detail methods for directed evolution and provided many examples for its successful application.

Up to now, there is only one example where directed evolution has been used to successfully increase the enantioselectivity of a lipase. Reetz and coworkers demonstrated a few years ago that the enantioselectivity of a lipase from *Pseudomonas aeruginosa* PAO1 towards 2-methyldecanoate could be enhanced by means of directed evolution from $E = 1$ to $E \sim 26$ for a mutant bearing five amino acid substitutions [33]. More recently, a combinatorial approach using cassette mutagenesis gave E -values above 50 [34].

Figure 3

Fluorinated acyl donors in combination with extraction using perfluorinated solvents facilitate efficient resolution and downstream processing in the lipase-catalyzed resolution of racemic secondary alcohols like α -phenyl ethanol [46]. CAL-B, lipase from *C. antarctica*; ee, enantiomeric excess; MeCN, acetonitrile; RT, room temperature.



In addition, mutants were identified having opposite stereopreference.

Substrate and medium engineering

It was shown in the 1980s and 1990s that variations of the solvent system can be used to influence the enantioselectivity of enzymatic reactions (for one example in which the enantioselectivity was reversed when changing the solvent, see [35]).

Although the effect of an organic solvent or an additive can hardly be predicted, it still can be a useful approach for increasing enantioselectivity. For instance, enantioselectivity was increased from $E < 4$ to $E > 100$ by the addition of 30–70% dimethyl sulfoxide (DMSO) in the hydrolysis of various substituted phenoxypropionates catalyzed by lipases from *Candida rugosa* [36]. For transesterifications, similar effects were observed but only 0.05–0.3% DMSO was found sufficient as additive to the organic solvent system. Similar effects were also reported for the same reaction by the addition of aqueous sodium dodecyl sulfate (SDS), which increased E from 3.8 to >100 [37], or by chemical modification of a lipase [38] (Figure 2).

Probably the most striking recent achievement in the field of medium engineering was the discovery that enzyme-catalyzed reactions can be performed in the presence of ionic liquids (ILs). The most commonly used ILs are 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM]BF₄) and the hexafluorophosphate ([BMIM]PF₆). Furthermore, the solvation properties of ILs can be modulated using different cations or anions resulting in water miscible and immiscible systems [39].

The Sheldon group published the first paper describing the successful use of ILs and found similar performance (i.e. activity and stability) of lipase from *Candida antarctica* (CAL-B) compared with conventional organic solvents in alcoholysis, ammoniolysis and perhydrolysis reactions [40]. Furthermore, it could be shown that the enantioselectivity of CAL-B and lipase from *Pseudomonas cepacia* (also called *Burkholderia cepacia*; PCL) can be maintained or even substantially enhanced in the presence of ILs in the

resolution of several secondary alcohols [41,42,43]. For α -phenylethanol, a kinetic resolution catalyzed by a lipase from *Pseudomonas* sp. using vinyl acetate at controlled water activity gave $E = 4$ in methyl-*t*-butyl ether at temperatures between 60–90°C, whereas a reaction in 1-butyl-3-methylimidazolium-bis[(trifluoromethyl)sulfonyl] amide proceeded at $E > 150$ [44]. More information about the properties and application of ILs in biocatalysis can be found in a recent review [45].

Related to this field is the discovery that an efficient separation of substrate and product of lipase-catalyzed acylations can be achieved by using fluorinated acyl donors in combination with an extraction step using perfluorinated solvents [46] (Figure 3). Besides the advantage of an easy up-scalable process, enantioselectivity was slightly higher compared with reactions using vinyl acetate for acylation and a simple flash chromatography for separation. This concept was later adapted for the resolution of 1-(2-naphthyl)ethanol in a triphasic separative reaction [47].

Another alternative is the variation of the substrate structure. Enantioselectivity could be enhanced by the use of vinyl esters of chiral carboxylic acids instead of commonly used ethyl esters. In the CAL-B catalyzed resolution of ibuprofen, the E -value increased from $E < 2$ (ethyl ester) to $E = 39$ (vinyl ester) [48]. Variation of the chain-length of the acyl donor in the resolution of secondary alcohols also changed the E -value remarkably. Ottosson and Hult [49] found that the E -value decreased for shorter vinyl esters in the transesterification of 3-methyl-2-butanol. This was mainly attributed to changes in the entropic component except for the reaction with vinyl propionate, which differed from the other acyl donors also in the enthalpic component [49]. Similar findings were made in the resolution of a diester using a lipase from *Chromobacterium viscosum* [50]. The highest E -value was found for the dibutanoate ester ($E = 48$), shorter or longer acid chains led to a substantial decrease in E -value (e.g. $E = 5$ for the diacetate and the didecanoate). This observation can be explained by computer modeling: only the (*S*)-enantiomer of the butanoate fits well into the binding pocket of the *C. viscosum* lipase, but the longer or shorter residues did not fit.

Conclusions

Enantioselectivity is one of the key features of enzymes making them attractive for biocatalysis. It is still not well understood, however, why a certain lipase or esterase shows a certain enantioselectivity in a given reaction. As summarized in this article, many different approaches can be used to influence the selectivity. Substrate engineering or modification of the reaction system remain rather unpredictable approaches. By contrast, recent work in protein engineering shows that the understanding of the basis of enantioselectivity on a molecular level is steadily increasing and, consequently, a number of successful examples for increased enantioselectivity can be found.

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