$[Zn_4(L^2)_4](PF_6)_8$ (**2**) was prepared similarly, but the reaction mixture needed to be refluxed and the subsequent PF_6^- salt recrystallized from $MeCN/Et_2O$. The yields in both cases exceeded 95%. **ATTENTION**: The intermediate perchlorate salts of the complexes are explosive.

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By Overexpression in the Yeast *Pichia pastoris* to Enhanced Enantioselectivity: New Aspects in the Application of Pig Liver Esterase**

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Dedicated to Professor Günter Schmidt-Kastner on the occasion of his 75th birthday

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 has been demonstrated that this preparation can convert a broad range of compounds at partially very high stereoselectivity, its application is connected with a number of disadvantages. Besides a variation of the esterase content between different batches, the presence of other hydrolases particularly has to be considered as problematic with respect to stereoselectivity.[3] Furthermore, it has been 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 that,

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besides other substances, preferentially converted butyrylcholine (an action comparable to butryrylcholine esterase), proline-β-naphthylamide, and methylbutyrate.[5a,b] Öhrner and co-workers[5c] not only found an influence by the chainlength of *p*-nitrophenyl esters on the activity of different PLE fractions, but also a significant change in enantioselectivity in the hydrolysis of prostereogenic substrates. In contrast, Jones and co-workers 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 has already been described: Takahashi and co-workers[9a,b] cloned a gene, isolated from pig liver, which encoded for a proline-β-naphthylamidase. Later, the same group[10] and Heymann's group[11] demonstrated that, with high probability, it was actually PLE that was cloned. The same holds true for a glycerolester hydrolase,[12] which is distinguished only by 16 amino acids from the protein sequence published by Takahashi and co-workers. However, the functional expression of active enzymes was not reported by any of the research groups.

We have 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 temperature profiles[13] and a similar substrate spectrum in the hydrolysis of simple (achiral) esters and triacylglycerides compared to nonrecombinant 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 characterization, we were especially interested in the stereoselectivity of rPLE in the conversion of chiral substrates in comparison to commercial preparations,[14] as this represents by far the most important area of application for PLE.

Whereas in the PLE-catalyzed kinetic resolution of (*R*,*S*)-(1-phenylethyl)acetate (**1**) similar enantioselectivities[15] between $E = 5.7$ and 7.9 were determined in all cases (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) with the recombinant PLE in comparison to reactions with PLE preparations from Fluka, Sigma, or Roche Diagnostics (Chirazyme E1 or E2). In the hydrolysis of **3**, these preparations showed only very low enantioselectivities ($E = 1.4 - 4.0$), whereas *E* values much greater than 100 were determined with recombinant PLE (Table 3). For **2**, this increase of enantioselectivitiy was less pronounced, but here we observed an inversion of enantiopreference (Table 2). With commercial esterases the *R* alcohol was preferentially formed, whereas 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

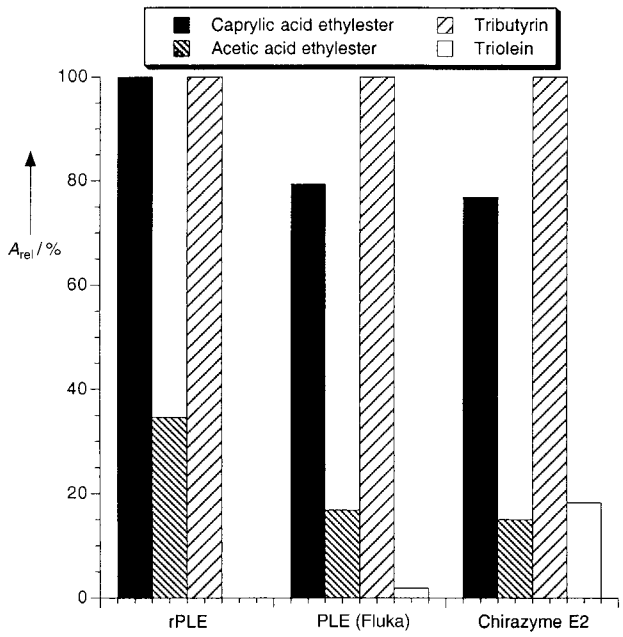

Figure 1. Comparison of the hydrolytic activity (A_{rel}) of recombinant PLE (rPLE) with commercial preparations from Fluka and Roche Diagnostics (Chirazyme E2) towards different achiral esters.

Table 1. Enantioselectivity of different pig liver esterases in the kinetic resolution of **1**.

PLE[a]	*t* [h]	Enantiomeric excess (*ee*)		Conversion [%]	*E*[b]
		ee_S [%][c]	ee_P [%][c]		
recombinant	1	58	53	53	5.7
Fluka	1.5	65	56	54	6.8
Sigma	1	72	58	55	7.8
Chirazyme E1	5	73	58	56	7.9

[a] In all reactions 0.5 U of esterase (based on pNPA-assay) were used. [b] The enantioselectivity *E* was calculated according to the method of Chen et al. (1982).[15] [c] ee_S = enantiomeric excess of the nonconverted substrate, ee_P = enantiomeric excess of the product. In all cases the product alcohol **1a** had *R* configuration and the nonconverted acetate **1** had *S* configuration.

Table 2. Enantioselectivity of different pig liver esterases in the kinetic resolution of **2**.

PLE[a]	*t* [h]	Enantiomeric excess		Conversion [%]	*E*[b]
		ee_S [%]	ee_P [%]		
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 E1	1.5	22 (*S*)	43 (*R*)	34	3.1
Chirazyme E2	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 **3**.

PLE[a]	t [h]	Enantiomeric excess		Conversion [%]	*E*[b]
		ee_S [%][c]	ee_P [%][c]		
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 nonconverted acetate **3** had *R* configuration.

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preparations are a mixture of subunits, which differ considerably in sequence and molecular weight.[5] This, of course, can also effect substrate selectivity and enantioselectivity.

Thus, the application of recombinant PLE now also allows the synthesis, in high optical purity, of compounds 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 Section

Production of recombinant pig liver esterase (rPLE): For expression of rPLE, the yeast *Pichia pastoris*—bearing the genomic-integrated gene that encodes the esterase under the control of the methanol-inducible alcohol oxidase 1 promotor (AOX1)—was grown according to the protocol given by the manufacturer (Invitrogen, Carlsbad, CA, USA), first in BMGY media containing glycerol (1% (v/v)) and then in BMMY media that contained methanol (0.5% (v/v)) as the carbon source and inducer. Induction of rPLE expression was maintained by daily addition of methanol (0.5% (v/v)). After 96 h, the cells were removed by centrifugation and the supernatant was concentrated for 15 min at 4000 g with 20 mL Centricons (NMWL 30000, Ultracel-PL membrane, Millipore). The activity thus obtained was 10 U mL^{-1} (pNPA-assay), which corresponds to $\sim$500 U mg^{-1} protein.

Determination of esterase activity: Photometric determination of esterase activity was performed by hydrolysis of *p*-nitrophenyl acetate (pNPA).[7d] One unit (U) of esterase corresponds to the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min.

The substrate spectrum of PLE was measured with a pH-Stat (Schott, Mainz, Germany) by hydrolysis of different esters (caprylic acid ethylester, acetic acid ethylester, tributyrin, or triolein) at 37 °C and pH 7.5.[7d] One unit (U) of esterase corresponds to the amount of enzyme that releases 1 μmol of acid per min. The thus-determined highest activity was set as the 100% value.

Esterase-catalyzed kinetic resolution of the acetates: Acetates **1**–**3** (10 mM) were dissolved in sodium phosphate buffer (pH 7.5, 50 mM) in 1-mL reaction vials and the kinetic resolution was started by addition of esterase (0.5 U, based on the pNPA assay). To stop the reaction, the mixture was extracted with dichloromethane and the organic phase was dried over anhydrous sodium sulfate. The determination of enantiomeric purity and conversion was performed by gas chromatography (column: heptakis(2,6-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin; carrier gas: H_2; flame ionization detector).

Retention times [min]: **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 configurations given for **1** are based on comparison with commercial (*R*)-**1a**. In case of **2** and **3** the *R* preference of the lipase Amano PS, as recorded in the literature,[17] served as the reference.

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Hydrolysis/Polycondensation in the Solid State: Access to Crystalline Silica-Based Hybrid Materials**

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Silicon-based hybrid materials allow the association at the molecular level of the properties of an organic group with the specific properties of a Si–O–Si network (transparency, chemical and thermal stability).[1–6] They are generally prepared by hydrolytic polycondensation from organic units covalently bound to trialkoxysilyl groups ($Si(OR')_3$, R′ = Me

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