Enzyme Catalysis

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

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Esterases and lipases are frequently used biocatalysts because they accept a broad range of substrates, are usually stable in organic solvents, and often show high stereoselectivities even towards non-natural substrates.[1] While a large number of lipases is commercially available, there are only few well explored carboxylesterases, among which pig-liver esterase (PLE) plays the most important role in industrial processes owing to its high versatility.[2] One major drawback in the application of PLE is its natural heterogeneity as it consists of several isoenzymes.[3] These differ in isoelectric point, molecular weight, sensitivity towards inhibitors and—most importantly—substrate specificity.[3b]

Several years ago, we reported the cloning and recombinant expression of the γ-isoenzyme of PLE (γ-PLE) in *Pichia pastoris*[4] and more recently in *E. coli*[5] thus overcoming the undesirable presence of several PLE isoenzymes and of interfering other hydrolases in the commercial preparations. Furthermore, we could demonstrate that the recombinant γ-PLE shows considerable differences in enantioselectivity towards esters of secondary alcohols in comparison with the naturally occurring mixture of isoenzymes.[6] This encouraged us to identify the then unknown sequences encoding the other isoenzymes of PLE. Initially, we used tandem mass spectrometry[7] of PLE samples separated by 2D gel electrophoresis. Indeed, this led to the discovery of certain amino acid positions, such as V236P/A237G, which impart enhanced enantioselectivity. However, the elucidation of the complete protein sequences appears impossible using this approach.

To access the genes encoding for unknown isoenzymes of PLE, first, the cDNA of pig-liver RNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR). The cDNA served as the template for the amplification of PLE homologous genes using primers derived from the known γ-PLE sequence (GenBank accession code X63323). To enable functional expression in *E. coli*, the N-terminal signal sequence (18 amino acids) and the C-terminal ER-retention signal (four amino acids, HAEL; ER = endoplasmic reticulum) were omitted. Amplification by PCR resulted in a single DNA band of approximately 1.6 kbp (bp = base pairs) in the agarose gel, matching the size of the γ-PLE gene. The fragments were cloned first into the pET101/D-TOPO vector and later, for functional expression, into pET15b, and sequenced. This resulted in the identification of four novel sequences (named PLE 2 to PLE 5), bearing 3–21 amino acid exchanges[8] compared to γ-PLE (now renamed PLE 1). Figure 1 schematically shows that the amino acid exchanges are not randomly distributed along the protein, but can be found in distinct regions.

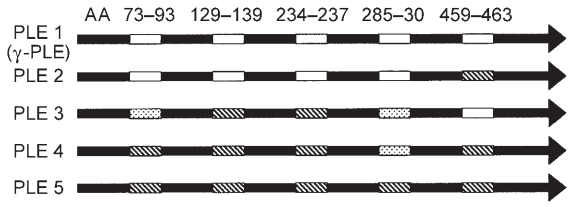

Figure 1. Differences between the isoenzymes are not randomly distributed, but occur in conserved areas. Black: homologous regions, white: variations in PLE 1 (γ-PLE), hashed: variations in PLE 5, dotted: variations, which occur neither in PLE 1 nor PLE 5; AA = amino acid.

After functional expression in *E. coli*, we observed that the novel isoenzymes show distinct differences in their characteristics, amongst others in the specific activity towards achiral esters: All of them preferentially cleave tributyrin, but PLE 4 and PLE 5 also show a high activity for methyl butyrate and ethyl caprylate.[8] Similarly, the sensitivity of the isoenzymes towards certain inhibitors varied considerably: PLE 3–5 are less sensitive than the others towards sodium fluoride and physostigmin, but are more strongly inhibited by phenyl methyl sulfonylfluoride.[8] The ratio in the specific activities against methyl butyrate and tributyrin as well as the sensitivity against the chosen inhibitors has been reported to be characteristic for distinguishing between the main isoenzyme fractions in the natural PLE mixture, α-PLE, and γ-PLE,[3b] so that it can be suggested that PLE 4 or PLE 5 represent the so-called α-PLE.

Most importantly for organic synthesis the enantioselectivity of the PLE isoenzymes differed substantially as exemplified for the kinetic resolution of esters of secondary

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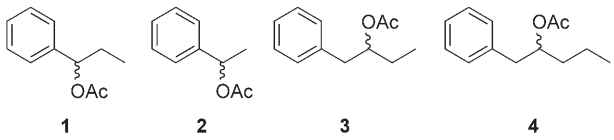
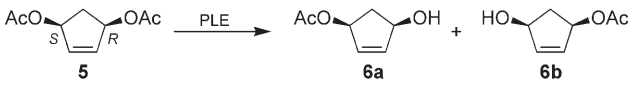
alcohols **1**–**4** (Scheme 1) and the desymmetrization of the *meso*-diacetate **5** (Scheme 2).

In previous studies,[6] we reported that the recombinant PLE 1 (γ-PLE) isoenzyme showed increased enantioselectivity (E) towards **3** ($E > 100$) and **4** ($E = 17$) in contrast to the



Scheme 1. Acetates **1**–**4** of secondary alcohols used in the kinetic resolution with the PLE isoenzymes.



Scheme 2. PLE-catalyzed desymmetrization of **5** yielding **6a** or **6b**.

naturally occurring PLE mixture ($E < 5$). The comparison of the enantioselectivities and the enantiopreferences of the novel PLE isoenzymes (PLE 2–5) with PLE 1 and the commercial enzyme from Fluka in the kinetic resolutions of **1**–**4** clearly shows striking differences in their properties (Figure 2).

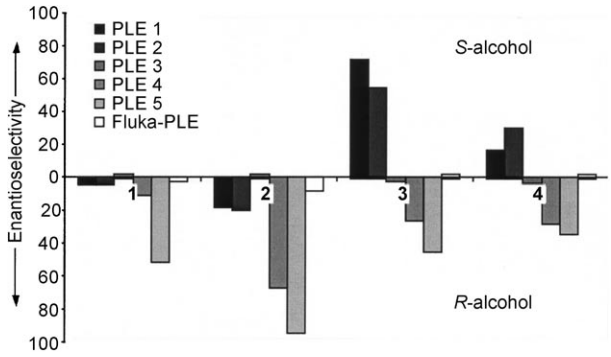


Figure 2. Enantioselectivity and enantiopreference of the recombinant PLE isoenzymes and commercial PLE isoenzyme mixture in the kinetic resolution of acetates **1**–**4**.

PLE 1 (γ-PLE) and PLE 2 differ by only three amino acids and it is not surprising, that their selectivities are highly similar. In contrast, drastic changes are clearly seen for PLE 3–5, which differ by 20 or 21 amino acid exchanges[8] from γ-PLE: For **1** and **2** notably higher enantioselectivities were found using enzymes PLE 4 and PLE 5 (Table 1 and Supporting Information) with the E-value towards **2** increasing from 17 (PLE 1, γ-PLE) to 66 (PLE 4) and 94 (PLE 5). In the kinetic resolution of acetate **1**, PLE 5 shows a more than tenfold increase in enantioselectivity compared to PLE 1.

For the other two acetates (**3** and **4**), even a switch in enantiopreference takes place: while PLE 1 and PLE 2 preferentially converted the (*S*)-enantiomer, the other three

Table 1: Enantioselectivity of different recombinant PLE isoenzymes and a commercial PLE preparation in the kinetic resolution of **2**.

PLE[a]	t[h]	ee_S [%][b]	ee_P [%][b]	Conv. [%]	E[c]	Preference
PLE 1	2	74	77	49	17	*R*
PLE 2	2	67	81	45	19	*R*
PLE 3	1.5	18	24	43	2	*S*
PLE 4	3	68	94	42	66	*R*
PLE 5	2	79	95	45	94	*R*
Fluka-PLE[d,e]	1.5	65	56	54	7	*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.[9] [d] Commercially available PLE preparation from Fluka. [e] Data for Fluka-PLE taken from literature.[6a]

isoenzymes preferred the (*R*)-enantiomers (Figure 2, Supporting Information). Although enantioselectivity is well pronounced for all the other isoenzymes towards all acetates studied, PLE 3 shows nearly no preference.

Analogously, we found a change in enantiopreference in the desymmetrization of **5** (Figure 3, Supporting Information). The resulting cyclopentene monoesters are

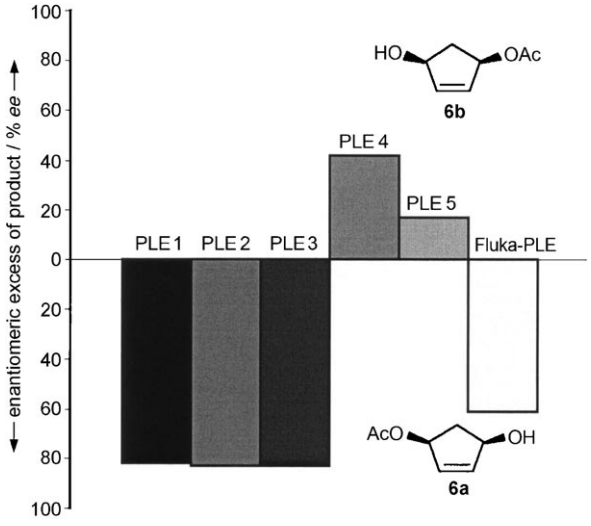


Figure 3. The enantiopreference of the recombinant PLE isoenzymes and of a commercial PLE isoenzyme mixture in the desymmetrization of **5**.

important chiral building blocks in the synthesis of prostaglandins and their derivatives.[10] The commercial PLE (mixture) shows *pro*-(*R*) selectivity yielding 80% *ee*.[11] Figure 3 shows, that the same selectivity was found using Fluka PLE, but only 60% *ee* was achieved for **6a**. Most importantly, whereas PLE 1–3 show the same preference and gave up to 80% *ee*, isoenzymes PLE 4 and 5 favored the *pro*-(*S*) acetoxy group yielding monoacetate **6b** with 42% *ee* (PLE 4) and 17% *ee* (PLE 5).

PLE 4 and PLE 5 do not only show altered enantioselectivities, but also exhibit higher kinetic constants in the hydrolysis of *p*-nitrophenyl acetate (Table 2). Owing to a

Table 2: Kinetic data of the different isoenzymes towards pNPA.[a]

Isoenzyme[b]	V_{max} [U mg^{-1}]	K_m [mM]	k_{cat}/K_m [M^{-1} s^{-1}][c]
PLE 1	149	1.57	3.0×10^5
PLE 3	110	0.96	3.6×10^5
PLE 4	133	0.81	5.2×10^5
PLE 5	217	0.76	9.1×10^5

[a] pNPA, *p*-nitrophenyl acetate; activity measured at pH 7.5 and room temperature. [b] PLE 2 was not measured as its properties are very close to PLE 1. [c] To calculate k_{cat}, the PLE trimer was regarded as one catalytically active unit.

higher v_{max} and a lower K_m value, the catalytic efficiency (k_{cat}/K_m) of PLE 5 is about threefold higher than that of PLE 1.

These results emphasize that the differences in protein sequences between the naturally occurring isoenzymes have a strong impact on the enantioselectivity and enantiopreference of pig-liver esterase. The availability of individual PLE isoenzymes now provides a versatile source for the application of this very important esterase. Thus, well-defined biocatalysts with distinct properties can be selected for a given synthetic problem.

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