Via overexpression in the yeast *Pichia pastoris* to enhanced enantioselectivity: A new chapter in the application of pig liver esterase**

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Lipases and Esterases can be used as efficient biocatalysts for the preparation of a wide variety of optically pure compounds.[1] Whereas a range of lipases - especially of microbial origin - are commercially available, only a few esterases can be obtained for the kinetic resolution of racemates or desymmetrization. In the majority of publications, pig liver esterase[2] (PLE) is used, which is isolated from pig liver by extraction. Although it could be demonstrated, that this preparation can convert a broad range of compounds at partially very high stereoselectivity, its application is encountered with a number of disadvantages. Beside a variation of the esterase content between different charges, especially the presence of other hydrolases has to be considered as problematic

with respect to stereoselectivity.[3] Furthermore, it could be shown, that PLE consists of several isoenzymes,[4] which in part differ considerably in their substrate specificity. Thus, electrophoretic separation by isoelectric focusing enabled access to PLE fractions, which beside other substances preferentially converted butyrylcholine (comparable to butryrylcholine esterase) and prolin-β-naphthylamide.[5a-b] Öhrner and coworkers[5c] did not only found an influence of the chain-length of *p*-nitrophenyl esters on activity of different PLE fractions, but also a significant change in enantioselectivity in the hydrolysis of prostereogenic substrates. In contrast, Jones and coworkers reported that different isoenzymes show almost no differences in the stereoselective hydrolysis of several monocyclic and acyclic diesters. Only differences in activity were reported.[6]

The production of enzymes at stable and defined composition can be achieved by overexpression of the encoding genes in suitable host organisms such as *Escherichia coli*, *Pichia pastoris* or *Aspergillus oryzae*. Indeed, several esterases[7] and lipases[8] are thus produced. The cloning of putative pig liver esterase genes was already described: Takahashi and coworkers[9a-b] cloned a gene isolated from pig liver, which encoded for a prolin-β-naphthylamidase. Later, the same[10] and Heymanns[11] group demonstrated, that with high probability PLE was cloned. The same holds true for a glycerolester hydrolase[12], which distinguishes only by 16 amino acids from the protein sequence published by Takahashi. However, the functional expression of active enzyme was not reported by any research group.

We now succeeded for the first time in the overexpression of active PLE in the yeast *Pichia pastoris*.[13] Thus, we can now produce recombinant PLE (rPLE) at stable product quality without interfering influences of other isoenzymes and hydrolases. The rPLE shows almost identical pH- and temperatur profiles[13] and a similar substrate spectra in

the hydrolysis of simple (achiral) esters and triacylglycerides compared to non-recombinant PLE (Figure 1). It has to be emphasized, that only rPLE does not cleave triolein, which is a typical lipase substrate. This exemplifies the impurity of commercial preparations containing other hydrolases. Beside a biochemical characterisation, we were especially interested in the stereoselectivity of rPLE in the conversion of chiral substrates in comparison to commercial preparations, as this represents by far the most important area of application for PLE.

Whereas in the PLE-catalyzed kinetic resolution of (*R*,*S*)-1-phenyl-1-ethyl acetate **1** in all cases similar enantioselectivities[15] between E=5.7 and 7.9 were determined (Table 1), significantly higher enantioselectivities were found in the hydrolysis of (*R*,*S*)-1-phenyl-2-propyl acetate **2** (Table 2) or (*R*,*S*)-1-phenyl-2-butyl acetate **3** (Table 3) in comparison to reactions using PLE preparations from Fluka, Sigma or Roche (Chirazyme E-1 or E2). In the hydrolysis of **3**, these preparations showed only very low enantioselectivities (E=1.4-4.0), whereas with recombinant PLE E-values >> 100 were determined (Table 3). For **2**, this increase of enantioselectivitiy was less pronounced, but here we observed an inversion of enantiopreference (Table 2). With commercial esterases preferentially the (*R*)-alcohol was formed, instead with rPLE the (*S*)-alcohol was produced. These results can be explained by the fact, that we cloned the γ-subunit of PLE,[13] but commercial preparations are a mixture of subunits, which distinguish considerably in sequence and molecular weight.[5] This of course can also effect substrate- and enantioselectivity.

Thus, the application of recombinant PLE now also allows the synthesis of compounds at high optical purity, which have so far not been accessible by PLE-catalyzed reactions.

Furthermore, the successful functional expression now makes the manipulation of enzyme properties by site-directed mutagenesis or directed evolution[16] feasible.

Experimental

Production of recombinant pig liver esterase (rPLE)

For expression of rPLE, the yeast *Pichia pastoris* - bearing the genomic-integrated gene encoding the esterase under the control of the methanol-inducible alcohol oxidase 1 promotor (AOX1) - was grown according to the manufacturers (Invitrogen, Carlsbad, CA, USA) protocol first in glycerol-containing (1 % (v/v)) BMGY media and then in BMMY media, containing 0.5 % (v/v) methanol as carbon source and inducer. Induction of rPLE expression was maintained by daily addition of 0.5 % (v/v) methanol. After 96 h, the cells were removed by centrifugation and the supernatant was concentrated for 15 min at 4000 g using 20 ml Centricons (NMWL 30 000, Ultracel-PL Membran, Millipore). The activity thus obtained was 10 U/ml (pNPA-assay) corresponding to ~500 U/mg protein.

Determination of esterase activity

Photometric determination of esterase activity was performed by hydrolysis of *p*-nitrophenyl acetate (pNPA).[7d] One Unit (U) esterase corresponds to the amount of enzyme releasing 1 µmol *p*-nitrophenol per min.
The substrate spectra of PLE was measured using a pH-Stat (Schott, Mainz, Germany) by hydrolysis of different esters (caprylic acid ethylester, acetic acid ethylester, tributyrin, triolein) at 37°C and pH 7.5.[7d] One Unit (U) esterase corresponds to the amount of enzyme releasing 1 µmol acid per min. The thus determined highest activity was set as 100%-value.

Esterase-catalyzed kinetic resolution of the acetates

For this, 10 mM of acetates **1**-**3** was dissolved in sodium phosphate buffer (pH 7.5, 50 mM) in 1 ml reaction vials and the kinetic resolution was started by addition of 0.5 U

(based on the pNPA-assay) esterase. To stop the reaction, the mixture was extracted with methylen chloride and the organic phase was dried over anhydrous sodium sulfate. The determination of enantiomeric purity and conversion was performed by gaschromatography (column: Heptakis(2,6-*O*-methyl-3-*O*-pentyl)-β-cyclodextrine, carrier gas: H_2, flame ionisation detector).
Retention times: **1** (100°C isothermal): (*S*)-**1** 3.7 min; (*R*)-**1**, 5.8 min; (*R*)-**1a** 6.7 min; (*S*)-**1a**, 7.6 min; **2** (75°C isothermal): (*S*)-**2** 26.5 min; (*R*)-**2**, 42.3 min; (*S*)-**2a** 32.6 min; (*R*)-**2a**, 34.2 min. **3** (90°C isothermal): (*S*)-**3** 17.6 min; (*R*)-**3**, 20.2 min; (*S*)-**3a** 24.8 min; (*R*)-**3a**, 27.4 min. The absolute configuration was based for **1** on comparison with commercial (*R*)-**1a**. In case of **2** and **3** the literature-known[17] (*R*)-preference of the lipase Amano PS served as reference.

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For cloning and expression of rPLE the following strategy (in brief) was used: We isolated the mRNA from fresh pig liver and transcribed this into the corresponding cDNA using RT-PCR. Purification and blotting of a commercial PLE preparation allowed determination of the *N*-terminal protein sequence. This data was identical to the sequence published by Takahashi et al.[9] in the data base SWISS-PROT. The complete gene was then cloned from the mRNA based on this sequence. Only after removal of a leader sequence and a retention signal, rPLE could be functionally expressed in the yeast *Pichia pastoris.*

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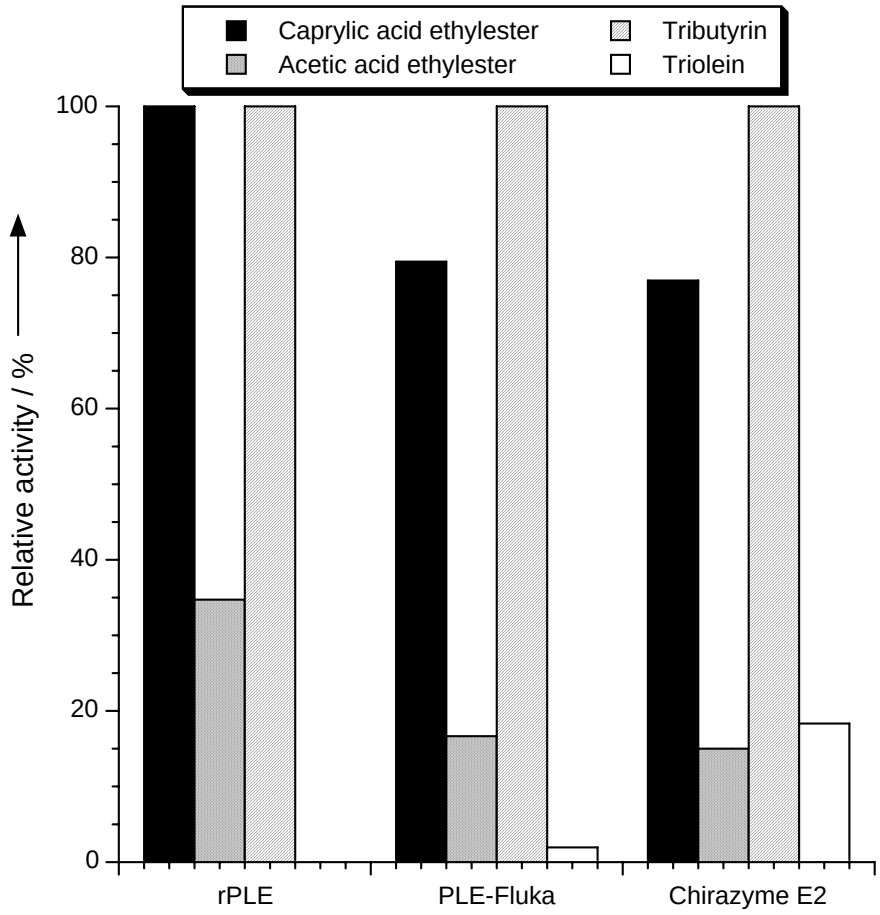
FIGURE CAPTION

Figure 1: Comparison of the hydrolytic activity of recombinant (rPLE) with commercial preparations from Fluka (PLE Fluka) and Roche Diagnostics (Chirazyme E-2) towards different achiral esters.

Figure 1, Musidlowska et al.



TABLES

Table 1: Enantioselectivity of different pig liver esterases in the kinetic resolution of (*R*,*S*)-1-phenyl-1-ethyl acetate **1**.

PLE[a]	**Time** [h]	**Enantiomeric excess** [%ee_S][c]	[%ee_P][c]	**Conversion** [%]	**E**[b]
Recombinant	1	58	53	53	5.7
Fluka	1.5	65	56	54	6.8
Sigma	1	72	58	55	7.8
Chirazyme E-1	5	73	58	56	7.9

[a] in all reactions 0.5 Units (based on pNPA-assay) were used.

[b] enantioselectivity E was calculated according to Chen et al. (1982).[15]

[c] in all cases the product alcohol **1a** had (*R*)-configuration and the non-converted acetate **1** (*S*)-configuration.

Table 2: Enantioselectivity of different pig liver esterases in the kinetic resolution of (*R*,*S*)-1-phenyl-2-propyl acetate **2**.

PLE[a]	**Time** [h]	**Enantiomeric excess** [%ee_S][c]	[%ee_P][c]	**Conversion** [%]	**E**[b]
Recombinant	2	75 (*R*)	70 (*S*)	52	12.6
Fluka	1.5	35 (*S*)	44 (*R*)	44	3.6
Sigma	1.5	24 (*S*)	32 (*R*)	43	2.4
Chirazyme E-1	1.5	22 (*S*)	43 (*R*)	34	3.1
Chirazyme E-2	1	9 (*S*)	9 (*R*)	50	1.3

[a], [b]see Table 1.

Table 3: Enantioselectivity of different pig liver esterases in the kinetic resolution of (*R*,*S*)-1-phenyl-2-butyl acetate **3**.

PLE[a]	**Time** [h]	**Enantiomeric excess** [%ee_S][c]	[%ee_P][c]	**Conversion** [%]	**E**[b]
Recombinant	2	57	>99	36	>>100
Fluka	2	12	12	49	1.4
Sigma	1	17	11	59	1.5
Chirazyme E-1	2	19	18	52	1.7
Chirazyme E-2	1	58	40	59	4.0

[a], [b]see Table 1.

[c]in all cases the product alcohol **3a** had (*S*)-configuration and the non-converted acetate **3** (*R*)-configuration.

Keywords: Enantiomeric resolution, enzyme catalysis, gene technology, hydrolases, pig liver esterase

Graphical Abstract

The application of recombinant pig liver esterase (rPLE) – for which the functional expression was now achieved for the first time – leads to substantially higher enantioselectivity in the hydrolysis of 1-phenyl-2-butyl acetate compared to commercial PLE preparations.