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Directed Evolution of An Esterase: Screening of Enzyme Libraries Based on pH-Indicators and a Growth Assay

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Abstract—In order to resolve a sterically hindered 3-hydroxy ethyl ester, which was not accepted as substrate by 20 wild-type hydrolases, a directed evolution of an esterase from *Pseudomonas fluorescens* (PFE) was performed. Mutations were introduced using the mutator strain *Epicurian coli* XL1-Red. Enzyme libraries derived from seven mutation cycles were assayed on minimal media agar plates supplemented with pH indicators (neutral red and crystal violet), thus allowing the identification of active esterase variants by the formation of a red color caused by a pH decrease due to the released acid. A further selection criteria was introduced by using the corresponding glycerol ester, because release of the carbon source glycerol facilitates growth on minimal media. By this strategy, one double mutant (A209D and L181V) of PFE was identified, which hydrolyzed the 3-hydroxy ethyl ester in a stereoselective manner (25% ee for the remaining ester, E∼5).

Introduction

Esterases as well as lipases accept a wide range of non-natural esters and also exhibit high activity in organic media. They usually show high stereoselectivity and as a consequence have been widely used in the preparation of several hundred optically pure substances.[1–4] However, in the case of sterically hindered compounds, resolutions using lipases or esterases often failed. For instance, dihydropyridine derivatives could only be resolved by introducing a sterically non-hindered cleavage site.[5,6] However, modification of the substrate structure is not always feasible, e.g. when the structure is fixed as in the total synthesis of natural products. In such a case, the problem of non-reactivity of the biocatalyst might be overcome by introducing mutations in order to broaden the substrate spectra. In principle, this could be done by either a rational protein design or by random mutagenesis. Major disadvantages of a rational design by site-directed mutagenesis are the need of structural data of the enzyme and detailed knowledge of the mechanism of catalysis. Furthermore, site-directed mutagenesis is a time-intensive method, which allows the production of relatively few enzyme variants at reasonable times.

Recently, the directed evolution of enzymes was described as a new and very elegant approach to generate and identify new enzyme variants. Principles and examples can be found in several recent reviews.[7–11] Prerequisites for a successful directed evolution are an effective mutation strategy for the improvement of the enzymes, the functional expression of the protein in a suitable microbial host, and a fast and reliable assay system for the identification of enzyme variants with desired properties out of a pool of 10^4 to more than 10^6 variants. A range of methods for the generation of enzyme libraries is available, and in most cases researchers use error-prone PCR for this purpose. However, the assay system usually represents the major bottle-neck. Desired hydrolase variants were identified by use of chromogenic esters to identify more active *p*-nitrobenzyl esterases[12] or to increase the stereoselectivity of a lipase.[13]

Our interest in directed evolution started from the attempt to resolve 3-hydroxy ester (**1**) (Scheme 1), which shows close structural similarity to a key building block in Epothilones, a new class of macrolides, showing taxol-like biological activity[14,15] Previously, we have shown the successful resolution of aliphatic[16] and

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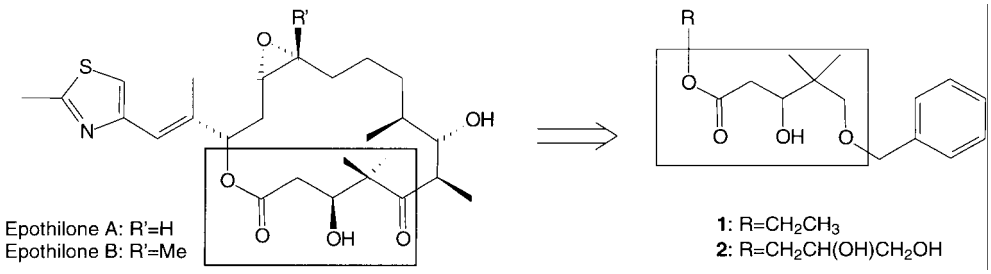


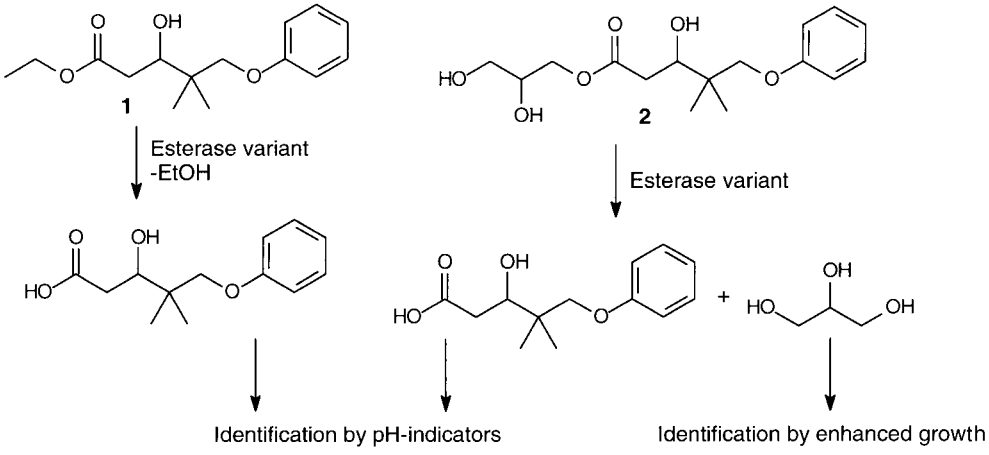
Scheme 1.

arylaliphatic[17] 3-hydroxy esters using lipases or esterases. Unfortunately out of 18 lipases and two esterases,[18] none showed any activity towards **1** either in hydrolysis in phosphate buffer or in acylation with vinyl acetate in toluene (data not shown). We considered several options to overcome this problem: (i) to screen for new enzymes, which is tedious and time-consuming, (ii) to alter the reaction conditions, e.g. change the solvent system or acyl donor, which is very unlikely to help in this specific case or (iii) to evolve new enzymes by directed evolution. Deciding for the last option, we created an enzyme library of an esterase from *Pseudomonas fluorescens* (PFE), by using a commercial mutator strain from *Epicurian coli* XL1-Red.[19] The gene encoding PFE could be easily expressed in *Escherichia coli* and facile protocols for the isolation and purification of this esterase have been developed already in our laboratory [20,21] These libraries were then assayed by means of an agar plates assay in the presence of pH indicators in combination with a growth assay.

Results

In order to create an enzyme library of the esterase from *Pseudomonas fluorescens* (PFE), the plasmid containing the wild-type gene was transferred into the mutator strain *Epicurian coli* XL1-Red and incubated overnight in LB media. An aliquot of the overnight culture was used for plasmid isolation using a kit. A second aliquot was diluted 1:50 with fresh LB media and subjected to a subsequent mutation cycle. These steps were repeated seven times. Plasmids collected during these cycles were transferred into *Escherichia coli* DH5α, and 50–100 μL aliquots were plated onto agar plates based on LB media to facilitate growth. These plates served as master plates for the screening of new esterase variants.[22,23]

The principle of the assay system is depicted in Scheme 2. Identification of desired esterase variants, which are capable to hydrolyze the sterically hindered 3-hydroxy esters (**1**) and (**2**), was performed by supplementing minimal media agar plates with pH indicators. PFE shows highest activity at pH values between 6 and 7.5. A preliminary test of different indicators revealed that a combination of neutral red and crystal violet gave best results. A further selection criteria was introduced by supplementing agar plates with glycerol ester (**2**). Active variants would release the carbon source glycerol, which facilitates growth on minimal media. Because a rhamnose promotor is located upstream of the esterase gene, L-rhamnose was spread onto the plates for the induction of esterase production. Minimal media was used instead of rich LB media in the preparation of agar plates for two reasons: (i) false positive results were obtained when the screening was performed with LB media, and (ii) an additional growth assay can be performed using minimal media.



Scheme 2.

Colonies from each master plate were then replicated onto two minimal media agar plates prepared as outlined above (see also Experimental) and incubated at 37°C. One set of plates contained ethyl ester (**1**), the second set glycerol ester (**2**). After incubation for 2–6 days, two colonies which had turned red on both substrates, were identified. Moreover, the red colony on the plate containing the glycerol ester was significantly larger (Ø ~3 mm) compared to non-red colonies (Ø < 1 mm) on the same plate. The plasmid was isolated using the colony from the master plate, transformed into *Escherichia coli* and the esterase was produced and isolated according to the general protocol. The esterase was then subjected to a preparative hydrolysis of ethyl ester (**1**), and samples were analyzed by gas chromatography using a chiral column. Indeed, this esterase variant stereoselectively hydrolyzed **1** resulting in 25%ee for the remaining substrate, which corresponds to an enantioselectivity[24] of approx. $E = 5$. This is in accordance with the low enantioselectivity observed with PFE towards other chiral substrates.[25]

Sequencing of this esterase variant revealed that two point mutations (A209D, L181V) were introduced. The structure of PFE is unknown, but the enzyme shows approx. 55% identity to a haloperoxidase from *Streptomyces aureofaciens* with the determined structure.[26] This haloperoxidase also shows the characteristic α/β-hydrolase fold of lipases and esterases. Thus, a preliminary homology modeling of PFE based on the haloperoxidase structure using the program InsightII was feasible. This modeling showed that both mutations are not near or in the active site of PFE, but at the periphery of the enzyme (Fig. 1).

Mutant A209D/L181V was subjected to a second mutation cycle, and 10 000 colonies were assayed using the assay system described above. Because activity was already observed with the parent mutant, only those colonies which turned red after 2 days of incubation, were selected. From a total number of 12 selected clones, none exhibited higher enantioselectivity compared to mutant A209D/L181V, but specific activity towards ethyl acetate serving as standard substrate for activity determination (see Experimental) differed significantly.[22,23]

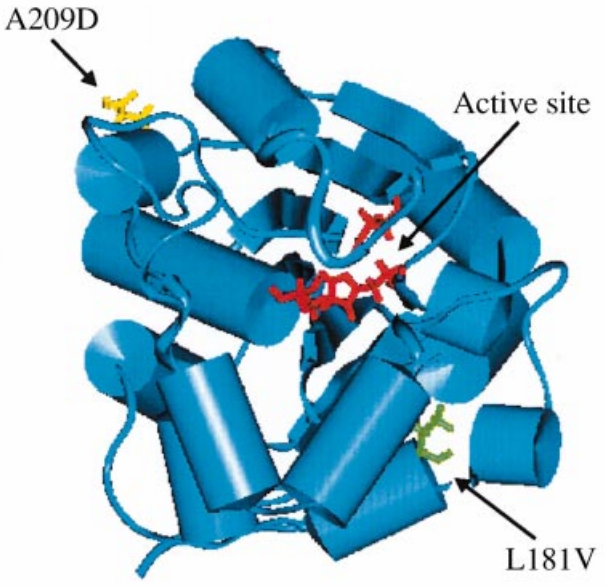


Figure 1.

Discussion

We demonstrated that the substrate specificity of an esterase by means of directed evolution can be altered. Only two mutations were necessary to tip the balance from a non-substrate to a stereoselective hydrolysis of the sterically-hindered 3-hydroxy ester (**1**). However, in this specific case, further improvements in the enantioselectivity of PFE are required to allow the preparation of optically pure 3-hydroxy ester. This is under current investigation. Alternatively, if the gene and a suitable expression system are available, a more stereoselective hydrolase might be subjected to rounds of mutagenesis and screening. Another option could be the application of DNA-shuffling.

In order to identify desired esterase variants within the enzyme library, a simple, reliable and easy-to-use assay system was developed. Major advantages of the use of pH indicators are, that there is no need for special substrates, e.g. chromogenic esters and a simple visual detection is possible. The wide variety of available pH indicators allows the adaption of this assay to the optimum pH range of the enzyme of interest. Currently, we are trying to refine the pH-indicator assay to allow the quantitative determination of hydrolytic activity in a microtiter plate format. We believe that our assay system could be of general use, where the substrate specificity of hydrolases or other enzymes needs to be altered, which leads to a change in pH during catalysis. Furthermore, a large number of clones can be examined within a short time. For instance, we have usually assayed around 300–500 colonies per plate. In the growth assay, a glycerol ester was successfully employed, but the principle of selection by growth is not restricted to glycerol esters of carboxylic acids. It should be easily transferable to esters, where the reactivity towards an alcohol is of interest and the released carboxylic acid serves as a carbon source (e.g. acetic acid, butyric acid).

Experimental

Cloning, expression, isolation and pH-stat assay of esterase from *Pseudomonas fluorescens* (PFE)

This has been performed as described previously.[20,21,25] Esterase activity was determined in a pH-stat assay with 5% (w/v) ethyl acetate emulsion using distilled water containing 2% (w/v) gum arabic at 37°C at pH 7.5. Different amounts of esterase were added to 20 mL of the emulsion. In order to maintain the pH constant, liberated acetic acid was titrated automatically in a pH-stat (Metrohm, Herisau, Switzerland) with 0.1 N

NaOH. One unit (U) of esterase activity was defined as the amount of enzyme, that liberates 1 μmol acetic acid per min under assay conditions. Units were corrected for autohydrolysis of ethyl acetate, which was 0.25 μmol per min at 37°C. Wild-type PFE served as a control in all experiments including those described below.

Biotranstransformation and analysis

3-Hydroxy esters (**1**) and (**2**) have been chemically synthesized as described previously.[22] Preparative hydrolysis of **1** was performed in round bottom flasks by dissolving 0.5 mmol of ester **1** in 1 mL toluene at 37°C and 700 rpm. The reaction was started by addition of 3 mL cell extract containing PFE. Reactions were stopped by extraction with diethyl ether, the organic phase was dried over $MgSO_4$ and solvents were evaporated in vacuo. Substrate and product were separated by silica gel column chromatography (petrol ether:ether, 2:1). Enzymatic hydrolysis of **1** using mutant A209D/L181V gave the corresponding acid $[\alpha]_D^{20} = -11.01°$ ($c = 0.965$, $CHCl_3$), oil, 11.6% y, and remaining starting material (25%ee), $[\alpha]_D^{20} = +0.97°$ ($c = 3.071$, $CHCl_3$), oil, 61.4% y. Enantiomeric excess of remaining ester was determined by gas chromatography (Hewlett–Packard, Model HP5890 Series II) using a chiral column (heptakis (6-*O*-thexyldimethyl-silyl)-2,3-di-*O*-methyl)-β-cyclodextrin, 25×0.25 mm, 15 m, Prof. W. König, Institute for Organic Chemistry, University of Hamburg, Germany).

Directed evolution of PFE

This has been performed as described previously.[22,23] Briefly, the plasmid containing the PFE wild-type gene was transformed in the mutator strain *Epicurian coli* XL1-Red competent cells (Stratagene, La Jolla, USA) according to the manufacturers protocol and grown overnight in 50 mL LB media supplemented with $MgCl_2$ (20 mM), glucose (20 mM) and ampicillin (100 μg/ml) at 37°C. 500 μL of this culture were added to fresh LB-media (50 mL) and subjected to another mutation cycle. From the rest, 2 mL were centrifuged (3000 rpm, 10 min, 4°C) and the plasmid was isolated. This mutation cycle was repeated up to seven times. Isolated plasmids were transferred into *Escherichia coli* DH5α, grown for 1 h at 37°C in LB media. Then, an aliquot (50–100 μL) of the culture was spread on LB agar plates (serving as master plates) and incubated overnight at 37°C.

Screening system

Colonies were replica plated using autoclaved velvet from master plates onto minimal media agar plates supplemented with ampicillin (100 μg/ml), crystal violet (1 mg/L), neutral red (30 mg/L) and rhamnose (0.2% w/v). In addition, plates contained either 0.1% (w/v) of 3-hydroxy ester (**1**) or (**3**) (dissolved in ethyl ether for easy distribution of substrate and quick evaporation of solvent). Plates were incubated at 37°C. Positive clones were selected by the formation of red spots *and* faster growth as detectable using ester **2**. Then, plasmids were isolated and transferred into *Escherichia coli* DH5α, followed by expression and isolation of PFE according to the general procedure.

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