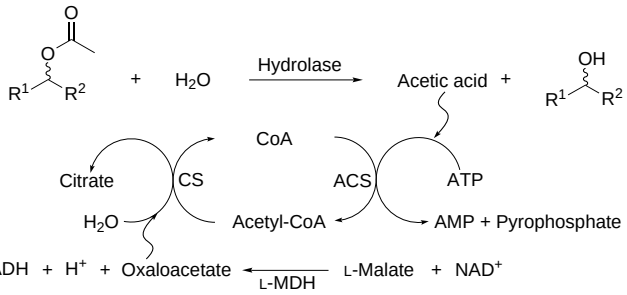
A High-Throughput-Screening Method for the Identification of Active and Enantioselective Hydrolases**

Markus Baumann, Rainer Stürmer, and Uwe T. Bornscheuer*

Hydrolytic enzymes are versatile biocatalysts and find increasing application in organic syntheses.[1] Within this class of enzymes, lipases and esterases are frequently used as they accept a broad range of nonnatural substrates, are usually very stable in organic solvents, and exhibit good to excellent stereoselectivity in the kinetic resolution of racemates or desymmetrization of prostereogenic compounds. Owing to the vast progress in genetic engineering, the number of (commercially) available biocatalysts is rapidly increasing. However, this makes the identification of suitable enzymes by laboratory-scale reactions followed by, for example, GC or HPLC-based determination of optical purities a tedious and time-consuming task. In addition, the recently developed techniques for directed (molecular) evolution[2] require highly sophisticated assays for high-throughput screening (HTS) of large libraries usually containing 10^4–10^6 mutants.

For the rapid determination of enantioselectivity E (also named enantiomeric ratio)[3] several assay formats have been delevoped. Kazlauskas and Janes measured initial rates of hydrolase-catalyzed hydrolysis of *p*-nitrophenyl derivatives of pure chiral carboxylic acids ("Quick E") in the presence of resorufin tetradecanoate to introduce competition yielding more exact E values.[4] Other groups used chromogenic[5] or fluorogenic[6] substrates for the identification of more enantioselective hydrolases. Although measurements with these substances are usually very sensitive, a major disadvantage resides in the presence of bulky groups, which usually differ considerably from the "true" desired substrate, for example an acetate. As a consequence, the risk is high that the identified "suitable" enzymes show different selectivities towards the "true" substrate. To overcome this problem, Kazlauskas and co-workers reported a more general applicable assay in which both acetates of chiral alcohols and esters of chiral carboxylic acids were hydrolyzed in the presence of *p*-nitrophenol, which served as a pH-indicator.[7] A stepwise assay for screening of enantioselective hydrolases by using bromothymol blue as pH-indicator to screen for activity followed by gas chromatography to measure enantioselectivity was reported recently by us.[8] Other methods are based on time-resolved IR-thermographic determination of enantioselectivity[9] or use of "pseudo"-enantiomers in which one form is isotopically labeled in combination with mass spectrometric analysis.[10] The major advantage is that true E values are determined, but the method needs deuterated pure enantiomers and equipment costs are very high. Recently, a "super-HTS" by parallel capillary electrophoresis with chiral cyclodextrins was reported, in which optical purities of fluorescein isothiocyanate (FITC)-labeled amines were measured.[11] So far this system is restricted to amines and also requires investments for expensive equipment.

We now describe a new assay format based on a coupled enzymatic conversion (Scheme 1). First, acetic acid is released by lipase- or esterase-catalyzed hydrolysis from the corresponding chiral ester followed by a cascade of enzymatic reactions (Scheme 1) based on a readily available and cheap test-kit initially developed for food analysis.[12] Thus, acetic acid released in the initial hydrolytic reaction is stoichiometrically "transformed" into NADH, which can be easily quantified by spectrophotometric measurements at 340 nm.



Scheme 1. The hydrolase-catalyzed reaction releases acetic acid, which is converted by acetyl-CoA synthetase (ACS) to acetyl-CoA in the presence of (ATP) and coenzyme A (CoA). Citrate synthase (CS) catalyzes the reaction between acetyl-CoA and oxaloacetate to give citrate. The oxaloacetate required for this reaction is formed from L-malate and NAD^+ in the presence of L-malate dehydrogenase (L-MDH). Initial rates of acetic acid formation can thus be determined by the increase in adsorption at 340 nm due to the increase in NADH concentration. Use of optically pure (*R*)- or (*S*)-acetates allows the determination of the apparent enantioselectivity E_{app}.

To evaluate this assay format, the hydrolysis of (*R*,*S*)-α-phenyl ethyl acetate (**1**) by a recombinant esterase from *Pseudomonas fluorescens* (PFE)[13a] was chosen as a model reaction (Scheme 2). Earlier results have already shown that PFE converts this substrate with good enantioselectivity.[14]

Preliminary hydrolysis reactions were performed on a mL scale by using lyophilized PFE (1 mg mL^{-1}), 50 mM (*R*,*S*)-**1**, and the acetic acid test-kit as described in the Experimental Section. In addition, conversion and enantiomeric excess were determined by gas chromatographic analysis (Scheme 2). Incubation of (*R*,*S*)-**1** at pH 8.1 in the presence of the test-kit, but in the absence of PFE revealed no NADH formation, thus auto-hydrolysis of **1** can be excluded. Furthermore, neither activity nor enantioselectivity of PFE were affected by

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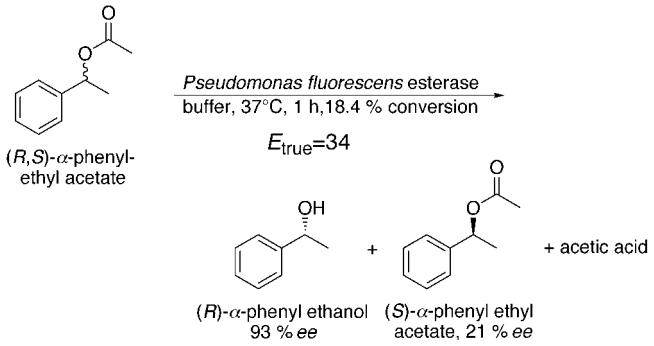


Scheme 2. Model reaction for the evaluation of the coupled-enzymatic acetate assay. Under these conditions, the esterase from *Pseudomonas fluorescens* exhibited high *R* selectivity ($E_{true} = 34$) as determined by GC analysis.

test-kit components, as confirmed by control experiments (data not shown). The maximum increase in absorption at 340 nm was about 0.3 per min, which corresponds to the maximum reaction rate that can be determined with the coupled esterase/test-kit system. However, for higher accuracy, a lower rate is recommended. Reactions in microtiter plates (MTP) revealed an almost linear[15] increase in absorbance at 340 nm over 10 min using an esterase concentration of 2 mg mL^{-1} PFE (lyophilized crude cell extract) albeit the concentration of (*R*,*S*)-**1** was varied over a broad range between 5 and 50 mg mL^{-1} (Figure 1). Beside model compound **1**, the assay format is also useful to monitor the

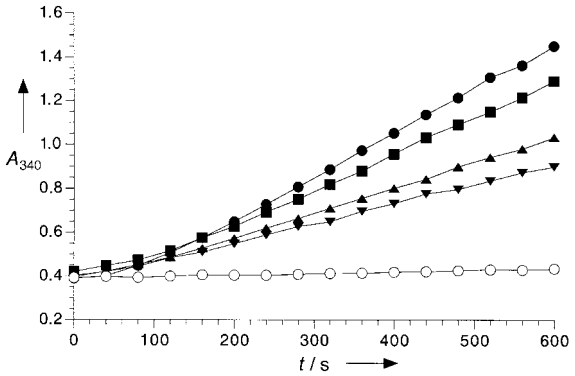
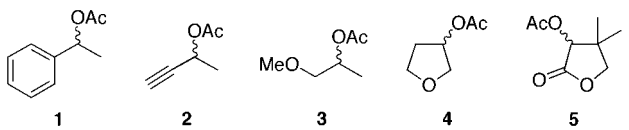


Figure 1. An almost linear increase in NADH concentration with time was observed in the conversion of acetic acid released from PFE-catalyzed hydrolysis of (*R*,*S*)-**1** (none (○), 5 (▼), 10 (▲), 25 (■), 50 mg mL^{-1} (●)). No hydrolysis was observed in the absence of esterase.

conversion of other chiral esters of secondary alcohols (Scheme 3). Here, lipase B from *Candida antarctica* (CAL-B) was chosen as catalyst, as this enzyme represents a frequently used lipase.[16] The coupled conversion of acetates



Scheme 3. Acetates **1**–**5** of secondary alcohols used for evaluation of the assay.

(*R*,*S*)-**2**–(*R*,*S*)-**5** shows that the reaction rates decreased in the order **2** > **3** > **4** > **5** (Figure 2). This observation is consistent with our previous results[8] and also proves that the assay format can be used to identify whether a certain hydrolase is active or not.

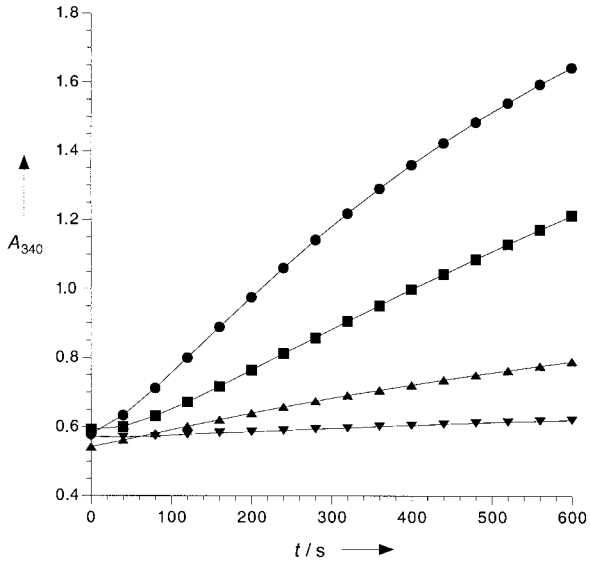


Figure 2. Transfer of the assay format to CAL-B-catalyzed hydrolysis of acetates (*R*,*S*)-**2**–(*R*,*S*)-**5** (0.5 mg mL^{-1}); **2** (●), **3** (■), **4** (▲), **5** (▼).

Measuring initial rates of the acetic acid release from pure enantiomers of (*R*)- or (*S*)-**1** (5 mg mL^{-1}) at an esterase concentration of 1 mg mL^{-1} PFE (lyophilized crude cell extract) in MTP format revealed an almost sole increase in absorption for the *R* enantiomer reflecting the high enantioselectivity of PFE (Figure 3A). The apparent enantioselectivity was calculated to be $E_{app} = 30$,[17] which closely matches the value of $E_{true} = 34$ determined by GC analysis (Scheme 2). Next, a culture supernatant containing PFE was split from one well into two portions to ensure that the same enzyme concentration is used for both enantiomers. In this case, decreased initial rates were observed and a lowered enantioselectivity ($E_{app} = 21$) was calculated (Figure 3B). This can be related to impurities from the cultivation such as cell debris or media components disturbing the determination of released acetic acid. However, it must be emphasized that this test format is useful for the direct determination of hydrolase enantioselectivity in crude cell extracts. This has the advantage that the assay format can be directly transferred to high-throughput screening in libraries obtained by directed evolution. The lowered accuracy of the assay using the crude culture supernatant might be solved by integrating a microtiter plate based purification step, for example, taking advantage of the His_6-tag cloned to the mature PFE. However, this will make the assay more time-consuming and expensive.

Next, we investigated the applicability of the assay format for the determination of *E* values in the resolution of **3** using 16 lipases and esterases. The data in Table 1 show that E_{app} values match closely to the E_{true} values determined by gas chromatography using a chiral column. In accordance to our

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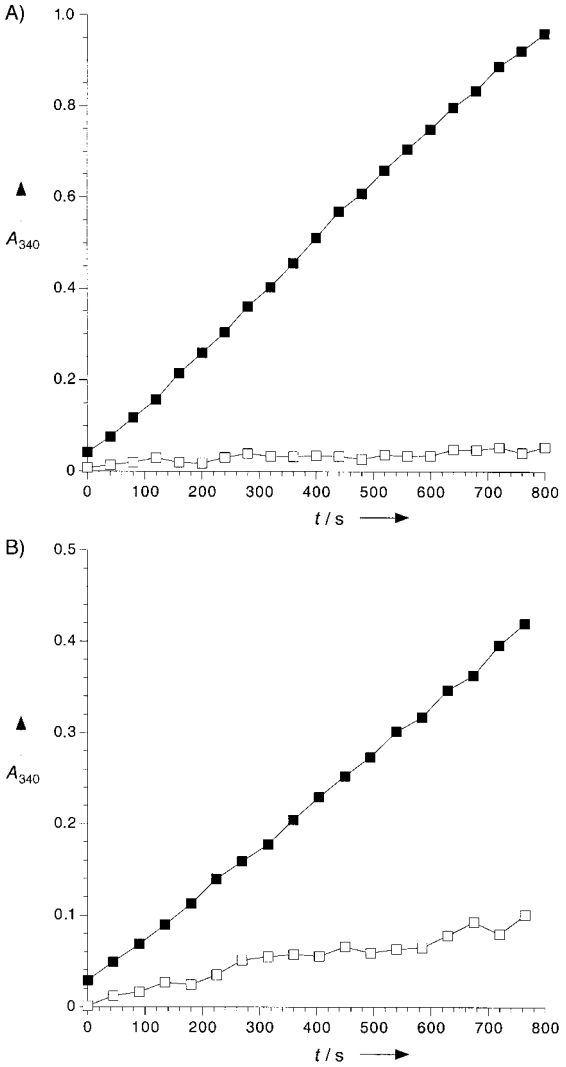

Figure 3. Initial rates determined by using optically pure (*R*)- or (*S*)-**1** (5 mg mL^{-1}; ■ and □, respectively). Reactions were performed by using lyophilized crude cell extract of PFE (A) or a culture supernatant containing PFE (B). Please note the different scaling on the *y* axes.

Table 1. Comparison of enantioselectivity determined by the enzyme cascade method (E_{app}) and by GC analysis (E_{true}) in the kinetic resolution of (*R*,*S*)-1-methoxy-2-propyl acetate by various lipases and esterases.

Enzyme[a]	E_{app} [%]	E_{true} [%]	Configuration[b]
CVL	7.5	4.9	*R*
Chirazyme L6	13.7	12.1	*R*
Chirazyme L10	3.3	2.4	*R*
Chirazyme L12	4.0	5.1	*R*
Chirazyme E1	2.1	2.6	*S*
Chirazyme E2	2.2	1.9	*S*
Chirazyme E3	1.1	1.4	*R*/*S*[c]
Chirazyme E4	2.0	2.3	*R*
PFE	1.4	1.2	*R*
CAL-B	80	100	*R*

[a] CVL = lipase from *Chromobacterium viscosum*; PFE = recombinant esterase from *Pseudomonas fluorescens*;[13a] CAL-B = lipase B from *Candida antarctica*; the following enzymes showed no or very little activity: "acid esterase" (Amano), "acylase" (Amano ACS), lipase from *Aspergillus niger* (Amano A), esterase from *Bacillus stearothermophilus*,[13b] as well as Chirazyme L7 and L8. [b] Configuration of faster reacting enantiomer. [c] *R* selectivity according to MTP assay; *S* selectivity according to GC analysis; note that substrate and product were almost racemic.

previous findings,[8] only CAL-B was found suitable for the efficient resolution of **3**.

As shown in Figure 3, an analysis time of less than 3 – 4 min per assay (plus 1 – 2 min for the pipetting steps) is sufficient to determine enantioselectivities. Thus, in a 96-well plate about 45 enzymes (two wells per biocatalyst, six wells for controls) can be analyzed. This amounts to at least 540 E_{app} values per hour corresponding to about 13000 mutants screened per day.[18] In addition, the assay is rather cheap, as one test reaction costs less than 0.1 € (catalogue price).

Moreover, the method should not be restricted to the measurement of esterase or lipase activity and enantioselectivity. Several other enzyme reactions, which proceed by releasing acetic acid, such as the protease- or amidase-catalyzed hydrolyses of amides should be accessible using the principle described here.

Experimental Section

Materials: Racemic acetates (*R*,*S*)-**1** – (*R*,*S*)-**3** were obtained from commercial suppliers at the highest purity available. Acetates (*R*,*S*)-**4** and -**5** and optically pure (*R*)- and (*S*)-**3** were synthesized from the corresponding alcohols by using standard procedures. Optically pure (*R*)- and (*S*)-**1** were provided by the BASF AG (Ludwigshafen, Germany). All enzymes—except PFE and BSE, which were produced by expression in *E. coli* as described in reference [13]—were obtained from commercial suppliers.

The test-kit for the determination of acetic acid released (initally produced and distributed by Roche Diagnostics, Penzberg, Germany) was purchased from R-Biopharm GmbH (Darmstadt, Germany) and applied in accord with the manufacturers protocol (see also below). Spectrophotometric determination of NADH concentration was performed at 340 nm in the mL-scale on a Ultrospec 3000 photometer (Pharmacia Biotech Ltd., Uppsala, Sweden) and in the µL-scale using a fluorimeter (FLUOstar, BMG LabTechnologies, Offenburg, Germany).

Conversion of racemic substrates for the determination of E_{true} values: For this, enzyme-catalyzed hydrolyses of (*R*,*S*)-**1** and (*R*,*S*)-**3** were performed as described in reference [8]. Conversion and enantiomeric excess (% *ee*) were then determined by GC analysis of samples from the reaction mixture. Enantioselectivities (E_{true} values) were calculated from these according to the method developed by Chen et al.[3]

General procedure for the determination of acetic acid in a MTP assay: A solution of PFE (20 µL, 2 mg mL^{-1}, unless stated otherwise) or CAL-B (2 mg mL^{-1}) was added to a mixture of the test-kit components (150 µL). The reactions were started by adding a solution of **1** – **5** (20 µL, concentrations are indicated in the text and in figure legends as mg mL^{-1}) in sodium phosphate buffer (10 mM, pH 7.3). Mixtures of the test-kit with buffer or cell lysates of noninduced *E. coli* harboring the gene encoding recombinant PFE served as controls. In a similar manner, reaction rates were determined by using optically pure (*R*)- and (*S*)-**1** as well as (*R*)- and (*S*)-**3**. For reactions with crude cell extract, PFE was produced in microtiter plates similar to the published protocol for shake-flask cultivation.[13a] However, the cultivation volume was 200 µL per well and cells were disrupted by two freeze-thaw cycles. Finally, cell debris was removed by centrifugation and the supernatants were used for the assay.

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[17] E_{app} values are the ratio of initial rates determined from the hydrolysis of pure *R* or *S* enantiomers. E_{true} values are derived from resolutions of racemates and therefore also include competition between the two enantiomers for the active site of the enzyme.
[18] More than 50000 mutants can be screened per day, if the analysis time can be reduced to about 1 min per test and faster pipetting steps are possible.

Pore-Size Engineering of Silicon Imido Nitride for Catalytic Applications**

David Farrusseng, Klaus Schlichte, Bernd Spliethoff, Annette Wingen, Stefan Kaskel,* John S. Bradley, and Ferdi Schüth

Microporous and mesoporous materials with high internal surface area and pore volume play a key role in the development of new heterogeneous catalysts and solid membranes.[1] The strategy in the synthesis of materials with well defined pore structure is to direct the networking of molecular or ionic precursors by a templating agent, which can be a molecule, an ion, a polymer, or a supramolecular assembly.[2] Removal of the occluded template produces materials with patterns of characteristic pore size, shape, and structure. Although there is a growing interest in expanding these templating routes to materials other than oxides, only in the case of sulfides and some super-Prussian-blue compounds have micro- and mesoporous inorganic nonoxide materials been synthesized.[3] Nitrido-sodalites with very small pores, probably inaccessible to organic substrates, were obtained from the solid-state reaction of HPN_2 and divalent metal salts.[4]

Several methods for the synthesis of dense silicon nitride starting from elemental silicon, silicon chloride, or even carbodiimide have been reported.[5] However, the first microporous silicon imido nitrides with a mean pore size of 0.7 nm were synthesized by Bradley and Dismukes in pyrolysis reactions of polysilazanes.[6] Mesoporous silicon imido nitride with a narrow pore-size distribution ($d_{av} = 5.6$ nm) and a high specific surface area (up to 1000 $m^2 g^{-1}$) was obtained recently using the ammonolysis of silicon tetrachloride in organic solvents.[7]

We describe herein a template-assisted method which allows, for the first time, tailoring the pore size of microporous silicon nitride materials in a wide range from primary to secondary micropores. In this procedure, tris(dimethylamino)silylamine $[(CH_3)_2N]_3SiNH_2$ **1**[8] is ammonolyzed in a concentrated solution of $CH_3(CH_2)_nNH_2$ ($n = 11 – 17$) in hot acetonitrile. The ammonolysis can be considered as an analogue of silicon oxide manufacture by sol – gel methods using prehydrolyzed tetramethoxysilane and water. After cooling to room temperature, a gel forms which is dried and heated slowly to 823 K in flowing ammonia. According to nitrogen physisorption experiments (Figure 1), such materials prepared using alkylamines of different chain length

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