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Supporting Information

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Novel Isoenzymes of Pig Liver Esterase Reveal Striking Differences in Enantioselectivities**

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Additional results

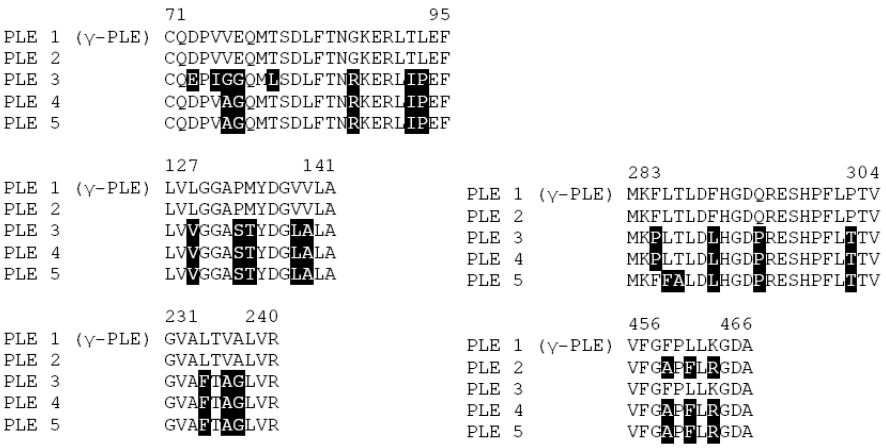

Figure 1. Alignment of sequence fragments differing between PLE 1 (γ-PLE) and the newly identified isoenzymes PLE 2-5.

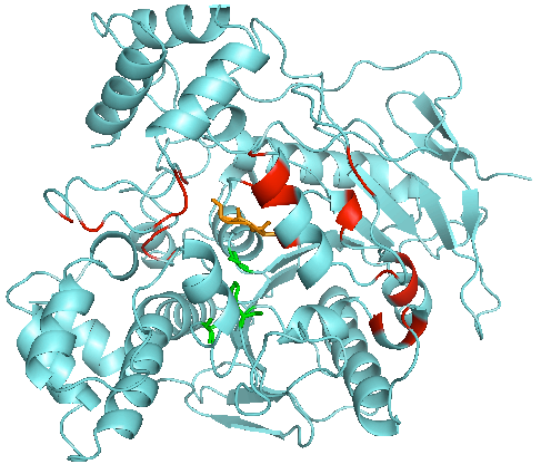

Figure 2. Model of the 3D-structure of pig liver esterase based on the X-ray structure of human carboxylesterase (pdb-code 1MX9).[1] For clarity, only sites of mutations are highlighted in addition to active site (green) and GGG-motif (orange) in the oxyanion binding pocket.

As expected from the sequence comparison shown in Figure 1, the differences between the isoenzymes PLE1-5 are distributed throughout the 3D structure of the protein as suggested by a preliminary modeling based on the 3D-structure of human carboxylesterase (Figure 2).

Table 1. Remaining activities [%] of the PLE isoenzymes after preincubation with the esterase inhibitors sodium fluoride, phenylmethyl-sulfonylfluoride (PMSF) and physostigmine at 25 °C. Activities were determined spectrophotometrically with the pNPA assay (see experimental section below).

Inhibitor and preincubation time [min]	Conc. [mM]	Remaining activity [%]				
		PLE 1 (γ-PLE)	PLE 2	PLE 3	PLE 4	PLE 5
NaF	1					
5		20	17	44	64	83
30		21	15	46	66	88
PMSF	0.01					
1		97	87	77	78	88
5		85	82	51	56	76
30		55	50	7	7	24
60		46	36	5	2	3
Physostigmine	0.01					
1		41	61	83	90	94
5		12	25	81	82	98
30		7	6	72	66	75

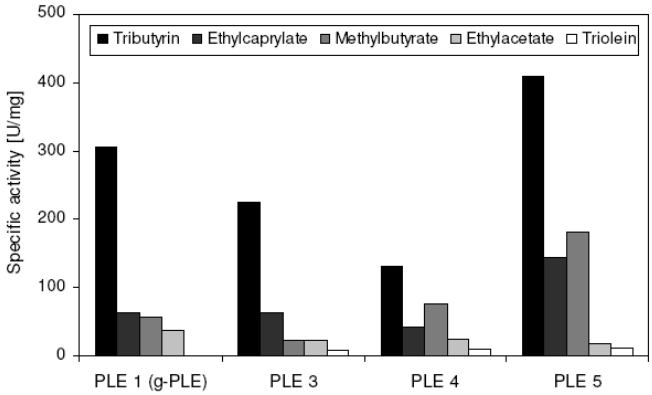

Figure 3. Specific activities of PLE isoenzymes towards several achiral esters determined with pH-Stat at 37°C and pH 7.5.

Additional and more detailed data for the resolution of compounds **1**, **3** and **4** and the desymmetrization of compound **5** using isoenzymes of PLE:

Table 2. Enantioselectivity of different recombinant pig liver esterase isoenzymes and a commercial PLE preparation in the kinetic resolution of *(R,S)*-1-phenyl-1-propyl acetate **1**.

PLE[a]	Time [h]	ee_S [%][b]	ee_P [%][b]	Conv. [%]	E[c]	Prefe-rence
PLE 1 (γ-PLE)	4	41	45	48	4	R
PLE 2	2	38	49	44	4	R
PLE 3	1	25	34	43	3	S
PLE 4	1.5	51	71	42	10	R
PLE 5	1	61	93	40	51	R
Fluka-PLE[d, e]	1	21	28	43	2.2	R

[a] In all reactions 0.5 U of esterase (based on pNPA assay) were used. [b] ee_S = enantiomeric excess of the non-converted substrate, ee_P = enantiomeric excess of the product as determined by GC analysis on a chiral stationary phase. [c] calculated according to Chen et al.[2] [d] commercially available PLE preparation from Fluka. [e] Data for Fluka-PLE taken from literature.[3a]

Table 3. Enantioselectivity of different recombinant pig liver esterase isoenzymes and a commercial PLE preparation in the kinetic resolution of **3** *(R,S)*-1-phenyl-2-butyl acetate.

PLE[a]	Time [h]	ee_S [%][b]	ee_P [%][b]	Conv. [%]	E[c]	Prefe-rence
PLE 1 (γ-PLE)	4	83	93	47	72	S
PLE 2	4	67	93	42	55	S
PLE 3	4	26	32	45	2	R
PLE 4	3	65	83	43	25	R
PLE 5	4	82	89	48	45	R
Fluka-PLE[d, e]	2	12	12	49	1.4	S

[a]–[d]: see Table 2. [e] Data for Fluka-PLE taken from literature.[3b]

Table 4. Enantioselectivity of different recombinant pig liver esterase isoenzymes and a commercial PLE preparation in the kinetic resolution of **4** *(R,S)*-1-phenyl-2-pentyl acetate.

PLE[a]	Time [h]	ee_S [%][b]	ee_P [%][b]	Conv. [%]	E[c]	Prefe-rence
PLE 1 (γ-PLE)[e]	2	69	78	47	17	S
PLE 2	0.2	71	87	45	30	S
PLE 3	0.05	23	37	38	3	R
PLE 4	0.2	76	84	48	27	R
PLE 5	0.5	86	85	50	34	R
Fluka-PLE[d, e]	0.3	24	26	48	2.1	S

[a]–[d]: see Table 2. [e] Data for Fluka-PLE and for γ-PLE taken from literature.[3b]

Table 5. Desymmetrisation of *cis*-3,5-diacetoxycyclopent-1-ene **5** by different recombinant pig liver esterase isoenzymes and a commercial PLE preparation.

PLE[a]	Time [h]	ee_P [%][b]	Conv. [%]	Preference
PLE 1 (γ-PLE)	14	82	96	**6a** (3*S*,5*R*)
PLE 2	14	82	91	**6a** (3*S*,5*R*)
PLE 3	14	83	99	**6a** (3*S*,5*R*)
PLE 4	14	42	95	**6b** (3*R*,5*S*)
PLE 5	14	17	100	**6b** (3*R*,5*S*)
Fluka-PLE[c]	20	61	100	**6a** (3*S*,5*R*)

[a]–[b]: see Table 2, [c] commercially available PLE preparation from Fluka.

Experimental Section

All chemicals were purchased from Fluka (Buchs, Switzerland), Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) and Promega (Mannheim, Germany) at the highest purity available. Oligonucleotides were obtained from and DNA-sequencing was performed by MWG-Biotech (Ebersberg, Germany).

Microorganisms and growth conditions. E. coli One Shot TOP10 Competent Cells (Invitrogen, Carlsbad, CA, USA) [F^- mcrA D(mrr-hsdRMSmcrBC) (F80lacZDM15) DlacX74 recA1 deoR araD139 D(ara-leu) 7697 galU galK rpsL (Str^R) endA1 nupG] or *E. coli* DH5α (Clontech, Mountain View, CA, USA) [supE44ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1relA1] were used for the cloning experiments. *E. coli* Origami™ (DE3) (Novagen, Darmstadt, Germany) (Δ(ara–leu)7697 ΔlacX74 ΔphoA PvuII phoR araΔ139 ahpC galE galK rpsL F'[lac+ lacIq pro] (DE3) gor522::Tn10 trxB; Kan^R, Str^R, Tet^R) was used for recombinant expression of PLE isoenzymes. Cells were cultivated in Luria Bertani (LB) medium [yeast extract (5 g L^{-1}), peptone (10 g L^{-1}) and NaCl (10 g L^{-1})] supplemented with the appropriate antibiotics at 30°C.

Recombinant DNA technologies. Unless stated otherwise, standard DNA technologies were used.[4] The QIAprep Spin Miniprep kit and the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) were used for plasmid DNA isolation and DNA gel extractions, respectively. Restriction enzymes and other DNA modifying enzymes were used as specified by the suppliers (New England BioLabs, Beverly, MA, USA; Promega, Mannheim, Germany). Standard protocols were used for the preparation and transformation of competent *E. coli* cells.[5]

Isolation of pig liver RNA, construction of cDNA and cloning of PLE genes. Tissue of fresh pig liver (0.1 g) was supplemented with Trizol® reagent (TRIzol® Plus RNA Purification Kit, Invitrogen), homogenized (10 min at room temperature; Ultraturrax T25, IKA-Labortechnik, Staufen, Germany) and RNA was isolated according to the procedure given in the manual. cDNA synthesis by RT-PCR was performed using oligo(dT)15 primers and MMLV reverse transcriptase with RNase H activity (Promega) according to the protocol of the producer. RT-PCR products were used as templates for the cloning of PLE homologous genes using PLE specific primers (primer 1: 5'-CACC*CATATG*GGGCAGCCAGCCTCGC-3', with italicised restriction site for *Nde*I; primer 2: 5'-CCG*CTCGAG*TCACTTTATCTTGGGTGGCTTCTTTGC-3', the restriction site for *Xho*I is italized; underlined sections are start and stop codons). Primer 1 contains at its 5'-end the bases CACC allowing a subsequent cloning into pET101/D-TOPO vector (see below). Both primers were designed based on the sequence of the mature g-PLE gene eliminating the N-terminal signal sequence (18 amino acids) and the C-terminal retention signal for endoplasmic reticulum (4 amino acids, HAEL), which are present in the original gene in order to enable subsequent expression.[6] PCR was performed in a thermocycler (Techne Progene, Jepson Bolton Laboratory Equipment, Watford, United Kingdom) using Pfu Plus polymerase (Roboklon, Berlin, Germany) according to the producers manual. After an initial denaturation step (95°C for 5 min) the following temperature program was used (30 cycles): 95°C for 1 min, 60°C for 1 min, 72°C for 3 min, followed by a final extension of 72°C for 7 min. The purified PCR products were initially cloned into the pET101/D-TOPO vector according to the manual (Champion™ pET Directional TOPO® Expression Kit; Invitrogen). *E. coli* Top10 cells were transformed with the construct mixture and separated on agar plates. Single colonies were cultivated separately for plasmid isolation followed by restriction mapping and sequencing.

Construction of the expression system. Plasmid DNA of the pET101/D-TOPO_PLE constructs were cleaved with *Nde*I and *Xho*I according to standard protocol (New England Biolabs, Beverly, MA, USA). Products were purified on an agarose gel and the resulting 1.6 kbp fragment was inserted into the respective *Nde*I/*Xho*I-cleaved pET15b vector fusing the PLE gene to an N-terminal His_6-Tag. *E. coli* DH5a was transformed with the resulting plasmid DNA for propagation following plasmid isolation and an additional sequencing. The pET15b_PLE constructs were used for transformation of *E. coli* Origami™ (DE 3) for recombinant expression of PLE isoenzymes; *E. coli* Origami™ (DE 3) cells had previously been transformed with the plasmid pGro7 (Chaperone plasmid set, TaKaRa BIO Inc., Otsu, Shiga, Japan) encoding for L-arabinose induced expression of the chaperones GroEL and GroES.

Recombinant expression of PLE isoenzymes with coexpression of chaperone complex GroEL/GroES in E. coli Origami™. The expression of the PLE isoenzymes was carried out by coexpression of the chaperone complex GroEL/GroES in *E. coli* Origami™ (DE3) according to Böttcher et al.[7] using 0.5 g L^{-1} L-arabinose for the induction of the chaperone-expression (at the beginning of cultivation) and 40 µM IPTG for the induction of PLE-expression (at OD_{600} of 0.8). Cells were harvested after 24 h of total cultivation time by centrifugation, washed and resuspended in sodium phosphate buffer (50 mM, pH 7.5) and sonified. The supernatant after centrifugation of cell debris was used for further experiments (crude cell extract). The protein content was determined by the Bradford method.

Polyacrylamid gel electrophoresis (PAGE) and activity staining for esterase activity. PAGE was carried out according to a standard method. For both denaturating SDS and native polyacrylamid gels, 7.5 % separation gels were used with 4 % and 4.5 % stacking gels for SDS and native PAGE, respectively. Gels were stained for protein detection with Coomassie Brilliant Blue. For activity staining, native PAGEs were incubated in a freshly prepared mixture of solutions of a-naphthyl acetate and Fast Red according to Krebsfänger et al.[8] In the presence of hydrolytic activity, released a-naphthol forms a red complex with Fast Red.

Determination of esterase activity. Esterase activity was determined spectrophotometrically in sodium phosphate buffer (50 mM, pH 7.5) using *p*-nitrophenyl acetate (pNPA, 10 mM in DMSO) as substrate. The amount of *p*-nitrophenol released was determined at 410 nm ($e = 14.76*10^3$ M^{-1} cm^{-1}) and room temperature. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 µmol *p*-nitrophenol per min under assay conditions. All experiments were performed in duplicate. Substrate specificity of PLE was measured using a pH-stat technique. To an emulsion (30 mL, prepared with Ultra Turrax T25 basic homogenisator, IKA Labortechnik, Staufen, Germany) containing ester (5% (v/v) methyl butyrate, ethyl caprylate, ethyl acetate, tributyrin or triolein) and gum arabic (2% (w/v)), a known amount of esterase was added at 37°C. Liberated acid was titrated automatically in a pH-stat (Schott, Mainz, Germany) with 0.01 N NaOH in order to maintain the pH constant at 7.5. One unit of activity was defined as the amount of enzyme releasing 1 µmol acid per min under assay conditions. Autohydrolysis of the esters was determined and subtracted.

Purification. PLE isoenzymes carrying an N-terminal His_6-Tag were purified in two steps: first, a nickel ion chelating affinity chromatography was applied followed by a gelfiltration, mainly to separate residual chaperone protein from the PLE. The first step was carried out on IMAC Sepharose™ 6 Fast Flow (GE Healthcare Bio-Sciences AB; Uppsala, Sweden) according to the manufacturers' protocol. The beads were loaded into columns and the purification procedure was performed manually at 4°C. The beads were washed, loaded with Ni^{2+} and washed to remove excess nickel. The column was equilibrated with basis buffer (50 mM sodium phosphate, 100 mM NaCl, pH 7.5) and the sample was loaded. Non-binding proteins were washed from the column with washing buffer (basis buffer + 50 mM imidazol), followed by a washing step with washing buffer supplemented with 50 mM ATP (freshly prepared) to remove chaperone protein. PLE isoenzymes were eluted from the column with elution buffer (basis buffer + 250 mM imidazole). If necessary, eluted samples were concentrated with Centricons (30 kDa cut-off, Millipore; Billerica, MA, USA). Gelfiltration was performed on a Superdex™ 200 HiLoad™ 16/60 column connected to an Äkta Purifier (Amersham-Pharmacia, Uppsala, Sweden) at 4°C. Tris buffer (50 mM with 200 mM NaCl, pH 7.5) was used for the chromatography. Samples were concentrated to <2 mL with Centricons prior to purification. Flow rate was 0.3 mL min^{-1}, and chromatography was finished after 1.5 times column volume and protein content was monitored at 260 and 280 nm.

Inhibition studies. To determine the influence of the esterase inhibitors physostigmin, sodium fluoride and phenylmethylsulfonyl fluoride, the crude cell extract was incubated with the concentration of the inhibitors stated in Table 1 at room temperature with sampling at specified time intervals. Residual activity in the crude cell extract was then determined using the pNPA assay. Samples without inhibitor served as controls.

Enzyme-catalyzed kinetic resolution and desymmetrisation of the acetates: The acetates of secondary alcohols were synthesized as described.[3b] *Cis*-3,5-diacetoxycyclopent-1-ene was purchased from Fluka.

The acetates of secondary alcohols **1**-**4** or *cis*-3,5-diacetoxycyclopent-1-ene **5** (10 mM each) were dissolved in sodium phosphate buffer (50 mM, pH 7.5). The biocatalytic reaction was started by addition of esterase containing cell crude extract (0.5 U, based on pNPA assay) to 1 mL of substrate solution and hydrolysis reaction was performed at 37°C in a thermomixer (Eppendorf) at 1200 rpm. At certain time intervals samples (300 µL) were taken, extracted twice with dichloromethane, and the combined organic phases were dried over anhydrous sodium sulfate. Excess solvent was removed under nitrogen and the enantiomeric purity of substrate and product and the conversion were determined by gas chromatography (GC, Shimadzu GC-14A gas chromatograph, column: heptakis(2,6-*O*-methyl-3-*O*-pentyl)-b-cyclodextrin (Machery-Nagel, Düren, Germany); carrier gas: H_2; flame ionization detector).

GC analysis: Injection and detection temperatures were set to 220°C. Column temperature programs and retention times for the acetates of secondary alcohols were already published. [3] **5**, **6a** and **6b** were separated at 110°C (isothermal), retention times: **5**, 21.8 min; **6a**, 18.2 min; **6b**, 16.0 min. Absolute configurations were assigned after preparative biocatalysis and polarimetry according to literature.[9]

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