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# “Activity of lipases and esterases towards tertiary alcohols: New insights into structure-function relationships“

**E. Henke, J. Pleiss, U. T. Bornscheuer**

## Experimental

**Enzymes.** The following commercially available enzymes were donated by the named suppliers. From Amano (Nagoya, Japan): Amano AYS (Lipase from *Candida rugosa*), Lipase A Amano 6 (*Aspergillus niger* lipase (ANL)), Lipase AK Amano 20 (*Pseudomonas fluorescens* lipase (PFL)), Lipase D (*Rhizopus oryzae* lipase (ROL)), Lipase G Amano 50 (*Penicillium camembertii* lipase (PcamL)), Papain W40 (*Carica papaya*), Acylase (*Aspergillus* sp.); from Roche (Penzberg, Germany): Chirazyme L1-L12 (*Pseudomonas cepacia* lipase (PCL), *Candida antarctica* lipase B (CAL-B), *Candida antarctica* lipase A (CAL-A), *Pseudomonas* sp. lipase, Pig pancreatic lipase (PPL), *Thermomyces* sp. lipase, *Mucor miehei* lipase (MML), *Alcaligenes* sp. lipase). These enzyme preparations were used as delivered. Protein content was determined using the BCA

Kit (Pierce, Rockford, IL, USA). Enzyme activity was determined by the pH-Stat assay as described below.

**Cloning of the *p*-nitrobenzyl esterase from *Bacillus subtilis*.** Genomic DNA from *Bacillus subtilis* DSM 402 was isolated using a standard protocol.[1] BsubpNBE was cloned by amplification of the corresponding open reading frame from the genomic DNA using the primers EHE-pNBE-V2 (5´- ACT ACT ACT ACT CAT ATG ACT CAT CAA ATA GTA ACG -3´) and EHE-pNBE-R1 (5´- CTA CTA CTA CTA GGA TCC TTC TCC TTT TGA AGG-3´). The PCR product was digested by *BamH*I and *Nde*I and ligated into an expression vector, based on pUC19 under control of the strong regulating *rhaP* promotor[14], yielding plasmid pG-BsubpNBE.WT. This cloned BsubpNBE from DSM 402 differs in 11 residues from the BsubpNBE of strain NRRL B8079 described by Zock *et al.*[2]

**Protein expression system.** Esterases from *Pseudomonas fluorescens*[10], *Bacillus stearothermophilus*[11] and the *p*-nitrobenzyl esterase from *Bacillus subtilis* were expressed in *E. coli* using the corresponding plasmids (pG-PFEI.WT, pG-BsteE.WT and pG-BsubpNBE.WT) under control of a rhamnose inducible promoter, *rhaP*[14], an ampicillin resistance served as selection marker. Esterase production was performed as described previously.[3] Recombinant AChEs were produced by

expression in *Pichia pastoris* and the enzymes were kindly provided as crude extracts by S. Vorlova (Institute for Technical Biochemistry, Stuttgart University). Lipase B from *Geotrichum candidum* was expressed in *Pichia pastoris* as described by Catoni *et al.*[7]

**Directed evolution.** Random mutagenesis was performed by error-prone PCR[12] and DNA shuffling.[13] Mutated esterase encoding genes were ligated into the expression vector and transformed into *E. coli* DH5α. Colonies were transferred into 384 well microtiterplates using a picking robot (Biorobotics, Cambridge, UK) and grown in LB-Amp media supplemented with 5 % (v/v) DMSO. These plates were used as stocks enabling direct storage at –80°C. Expression of esterase variants was performed by replica plating in 96 well microtiterplates containing LB-amp-rhamnose broth followed by incubation at 37°C for 20 h. Cell were disrupted by two freeze / thawing cycles. After centrifugation (4000 rpm, 10 min, 4°C), the cell lysate was used directly for activity testing towards TAEs using a pH-assay (see below). In addition, activity was determined spectrophotometrically using *p*-nitrophenyl acetate as described previously.[10c]

**pH-Assay.** The assay was performed in 96 well microtiterplates. To a substrate emulsion (180 µl / well

containing 6 mM substrate, 10 % DMSO, 2 % gum arabic in sodium phosphate buffer 2 mM, pH 7.5) 6-carboxyfluorescein (10 µM) was added. Then cell lysate (20 µl / well) containing the recombinant esterase was added and fluorescence (ex.: 485 nm, em.: 538 nm) determined using the FLUOstar Galaxy fluorimeter from BMG Labtechnologies GmbH (Offenburg, Germany)) was measured immediately and after 20 h of incubation at 37°C.

**Synthesis of tertiary alcohol acetates.** Linalyl acetate (**2**) and *tert.*-butyl acetate (**1**) were purchased from Fluka (Buchs, Switzerland). All other acetates were prepared as follows.

**(*R*,*S*)-3-Methyl-1-pentin-3-yl acetate** (**3**)**:** To 17 ml (*R*,*S*)-3-methyl-1-pentin-3-ol (19.6 g, 200 mmol) 20 ml acetic anhydride (15.7g, 154 mmol) was added dropwise while the flask was cooled with an ice bath. After adding 50 mg phosphorus pentoxide and stirring for another 15 min the ice bath was removed and the mixture was stirred at room temperature for 16 h. The solution was washed twice with 100 ml water and extracted two times with 100 ml diethyl ether. The organic layers were combined and washed with saturated $NaHCO_3$ solution until no further formation of $CO_2$ was observed. The solution was washed again twice with water and dried with anhydrous $Na_2SO_4$ before the solvent

was removed in vacuum. The product was purified by flash chromatography on silica gel (petrol ether:ethyl acetate, 4:1) yielding the product as a colorless liquid (19.2 g, 68 % yield).

$^{1}$H-NMR (500,15 MHz; δ in ppm vs. TMS; in $CDCl_3$) δ: 1.03 (3 H; t; J = 7.5); 1.66 (3 H; s); 1.85 (1 H; m); 1.96 (1H; m); 2.03 (3 H; s); 2.55 (1 H; s). $^{13}$C-NMR (125.76 MHz; δ in ppm vs. TMS; in $CDCl_3$) δ: 8.40; 21.89; 25.90; 34.67; 73.18; 75.31; 83.75; 169.42.

**(*R*,*S*)-2-Phenyl-3-butin-2-yl acetate** (**4**)**:** 25 g (*R*,*S*)-2-phenyl-3-butin-2-ol (171 mmol) was dissolved in 300 ml freshly distilled dry tetrahydrofuran. The solution was cooled on ice and 80 ml of 2.5 M butyllithium (200 mmol) in toluene was added dropwise over a period of 10 min. The mixture was stirred for 15 min before 15 ml freshly distilled acetyl chloride (211 mmol) was added. After removal of the ice bath, the mixture was heated under reflux for 1 h and then cooled to room temperature. Non-reacted acetyl chloride was hydrolysed by addition of 150 ml water. The mixture was extracted three times with 300 ml diethyl ether, the collected organic phases were dried with anhydrous $Na_2SO_4$ and solvent was removed in vacuum. Distillation (114°C, 15 mbar) yielded the product as a colorless liquid (24.5 g, 76 % yield).

$^{1}$H-NMR (500.15 MHz; δ in ppm vs. TMS; in $CDCl_3$) δ: 1.89 (3 H; s); 2.07 (3 H; s); 2.80 (1 H; s); 7.34 (1H); 7.36 (2 H); 7.58 (2H). $^{13}$C-NMR (125.76 MHz; δ in ppm vs. TMS; in $CDCl_3$) δ: 21.71; 32.05; 75.31; 75.56; 124.75; 124.84; 127.91; 128.33; 128.38; 142.10; 168.62.

**Biotransformation.** Biotransformations were performed in a pH-Stat device (Metrohm, Switzerland) at 40°C. 20 ml of a preheated substrate emulsion (10 mM, stabilized with 2 % (w/v) gum arabic) were placed in the reaction chamber and the enzyme preparation was added. The pH-value was kept constant by automatic addition of NaOH solution. Esterase activity and conversion were calculated from NaOH consumption. In addition, samples (200 µl) were withdrawn from the reaction mixture and extracted with isohexane (1 ml). Enantiomeric excess of substrate and product were determined by gas chromatography.

**Gas chromatography.** Determination of optical purity was performed using gas chromatography on a chiral column (Heptakis-(6-*O*-pentyl-2,3-di-*O*-methyl)-γ-cyclodextrin in OV1701, 25 m; Prof. König, Hamburg University, Hamburg, Germany) with hydrogen (40 kPa) as carrier. Conditions: linalyl acetate/linalool: 90°C, 25 min; (*R*,*S*)-3-methyl-1-pentin-3-yl acetate/(*R*,*S*)-3-methyl-1-pentin-3-ol: 40°C, 0

min-2°C/min-55°C, 10 min; (*R*,*S*)-2-Phenyl-3-butin-2-yl acetate/(*R*,*S*)-2-phenyl-3-butin-2-ol: 120 °C, 15 min.

**Molecular modeling.**

**Software, force field:** Molecular modeling was performed using a Silicon Graphics Octane 2 workstation (SGI, Mountain View, CA) and the Sybyl 6.1 (Tripos, St. Louis, MO) software. For all calculations, the Tripos force field was utilized. Gasteiger-Hückel charges were used, the charges of the catalytic $His_{act.}$ and the tetrahedral $Ser_{act.}$-substrate complex were adjusted as described by Holzwarth *et al.*[4]

**Structures, homology modeling, substrate docking.** Experimentally determined X-ray structures of CRL (PDB entry 1LPM) and eeAChE (PDB entry 1C2B) from a homology model of hAChE (PDB entry 2CLJ) were used. A homology model of BsubpNBE DSM 402 was created using the X-ray structures of the corresponding enzyme from strain NRRL B8079 (PDB entries 1QE3, 1C7J, 1C7I).[5] The homology model for bAChE was based on the structure of AChE from *Torpedo californica* (PDB entry 1QIK, 1CFJ). The homology models were computed using the Swiss-Model automated modeling service of GlaxoSmithKline (http://www.expasy.ch/swissmod/SWISS-MODEL.html).

All possible solvent molecules included in the PDB files were removed before substrate docking. Substrates were manually docked into the binding site of the enzymes, mimicking the tetrahedral intermediate formed after the nucleophilic attack of the catalytic $Ser_{act.}$. This resembles the rate-limiting step of ester hydrolysis. The substrate was orientated with the oxyanion towards the oxyanion hole residues and the protonated $N_{\varepsilon}$ of $His_{act}$ embedded between the $O_{\gamma}$ and the $O_{ester}$ of the substrate. The substrates' substituents were orientated in the binding pocket to a minimum of repulsive interaction with the protein structure.

**Minimization, molecular dynamics simulations.** The enzyme-substrate complex was refined first by minimization. Next, a molecular dynamics simulation was performed for 18 ps (2 ps at 5K, 2 ps at 30 K, 2 ps at 70 K; 12 ps at 100 K). The coupling constant was adjusted to 10 fs. The non-bonded interaction cut-off was set to 8 Å, the dielectric constant to 1.0. The conformers were saved every 40 fs. An average structure over the last 2 ps was calculated and used for analysis. All minimization and MD simulations were performed *in vacuo*, with constrained protein backbone.

To ensure that the results do not depend on the choice of the initial conformation, additional simulations of

the same enzyme-substrate complex were performed using different initial conformations. Also, it was verified that a simulation time of 18 ps is sufficient by extending the simulations to 100 ps. This prolonged simulation time led to same results as the 18 ps runs.

**References**

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