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Substrate specificity of the γ-isoenzyme of recombinant pig liver esterase towards acetates of secondary alcohols

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Abstract

The γ-isoenzyme of pig liver esterase (rPLE) was produced recombinantly by expression in *Pichia pastoris*. A comparison of rPLE with commercial preparations of crude PLE revealed significant differences in the kinetic resolution of a series of acetates of secondary alcohols. With rPLE substantially higher enantioselectivities were observed in the hydrolysis of (*R*,*S*)-1-phenyl-3-butyl acetate, (*R*,*S*)-1-phenyl-2-propyl acetate, (*R*,*S*)-1-phenyl-2-pentyl acetate, and (*R*,*S*)-1-phenyl-2-butyl acetate. For the first two compounds, also an inversed stereopreference was found. This change in substrate specificity can be related to varying contents of the γ-isoenzyme in commercial PLE preparations and the presence of further isoenzymes with different properties.

Keywords: Kinetic resolution; Enzyme catalysis; Enantioselectivity; Hydrolases; Pig liver esterase

1. Introduction

Lipases and esterases can be used as efficient biocatalysts for the preparation of a wide variety of optically pure compounds [1,2]. In the group of carboxyl esterases, pig liver esterase (PLE) represents by far the most important enzyme and numerous examples for its efficient application in kinetic resolutions and de-symmetrizations can be found [3,4]. PLE is obtained by simple treatment of homogenized pig liver tissue with an organic solvent. The resulting crude powder can contain beside PLE also various other hydrolytic enzymes. Moreover, it could be shown that PLE consists of various isoenzymes, which can differ considerably in their substrate specificity and also stereoselectivity [5,6]. The most dominant fractions amenable by isoelectric focusing are the so-called α- and γ-isoenzymes [7,8]. The α-isoenzyme preferentially hydrolyzes methylbutyrate, whereas, the γ-isoenzyme exhibits specificity for proline-β-naphthylamide [9]. Öhrner et al. [6] did not only find an influence of the chain-length 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 coworkers reported that different isoenzymes show almost no differences in the stereoselective hydrolysis of several monocyclic and acyclic diesters [10].

We have recently achieved the functional expression of PLE in the yeast *Pichia pastoris* [11].

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Biochemical studies confirmed that the γ-isoenzyme of PLE was cloned and expressed, as the recombinant esterase shows high specificity towards proline-β-naphthylamide, a typical substrate of this isoenzyme (often also named γ-subunit). Key to success was the removal of an 18 amino acid N-terminal sequence and of a four amino acid C-terminal putative endoplasmic reticulum retention signal. Thus, we can now produce recombinant PLE (rPLE) without interfering influences of other isoenzymes and hydrolases. The rPLE shows almost identical pH- and temperature profiles and a similar substrate specificity in the hydrolysis of simple (achiral) esters and triglycerides compared to non-recombinant PLE [11].

Preliminary results revealed that rPLE shows striking differences in its stereoselectivity compared to PLE from Fluka, Sigma or Roche (Chirazyme E1 and E2) in the resolution of the acetates of some secondary alcohols [12]. In the present paper, we report on the hydrolysis of further substrates of this type and attempt to rationalize the results.

2. Experimental

2.1. General methods

^{1}H NMR spectra were recorded at 300 MHz, ^{13}C NMR spectra at 75 MHz, in $CDCl_3$ with tetramethylsilane as internal standard. All chemicals and acetate **1** were purchased at the highest purity available from Fluka. Acetates (**2**–**4**, **6**) were synthesized from commercially available alcohols (**2a**–**4a**, **6a**) using standard procedures as described below. Gas chromatographic analyses were conducted using a heptakis-(2,6-*O*-methyl-3-*O*-pentyl)-ß-cyclodextrin (25 m × 0.25 mm, Prof. W. A. König, University of Hamburg, Germany), 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** (80 °C isothermal): (*S*)-**2** 13.9 min; (*R*)-**2**, 20.9 min; (*S*)-**2a** 51.5 min; (*R*)-**2a** 44.6 min; **3** (100 °C isothermal): (*S*)-**3** 15.6 min; (*R*)-**3**, 28.7 min; (*S*)-**3a** 19.1 min; (*R*)-**3a**, 20.5 min; **4** (75 °C isothermal): (*S*)-**4** 26.5 min; (*R*)-**4**, 42.3 min; (*S*)-**4a** 32.6 min; (*R*)-**4a**, 34.2 min; **5** (90 °C, 30′; 5 °C, min; 110 °C): (*S*)-**5**: 29.1 min and (*R*)-**5** 30.4 min; (*S*)-**5a** 37.3 min; and (*R*)-**5a** 38.3 min; **6** (90 °C isothermal): (*S*)-**6** 17.6 min; (*R*)-**6**, 20.2 min; (*S*)-**6a** 24.8 min; (*R*)-**6a**, 27.4 min. The absolute configuration was based for **1** on comparison with commercial (*R*)-**1a**. In the case of **2** and **4**, the literature-known [13] (*R*)-preference of lipase from *Burkholderia cepacia* (Amano PS), for **3**, the literature-known [14] (*R*)-preference of lipase from *Pseudomonas* sp. (Amano AK), for **6**, (*R*)-preference of the lipoprotein P (LPL 1) [15] served as reference. For **5**, the absolute configuration was assigned from the sense of optical rotation based on literature values [16]. Commercial PLE preparations from Fluka (390 U/mg, based on *p*NPA-assay), Sigma (176 U/mg) and Roche (Chirazyme E1 (155 U/mg), E2 (170 U/mg)) served as controls. Recombinant PLE (500 U/mg) was produced by methanol induction in the yeast *Pichia pastoris*as described [11].

2.2. Synthesis of (R,S)-1-phenyl-2-pentanol (*5a*)

Phenylacetaldehyde (10.6 g, 90 mmol) was dissolved in 50 ml diethyl ether and added drop wise to a solution of propylmagnesium chloride (10.3 g, 50 ml, 2 M in diethyl ether). The reaction mixture was stirred under heating for 2 h and then cooled. Next, ice and half concentrated HCl was added until the precipitate dissolved. The organic layer was separated and the aqueous layer was extracted twice with diethyl ether. The ether layers were combined and washed with saturated Na_2SO_3, $NaHCO_3$ and a small amount of water. After drying over anhydrous Na_2SO_4 and evaporation of excess solvent, the product was isolated by low pressure distillation (the fraction containing the product was collected at 750 Torr, 162 °C) giving 6.9 g (42 mmol, 48%) **5a**. ^{1}H NMR (300 MHz; $CDCl_3$) δ 0.93 (t, J = 6.9, 3H, $-CH_3$), 1.42–1.51 (m, 4H, $-CH_2-CH_2-$), 1.69 (s, 1H, –OH), 2.59–2.83 (m, 2H, Ph–$C\underline{H}_2$–H_2–), 3.79–3.83 (m, 1H, –$C\underline{H}_2$–H–OH), 7.18–7.32 (m, 5H, –Ph); ^{13}C NMR (75 MHz; $CDCl_3$) δ 14.01, 18.88, 38.88, 44.01, 72.34, 76.58, 77.43, 126.33, 128.11, 128.45, 129.38, 138.64.

2.3. General procedure for the preparation of acetates (*2*–*6*)

An amount of 40 mmol acetyl chloride were added to 20 ml pyridine at 4 °C and the mixture was stirred thoroughly. An amount of 40 mmol alcohol **2a**–**6a** was added drop wise and the mixture was stirred for 20 h

at RT. The mixture was extracted twice with ether, twice with saturated sodium hydrogen carbonate solution and the combined organic layers were dried over anhydrous Na_2SO_4 followed by evaporation of excess solvent. The acetates were purified by silica gel chromatography (hexane:ethyl acetate = 5:1 (**2**–**4**, **6**) or 3:1 (**5**)). Chemical structures were confirmed by NMR spectroscopy and elementary analyses.

(R,S)-1-phenyl-1-propyl acetate (**2**): following the general procedure, 5.4 g of (*R,S*)-**2a** (40 mmol) yielded 3.1 g of (*R,S*)-**2** (17.4 mmol, 43%).

(R,S)-1-phenyl-3-butyl acetate (**3**): 0.5 g of (*R,S*)-**3a** (3.3 mmol) yielded 0.56 g of (*R,S*)-**3** (2.9 mmol, 87%).

(R,S)-1-phenyl-2-propyl acetate (**4**): 5.4 g of (*R,S*)-**4a** (40 mmol) yielded 6.5 g of (*R,S*)-**4** (36.5 mmol, 91%).

(R,S)-1-phenyl-2-pentyl acetate (**5**): 3.28 g of (*R,S*)-**5a** (20 mmol) yielded 1.02 g of (*R,S*)-**5** (4.9 mmol, 25%).

(R,S)-1-phenyl-2-butyl acetate (**6**): 0.5 g of (*R,S*)-**6a** (3.3 mmol) yielded 0.48 g of (*R,S*)-**6** (2.5 mmol, 74%).

2.4. Esterase-catalyzed kinetic resolution of the acetates

Acetates **1**–**6** were dissolved in sodium phosphate buffer (pH 7.5, 50 mM) giving 1 ml of a 10 mM solution. The hydrolysis was carried out in 1.5 ml reaction vials in a thermomixer (Eppendorf) at 37 °C. For each reaction, 0.5 U pig liver esterase (based on a spectrophotometric *p*-nitrophenyl acetate (*p*NPA) assay) was used. Reactions were terminated by extraction with methylene chloride and the organic phases were dried over anhydrous sodium sulfate. The determination of enantiomeric purity and conversion was performed by gas chromatography. Enantioselectivity *E* was calculated according to Chen et al.[17].

3. Results and discussion

Kinetic resolutions of six representative acetates of secondary alcohols **1**–**6** by hydrolysis in phosphate buffer using recombinant PLE and several crude commercial preparations were studied. As already found for substrates **1**, **4** and **6** [12], again enantioselectivity *E* and stereopreference were markedly influenced by the chain-length and the position of the hydroxyl function. In the case of 1-phenyl-1-propyl-acetate **2**, similar stereopreference and low enantioselectivities were found with all PLE preparations used (Table 1). In contrast, the (*S*)-alcohol was formed when rPLE catalyzed the hydrolysis of acetate **3** and *E* increased modestly (from 1.1 to 3.2 using commercial preparations) to $E = 6.3$ with recombinant PLE (Table 2). Interestingly, with Chirazyme E2 a similar reversal in stereopreference as for rPLE was observed. With acetate **5** all PLEs showed (*S*)-preference and acceptable *E*-values can only be achieved with recombinant PLE ($E = 16.7$) whilst all crude esterases gave unsatisfactory optical purities ($E = 1.3$–2.1) (Table 3).

Fig. 1 summarizes results in PLE-catalyzed hydrolyses of acetates **1**–**6** arranged by increasing preference for the (*S*)-enantiomer. For simplicity, only PLE

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

PLE[a]	Time (h)	Enantiomeric excess		Conversio (%)	E[b]
		(%ee$_S$)[c]	(%ee$_P$)[c]		
Recombinant	4	13	20	40	1.7
Fluka	1	21	28	43	2.2
Sigma	0.5	17	19	48	1.7
Chirazyme E1	0.5	9	13	41	1.4
Chirazyme E2	0.5	18	27	40	2.1

[a] In all the reactions, 0.5 U (based on *p*NPA-assay) were used.
[b] Enantioselectivity *E* was calculated according to Chen et al. [17].
[c] In all the cases, the product alcohol **2a** had (*R*)-configuration and the non-converted acetate **2** (*S*)-configuration.

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

PLE[a]	Time (h)	Enantiomeric excess		Conversion (%)	E[b]
		(%ee$_S$)	(%ee$_P$)		
Recombinant	2	52 (*R*)	59 (*S*)	47	6.3
Fluka	0.5	31 (*S*)	42 (*R*)	42	3.2
Sigma	0.25	22 (*S*)	25 (*R*)	47	2.1
Chirazyme E1	0.5	25 (*S*)	29 (*R*)	47	2.3
Chirazyme E2	0.25	1 (*R*)	2 (*S*)	43	1.1

[a] In all the reactions, 0.5 U (based on *p*NPA-assay) were used.
[b] Enantioselectivity *E* was calculated according to Chen et al. [17].

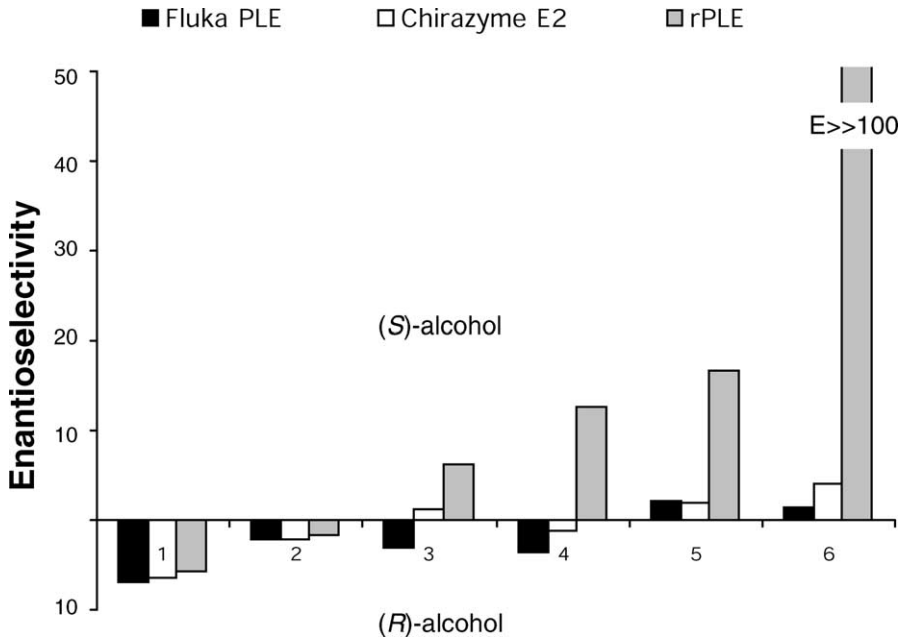


Fig. 1. Schematic presentation of enantioselectivity E and stereopreference of recombinant PLE (γ-isoenzyme) and commercial PLE preparations from Fluka (Fluka, PLE) and Roche (Chirazyme E2).

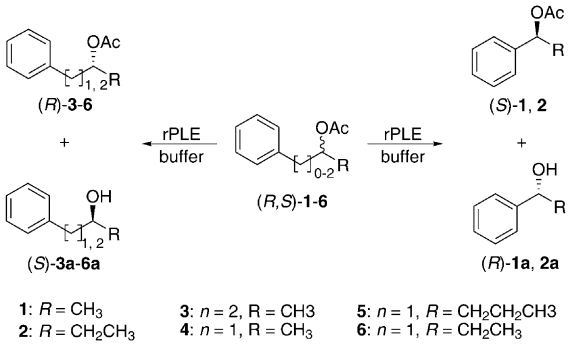
preparations from Fluka and Roche (Chirazyme E2) are compared with rPLE as here the largest differences in stereoselectivity among the commercial preparations were found. Linking this change in stereopreference with the position of the hydroxyl group shows a comparable pattern: at $n = 0$, (*R*)-preference and similar enantioselectivities for both acetates **1**–**2** independent of the PLE preparation occurred; at $n \geq 1$, only recombinant PLE preferentially hydrolyzes the (*S*)-enantiomer whilst commercial PLEs neither show a clear stereopreference nor satisfactory enantioselectivities (Scheme 1; Fig. 1).

The significant changes in substrate specificities and enantioselectivities of recombinant PLE in comparison to various crude commercial PLE preparations can be attributed to the sole presence of the γ-isoenzyme in the recombinant esterase and varying concentrations of it in the commercial ones [11].

Table 3
Enantioselectivities of different pig liver esterases in the kinetic resolution of (*R*,*S*)-1-phenyl-2-pentyl acetate **5**

PLE[a]	Time (h)	Enantiomeric excess		Conversion (%)	E[b]
		(%ee$_S$)[c]	(%ee$_P$)[c]		
Recombinant	2	69	78	47	16.7
Fluka	0.3	24	26	48	2.1
Sigma	0.5	15	13	52	1.5
Chirazyme E1	0.5	9	11	46	1.3
Chirazyme E2	0.3	21	24	46	2.0

[a] In all the reactions, 0.5 U (based on *p*NPA-assay) were used.
[b] Enantioselectivity E was calculated according to Chen et al. [17].
[c] In all the cases, the product alcohol **5a** had (*S*)-configuration and the non-converted acetate **5** (*R*)-configuration.



Scheme 1. Stereopreference of the recombinant PLE γ-isoenzyme in the kinetic resolution of acetates **1**–**6**.

This was confirmed by determination of the activity towards proline-β-naphthylamide—the specific substrate of the γ-isoenzyme—and methyl butyrate, which was reported to be cleaved preferentially by the α-isoenzyme. According to our experiments, PLE from Fluka showed lowest activity towards proline-β-naphthylamide and Chirazyme E2 the highest one. This observation might explain the pattern observed here for the six acetates investigated as preparations containing higher amounts of the α-isoenzyme show higher preference for (*R*)-alcohols **1**–**4** and lower selectivities towards the (*S*)-alcohols **5**–**6** compared to recombinant PLE.

4. Conclusions

It was shown in this study, that the presence of various isoenzymes in commercial PLEs substantially effects the stereopreference and enantioselectivity in the hydrolysis of a range of acetates of secondary alcohols. An efficient kinetic resolution cannot be achieved with commercial pig liver esterases, but the recombinant γ-isoenzyme allows the production of both enantiomers of acetate **6** and of the remaining (*R*)-acetate of **5**.

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