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Tetrahedron: *Asymmetry* 14 (2003) 1341–1344

TETRAHEDRON: *ASYMMETRY*

Site directed mutagenesis of recombinant pig liver esterase yields mutants with altered enantioselectivity

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Received 26 January 2003; revised 21 February 2003; accepted 26 February 2003

Abstract—The enantioselectivity of nine variants of recombinant pig liver esterase (rPLE) including recombinant porcine intestinal carboxyl esterase (rPICE) obtained by site-directed mutagenesis and heterologous expression in *Pichia pastoris* was investigated. A comparison of these mutants with rPLE revealed significant differences in the kinetic resolution of a series of acetates of secondary alcohols. A six-fold increase in enantioselectivity ($E=46$) compared to rPLE ($E=8$) was observed in the hydrolysis of (*RS*)-1-phenyl-1-ethyl acetate using a variant containing a single mutation (glutamic acid exchanged to glycine at position 77, E77G). No clear correlation between the number and type of amino acid substitutions was found, however a tendency was observed, that at high mutation levels enantioselectivity decreases, but at the same time a switch in enantiopreference occurred as observed for the resolution of (*RS*)-1-phenyl-3-butyl acetate and (*RS*)-1-phenyl-2-pentyl acetate.

1. Introduction

Lipases and esterases represent a group of biocatalysts widely used in organic chemistry[1,2] especially in the synthesis of enantiomerically pure compounds. Only a few carboxyl esterases are currently available, which is in contrast to the considerable numbers of commercial lipases. Amongst these, pig liver esterase (PLE), is commonly used and many successful applications are documented in literature.[3–5]

PLE is a heterogeneous enzyme composed of several isoenzymes, containing three kinds of subunits (α, β and γ), which in most cases are assembled as trimers.[6,7] Investigations using isoenzyme fractions separated by isoelectric focusing revealed that these particular isoenzymes have different or even opposite enantioselectivity and also show varying substrate spectra.[8] As a consequence, irreproducible results can be obtained depending on the commercial PLE preparation used. Recently, we showed, that this problem could be overcome by cloning and overexpression of the recombinant PLE γ-isoenzyme (rPLE) in the yeast *Pichia pastoris*.[9] Indeed, we observed, that the enantioselectivity of rPLE differs substantially when compared with commercially available heterogeneous preparations.[10,11]

The amino acid sequence of rPLE (γ-isoenzyme) shows very high identity (97%) to a porcine intestinal carboxyl esterase (PICE),[12] differing by only 17 out of 556 amino acids. As functional expression of PICE has not yet been reported and the gene encoding rPLE is readily available, we therefore step-wise introduced all 17 mutations into it by polymerase chain reaction (PCR) using the QuikChange™ protocol followed by expression in *P. pastoris*.[13] In addition to biochemical characterizations of these variants, we were especially interested in the influence of these mutations on the enantioselectivity, as described here.

2. Results

Variants of rPLE containing up to 17 amino acid substitutions were generated by site-directed mutagenesis using the QuikChange™ protocol (Table 1). Amino acids which are decoded by nucleotides being close on the DNA level, were exchanged in one PCR and thus only nine variants (including rPICE) of rPLE bearing increasing numbers of amino acid substitutions were obtained. The resulting gene products were then expressed in *P. pastoris* by induction with methanol and

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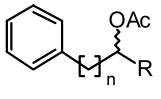
0957-4166/03/$ - see front matter
doi:10.1016/S0957-4166(03)00205-2

Table 1. New esterase variants obtained by site-directed mutagenesis of rPLE gene in the order to obtain recombinant porcine intestinal carboxyl esterase (rPICE)

Enzyme	Mutations
PLE-PICEa	V76A, E77G
PLE-PICEb	V76A, E77G, T92I, L93P
PLE-PICEc	V76A, E77G, T92I, L93P, P195T
PLE-PICEd	V76A, E77G, T92I, L93P, P195T, V236A, A237G
PLE-PICEe	V76A, E77G, T92I, L93P, P195T, V236A, A237G, F290L, Q294P
PLE-PICEf	V76A, E77G, T92I, L93P, P195T, V236A, A237G, F290L, Q294P, Y367F, A370T
PLE-PICEg	V76A, E77G, T92I, L93P, L129V, P133S, M134T, P195T, V236A, A237G, F290L, Q294P, Y367F, A370T
PLE-PICEh	V76A, E77G, T92I, L93P, L129V, P133S, M134T, V138L, V139A, P195T, V236A, A237G, F290L, Q294P, Y367F, A370T
rPICE	V76A, E77G, T92I, L93P, K112R, L129V, P133S, M134T, V138L, V139A, P195T, V236A, A237G, F290L, Q294P, Y367F, A370T

secreted into the culture supernatant. The proteins were concentrated from the cultivation media and directly used for biocatalysis reactions. The hydrolysis of six racemic acetates of secondary alcohols **1**–**6** (Scheme 1) was studied using these esterase variants (abbreviated PLE-PICEa to PLE-PICEh and rPICE).

The enantioselectivity of the esterases was very different and strongly depended on the substrate used (Fig. 1). First of all it was easy to observe that the enantioselectivity of the enzymes decreased when the number of mutations increased. PLE-PICEg, PLE-PICEh and rPICE were almost not enantioselective against the esters used. In the case of these three last mutants, highest enantioselectivity was observed in the hydrolysis of 1-phenyl-1-ethyl acetate **1** ($E=10$–12) and 1-phenyl-3-butyl acetate **3** ($E=5$–7). An inversion of enantiopreference was also observed for these three enzymes. They showed (*R*)-preference in the hydrolysis of **3** and 1-phenyl-2-pentyl acetate **5**, which was in clear contrast as found for rPLE and all other mutants. In the hydrolysis of 1-phenyl-1-propyl acetate **2** almost no differences between the various mutants were observed. For



(*R,S*)-**1-6**

1: n = 0, R = CH_3
2: n = 0, R = CH_2CH_3
3: n = 2, R = CH_3
4: n = 1, R = CH_3
5: n = 1, R = $CH_2CH_2CH_3$
6: n = 1, R = CH_2CH_3

Scheme 1. Acetates of secondary alcohols (**1**–**6**) used as substrates for kinetic resolution with the different rPLE variants. The alcohols obtained in the hydrolysis are named **1a**–**6a**.

all enzymes the *E*-value was between 2 and 6 and in all cases (*R*)-alcohol was formed in excess. A similar situation occured when 1-phenyl-2-propyl acetate **4** was used as substrate. The enantioselectivity reached values from 3 to 13 with (*S*)-preference, with the exception of PLE-PICEg, PLE-PICEh and rPICE, which were not selective in this reaction.

Very interesting results were obtained in the kinetic resolution of 1-phenyl-1-ethyl acetate **1** and 1-phenyl-2-butyl acetate **6**. When **1** was used as a substrate (Table 2), the enantioselectivity of esterase PLE-PICEa ($E=54$) increased 6–7-fold as compared to rPLE ($E=8$) and then decreased with the number of introduced mutations to $E=10$–12 for PLE-PICEg, PLE-PICEh and rPICE. In the case of **6**, the enantioselectivity of PLE-PICEa ($E=17$) decreased 4-fold as compared to rPLE ($E=53$, Table 3), stayed similar for the next five esterases ($E=11$–23) to decrease to only 1.3 for PLE-PICEg, PLE-PICEh and rPICE. The largest differences in the enantioselectivity towards substrates **1** and **6** were observed between the esterases rPLE and PLE-PICEa. The enzyme PLE-PICEa contains only two mutations: V76A and E77G. To verify if both of them or only one amino acid exchange are responsible for the changes in the enantioselectivity, two additional carboxyl esterase variants with the corresponding single mutations were produced and used for kinetic resolution of these two substrates.

The results shown in Figure 2, Tables 2 and 3 confirm that the exchange of glutamic acid at position 77 against glycine is responsible for the substantial change in the enantioselectivity of these enzymes.

3. Discussion

The site-directed mutagenesis of recombinant pig liver esterase created a group of artificial isoenzymes including recombinant porcine intestinal carboxyl esterase (rPICE). As variants of PLE, the new enzymes were assumed to differ in their enantioselectivity, similar to the case of natural PLE isoenzymes. This assumption was experimentally confirmed and considerable differences between the properties of the new carboxyl esterase variants were found.

The main observation is, that the enantioselectivity of the rPLE variants decreases in the direction to rPICE. The recombinant porcine intestinal carboxyl esterase is an enzyme with very low enantioselectivity. Maybe this can explain why no literature data concerning the enantioselectivity or application in organic synthesis of this esterase has been reported, although this enzyme was described a long time ago.[14] The most striking differences were observed between rPLE and PLE-PICEa (V76A/E77G). The mutations at position 76 and 77 were introduced separately and it was found, that the single exchange E77G has the strongest influence on enantioselectivity and almost no effect was observed with the introduction of the additional substitution at position 76 in the hydrolysis of **1** and **6**. The E77G

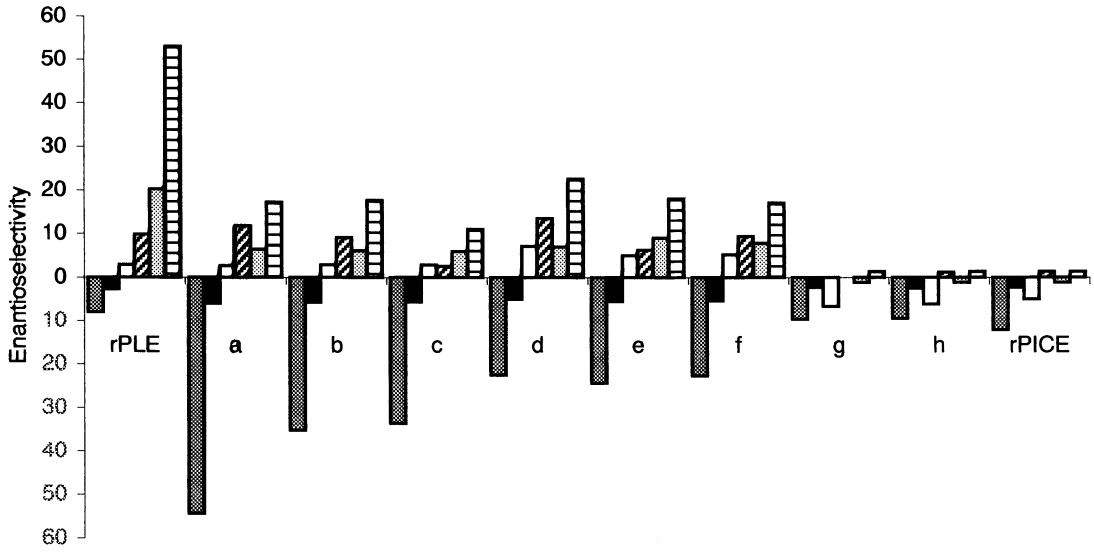


Figure 1. Enantioselectivity and stereopreference of the new esterase variants, obtained by site-directed mutagenesis from rPLE, in the kinetic resolution of acetates **1**–**6** as compared with rPLE. Above the x-axis: (*S*)-preference, below: (*R*)-preference. Substrates: 1-phenyl-1-ethyl acetate **1** (▩), 1-phenyl-1-propyl acetate **2** (■), 1-phenyl-3-butyl acetate **3** (□), 1-phenyl-2-propyl acetate **4** (▨), 1-phenyl-2-pentyl acetate **5** (□), 1-phenyl-2-butyl acetate **6** (⊟).

Table 2. Enantioselectivity of different esterases in the kinetic resolution of (*RS*)-1-phenyl-1-ethyl acetate **1**[a]

Enzyme[b]	Time (h)	Enantiomeric excess (%)		Conversion (%)	E[c]
		ee_S[d]	ee_P[d]		
rPLE	2.5	72	60	55	8
V76A	1	86	69	56	15
E77G	1	98	82	55	46
PLE-PICEa	1.5	96	87	53	54

[a] Substrate concentration 10 mM.
[b] In all reactions 0.5 Units (based on pNPA-assay) were used.
[c] Enantioselectivity E was calculated according to Chen et al.[15]
[d] In all cases the product alcohol **1a** had (*R*)-configuration and the non-converted acetate **1** (*S*)-configuration.

Table 3. Enantioselectivity of different esterases in the kinetic resolution of (*RS*)-1-phenyl-2-butyl acetate **6**[a]

Enzyme[b]	Time (h)	Enantiomeric excess (%)		Conversion (%)	E[c]
		ee_S[d]	ee_P[d]		
rPLE	2	60	93	39	53
V76A	0.5	85	99	46	>100
E77G	0.5	46	78	37	13
PLE-PICEa	2	89	71	56	17

[a] Substrate concentration 10 mM.
[b] In all reactions 0.5 Units (based on pNPA-assay) were used.
[c] Enantioselectivity E was calculated according to Chen et al.[15]
[d] In all cases the product alcohol **1a** had (*R*)-configuration and the non-converted acetate **1** (*S*)-configuration.

mutation also causes a change of the pI-value of the esterase variant and it can not be excluded, that this effect is responsible for the altered enantioselectivity.

These results show, that only small differences in the amino acid sequence of an enzyme can have very strong influences on its properties, which is confirmed here for substantial changes in the enantioselectivity and enantiopreference of variants of pig liver carboxyl esterase.

4. Experimental

All chemicals and acetate **1** were purchased at the highest purity available from Fluka. Acetates **2**–**6** were

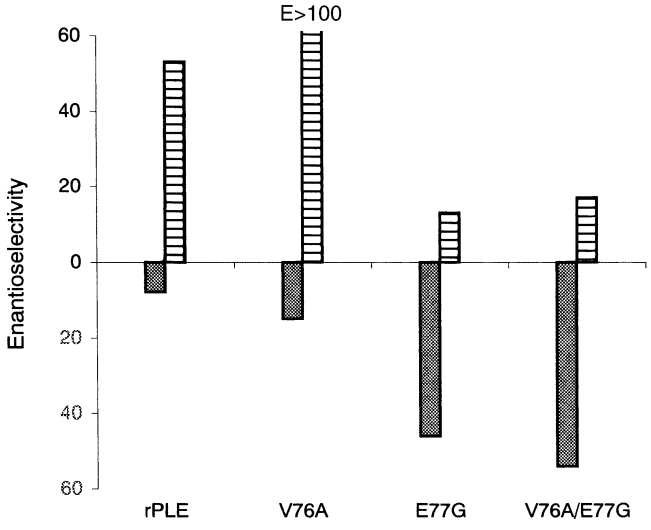


Figure 2. Enantioselectivity and stereopreference of the new esterase variants obtained by site-directed mutagenesis from rPLE: V76A, E77G, V76A/E77G (PLE-PICEa) in the kinetic resolution of acetates **1**–**6** as compared with rPLE. Above the x-axis: (*S*)-preference, below: (*R*)-preference. Substrates: 1-phenyl-1-ethyl acetate **1** (▩), 1-phenyl-2-butyl acetate **6** (⊟).

synthesized from commercially available alcohols **2a**–**4a**, **6a** using standard procedures as described.[11] Gas chromatographic analyses were conducted using a Hydrodex-β-3P (25 m×0.25 mm, Macherey-Nagel, Düren), carrier gas: H_2, flame ionisation detector. Retention times: **1** (110°C isothermal): (*S*)-**1** 7.2 min; (*R*)-**1**, 9.3 min; (*S*)-**1a**, 11.7 min; (*R*)-**1a** 10.7 min; **2** (120°C isothermal): (*S*)-**2** 6.9 min; (*R*)-**2**, 7.7 min; (*S*)-**2a** 12.3 min; (*R*)-**2a** 11.7 min; **3** (120°C isothermal): (*S*)-**3** 15.2 min; (*R*)-**3**, 20.2 min; (*S*)-**3a** 16.2 min; (*R*)-**3a**, 16.7 min; **4** (120°C isothermal): (*S*)-**4** 18.7 min; (*R*)-**4**, 22.9 min; (*S*)-**4a** 20.1 min; (*R*)-**4a**, 20.7 min; **5** (110°C isothermal): (*S*)-**5** 25.7min und (*R*)-**5** 26.3 min; (*S*)-**5a** 34.5 min und (*R*)-**5a** 36.1 min; **6** (110°C isothermal): (*S*)-**6** 17.5 min; (*R*)-**6**, 18.7 min; (*S*)-**6a** 20.7 min; (*R*)-**6a**, 21.9 min. Absolute configurations were assigned as already described.[11]

4.1. Enzymes

The recombinant enzymes: PLE-PICEa (63 U/mg), PLE-PICEb (59 U/mg), PLE-PICEc (23 U/mg), PLE-PICEd (377 U/mg), PLE-PICEe (35 U/mg), PLE-PICEf (20 U/mg), PLE-PICEg (16 U/mg), PLE-PICEh (4 U/mg), rPICE (58 U/mg), V76A (51 U/mg), E77G (71 U/mg) were obtained from the rPLE gene by site-directed mutagenesis and produced by methanol induction in the yeast *P. pastoris* as described elsewhere.[13]

4.2. Esterase activity

Esterase activity was determined photometrically in sodium phosphate buffer (50 mM, pH 7.5) with *p*-nitrophenyl acetate (pNPA, 10 mM dissolved in dimethyl sulfoxide) as the substrate at room temperature. The amount of *p*-nitrophenol released was routinely determined at 410 nm ($\varepsilon = 15.6\times10^3$ [M^{-1} cm^{-1}]). One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per min under assay conditions.

4.3. 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 esterase (based on the pNPA-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.[15] at a conversion close to 50% from the ee_P and ee_S values.

Acknowledgements

We thank the Konrad-Adenauer foundation (St. Augustin, Germany) for a stipend to A. Musidlowska-Persson. We are also grateful to B. Lange (Greifswald University) for her support during her practical course and to the Degussa AG (Project Houses Biotechnology and Catalysis) for financial support.

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