Cloning, Functional Expression, and Characterization of Recombinant Pig Liver Esterase

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The N-terminal amino acid sequence of pig liver esterase (PLE) from a commercial sample was determined and shown to match closely to a published sequence encoding a proline-β-naphthylamidase from pig liver. Next, mRNA isolated from pig liver was transcribed into cDNA and primers deduced from the N-terminal sequence were used to clone the 1698 base pairs of PLE cDNA. Initial attempts to express the cDNA in Escherichia coli *and* Pichia pastoris *with different expression vectors and secretion signal sequences failed. Only after deletion of the putative C-terminal sequence His-Ala-Glu-Leu, usually considered as an endoplasmic reticulum retention signal, could heterologous expression of PLE be readily achieved in the methylotrophic yeast* P. pastoris. *Recombinant PLE (rPLE) was secreted into the medium and exhibited a specific activity of approximately 600 U mg^{-1} and a V_{max}/K_m value of 139 μmol min^{-1} mM^{-1} with* p*-nitrophenyl acetate as a substrate. Activity staining of renatured sodium dodecylsulfate–polyacrylamide gels gave a single band with esterolytic activity for rPLE, whereas several bands are visible in crude commercial PLE preparations. This was confirmed by native gels, which also show that rPLE is active as a trimer. Biochemical characterization of the recombinant enzyme and comparison with properties of commercial PLE preparations as well as with published data confirmed that we expressed a single PLE isoenzyme which showed a high preference for proline-β-naphthylamide. This is a substrate specificity for the so-called γ subunit of PLE. The optimum pH value and temperature for the recombinant PLE were 8.0 and 60 °C, respectively. The determined molecular weight of the secreted enzyme was approximately 61–62 kDa, which closely matches the calculated value of 62.419 kDa. The active site residues are located at Ser_{203}, His_{448}, and Asp_{97}, and the typical consensus sequence motif for hydrolases was found around the active site serine (Gly-Glu-Ser-Ala-Gly).*

KEYWORDS:

enzyme catalysis · gene expression · hydrolases · *Pichia pastoris* · pig liver esterase

Introduction

Pig liver esterase (PLE, also named porcine liver carboxylesterase, EC 3.1.1.1) is a serine-type esterase. Its physiological role is believed to be the hydrolysis of various esters occurring in the pig diet, which might explain its wide substrate tolerance. Besides this possible natural function, PLE represents the most useful esterase for organic synthesis and numerous reports have shown the efficient application of this enzyme in kinetic resolutions and desymmetrizations of a wide range of organic compounds.[1] So far, PLE can only be obtained by the extraction of pig liver with organic solvents and, as a consequence, an ill-defined preparation is provided. Detailed studies revealed that PLE preparations consist of various PLE isoenzymes with considerably different substrate specificities.[2] Some fractions separated by isoelectric focusing preferred methyl butyrate or butanilicain (*N*-butylglycyl-2-chloro-6-methylanilide hydrochloride), whereas others hydrolyzed butyrylcholine and proline-β-naphthylamide with high activity. The three major isoenzyme fractions of nonrecombinant PLE preparations had apparent molecular weights of 58.2 kDa (α subunit), 59.7 kDa (β subunit), and 61.4 kDa (γ subunit) as determined by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE). PLE can consist of all three isoenzymes (α, β, γ subunits) and ααα, ααγ, αγγ, and γγγ trimers are believed to be major components. Amino acid analysis showed a lower content of aspartic acid and a higher content of arginine in the α subunit compared to the γ subunit.[2, 3]

So far, the cloning and expression of PLE has not been reported. However, evidence seems to exist that a proline-β-

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naphthylamidase—which was cloned from pig liver but not functionally expressed—is identical with pig liver esterase.[4] This was further underscored by Heymann and Peter,[5] who found that isoenzyme fraction I—the trimeric γ subunit—of a native PLE preparation is able to hydrolyze proline-β-naphthylamide. Another group purified and cloned a porcine intestinal glycerol ester hydrolase, but also did not report functional expression.[6] This hydrolase was found to share as much as 97% amino acid sequence identity with that reported by Matsushima et al.[4]

We now describe for the first time the cloning and functional expression of a PLE subunit – -which represents the major esterase in commercial PLE preparations—in the methylotrophic yeast *Pichia pastoris*. Recombinant PLE secreted into the medium was biochemically characterized and its properties were investigated in comparison to commercial samples of nonrecombinant esterase extracts from pig liver.

Results

Cloning and sequencing of the pig liver esterase

After electrophoretic separation of a commercial PLE sample obtained from Aldrich (data not shown) followed by blotting of the major protein band exhibiting esterase activity (ca. 60 kDa) on a polyvinylidene fluoride (PVDF) membrane, the N-terminal sequence was determined to be NH_2-G***T***PASP***L***VVDTAQGRVLGKYVS. A BLAST search in the SWISSPROT database using this sequence revealed an almost perfect match (20 out of 22 amino acids were identical, the mismatched residues are highlighted in the sequence above) to the mature protein of a proline-β-naphthylamidase (accession number: Q29550).[4] Assuming from these results that the major protein fraction of the commercial pig liver PLE preparations represents proline-β-naphthylamidase, polymerase chain reaction (PCR) primers were derived from the deposited nucleotide sequence for cloning of the PLE cDNA from pig liver. Following isolation of mRNA from fresh pig liver and transcription into cDNA by a reverse transcriptase PCR (RT-PCR), the PLE cDNA was amplified and the obtained PCR fragment was cloned into pUC19. As expected, the determined nucleotide sequence of the isolated PLE cDNA (1698 base pairs (bps)) was identical to the published gene sequence.[4] The deduced amino acid sequence of PLE consists of 566 residues including an 18 amino acid N-terminal signal peptide which after cleavage produces the 548 residue (60.073 kDa) mature PLE (mPLE). The tetrapeptide His-Ala-Glu-Leu located at the C terminus shows homology to known endoplasmic reticulum (ER) retention signal peptides,[7] suggesting that native PLE is a resident of the ER. Five Cys residues in mature PLE are present at positions 70, 71, 99, 256, and 267. One potential N-glycosylation site (Asn-Xxx-Ser/Thr[8]) has been identified at positions 62 – 64.

Expression of recombinant PLE

In a first attempt to express PLE functionally in *Escherichia coli*, the complete PLE gene including its N-terminal signal sequence was cloned into the temperature-inducible expression vector pCYTEXP1 to give pCYTEX – PLE. Similarly, expression vector pCYTEX – ompA – mPLE was generated by replacing the native signal sequence of PLE with the *E. coli* ompA leader sequence to facilitate efficient secretion of the expressed protein into the periplasmic space of *E. coli*.[9, 10] However, expression of PLE in *E. coli* failed and no formation of protein having the expected molecular weight was observed regardless of the constructs (Figure 1, **1 – 3**) used. This was confirmed by SDS-PAGE analysis and esterase activity assays (data not shown).

Due to the eucaryotic origin of PLE, we then considered expression of PLE in the methylotrophic yeast *Pichia pastoris*. The mPLE gene was therefore subcloned in-frame with the N-terminal α-factor signal sequence of the yeast expression vector pPICZαA to give pPICZα – mPLE (Figure 1, **4**). As the identified putative C-terminal ER retention signal His-Ala-Glu-Leu of the mammalian PLE[7] might function in yeasts too and, thus, interfere with the α-factor-mediated secretion of the protein in *P. pastoris*, a truncated variant (mPLE*) lacking this tetrapeptide was created and cloned into pPICZαA (Figure 1, pPICZα – mPLE*, **5**). To facilitate detection and purification of the expressed recombinant protein, a third construct (pPICZα – mPLE* – tag) was generated, where mPLE* is fused to a C-terminal His and myc tag provided by the vector (Figure 1, **6**).

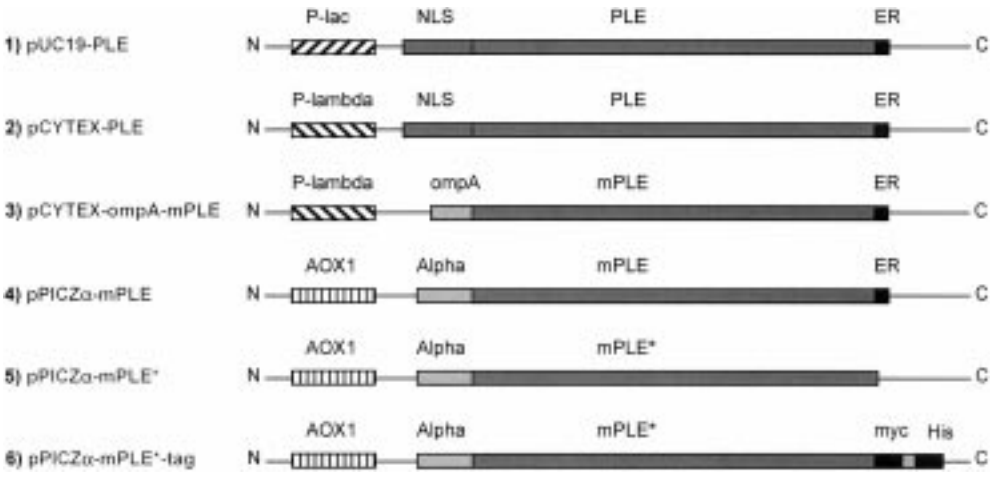

Figure 1. *Construction of different expression vectors derived from pUC19, pT – BTL2, pT – ompA – BTL2, pPICZαA. Expression cassettes containing the promotor, the leader sequence, the PLE gene, and the C-terminal tags are given. Vectors* ***1 – 3*** *were used for expression in* E. coli, *vectors* ***4 – 6*** *for expression in* P. pastoris. *P-lac: IPTG-inducible lac promotor; P-lambda: heat-inducible promotor from phage λ; AOX1: methanol-inducible alcohol oxidase 1 promotor; NLS: native leader sequence; ompA: outer membrane protein A secretion signal; Alpha: α-factor secretion signal from* Saccharomyces cerevisiae; *ER: C-terminal endoplasmatic reticulum retention signal; myc: myc tag; His: sixfold His tag.*

The resulting three linearized expression vectors (Figure 1, **4** – **6**) were transformed into *P. pastoris* X33, 30 transformants of each vector were randomly selected, and the culture supernatants were analyzed for secretion of active PLE using the rapid *p*-nitrophenyl acetate (pNPA) assay. While none of the tested transformants harboring the complete mature PLE gene on pPICZα – mPLE showed esterase activity, all of the tested transformants expressing the PLE gene with the truncated C terminus produced esterase activity in various amounts in the supernatant. Among these, two transformants, one of each construct with and without the C-terminal tag, showed the highest esterase activities with similar values. The transformant expressing the truncated PLE fused to the His and myc tag was chosen for further studies.

Characterization of recombinant mPLE

After cultivation for 96 h, 0.5 U mL^{-1} (based on the pNPA assay) of recombinant PLE were secreted into the supernatant. Concentration of the supernatant resulted in an enzyme preparation with 10 U mL^{-1}, which corresponds to a specific activity of approximately 600 U mg^{-1} protein; this was employed in subsequent characterizations. SDS-PAGE analysis (Figure 2, left)

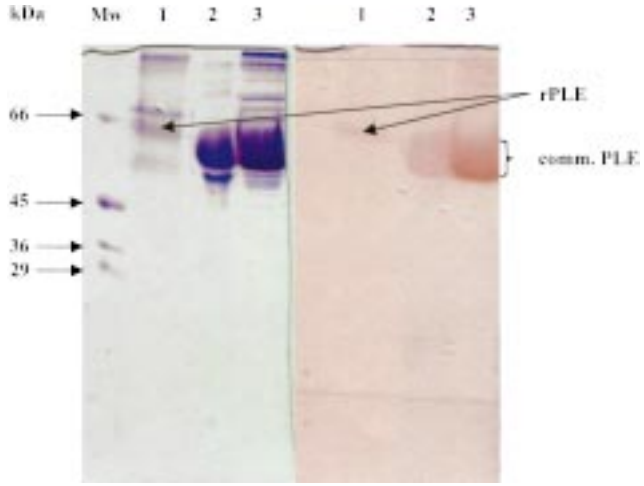

Figure 2. *SDS-PAGE analysis (left) and activity staining (right) of recombinant and commercial PLEs. Mw: molecular weight standard (66 kDa, bovine serum albumin; 45 kDa, chicken egg ovalbumin; 36 kDa, rabbit muscle glyceraldehyde-3 standard; phosphate dehydrogenase; 29 kDa, Bovine erthrocytes carbonic anhydrase); lane 1: 0.4 U recombinant PLE; lane 2: 4 U Fluka PLE; lane 3: 4 U Chirazyme E2; comm: commercial. Units are based on the pNPA assay.*

of the recombinant PLE preparation showed one major protein band with a molecular weight of approximately 61 – 62 kDa, together with some high molecular weight proteins. However, only the 61 – 62 kDa band showed esterase activity upon activity staining of renatured polyacrylamide gels with α-naphthyl acetate/Fast Red (Figure 2, right). This agrees closely with the calculated value of 62.419 kDa (for the mature truncated PLE fused to a His_6 and myc tag), as well as the 61.4 kDa reported for the PLE γ-subunit isoenzyme.[2, 3]

The commercial PLE preparations on the other hand appear to be much more inhomogeneous due to the presence of various protein bands, many of them exhibiting esterolytic activity (Figure 2). Activity staining of a native gel (Figure 3) confirms the presence of various isoenzymes of PLE in crude commercial extracts (especially the preparation from Fluka, lane 1) and

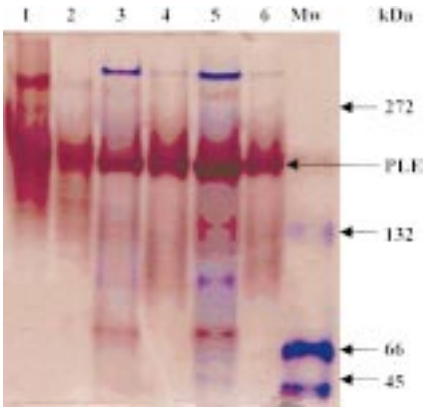

Figure 3. *Native PAGE analysis of recombinant and commercial PLEs. Gels were first activity stained followed by staining with Coomassie Brilliant Blue. Mw: molecular weight standard (272 kDa, Jack bean urease trimer standard; 132 kDa dimer and 66 kDa monomer, bovine serum albumin; 45 kDa, chicken egg albumin); lane 1: 0.1 U Fluka PLE; lane 2: 0.07 U Chirazyme E2; lane 3 – 6: 0.1, 0.045, 0.09, and 0.045 U recombinant PLE from different cultivations. Units are based on the pNPA assay.*

considerably higher purity of rPLE (Figure 3, lanes 3 – 6). Moreover, the native PLEs all have a molecular weight of approximately 180 kDa, which suggests the formation of trimers. Isoelectric focusing gave a pI of 4.78 for rPLE (data not shown), which closely matches the value reported for the γ subunit.

The V_{max}/K_m value of recombinant PLE was 139 μmol min^{-1} mM^{-1} with pNPA as the substrate, which is lower than the corresponding value for commercial PLE from Fluka (Table 1). Recombinant PLE cleaves ethyl caprylate and tributyrin with equal activity (47.3 U mg^{-1}) and ethyl acetate with moderate activity

Table 1. *Kinetic data (per milligram of protein) as determined by hydrolysis of pNPA with recombinant (rPLE) and commercial (Fluka PLE) enzymes.*

Esterase	V_{max} [U]	K_m [mM]	V_{max}/K_m [μmol min^{-1} mM^{-1}]
rPLE	0.74×10^3	5.32	139 (± 2.7)
Fluka PLE	1.58×10^3	1.82	868 (± 17.3)

(16.4 U mg^{-1}). As expected for an esterase, triolein is not accepted as substrate. However, we found that commercial preparations from Fluka and Roche Diagnostics (Chirazyme E2) do hydrolyze triolein,[11] suggesting the presence of lipases in these preparations.

Activity measurements of recombinant PLE and commercial PLE (Fluka) at different temperatures showed an almost identical behavior of the two enzyme preparations in the hydrolysis of ethyl caprylate. The highest activity was observed at 60 °C (Figure 4) but, in contrast to the commercial PLE, recombinant PLE was completely inactivated at 70 °C. Only minor deviations were found between the pH profiles of recombinant PLE and the

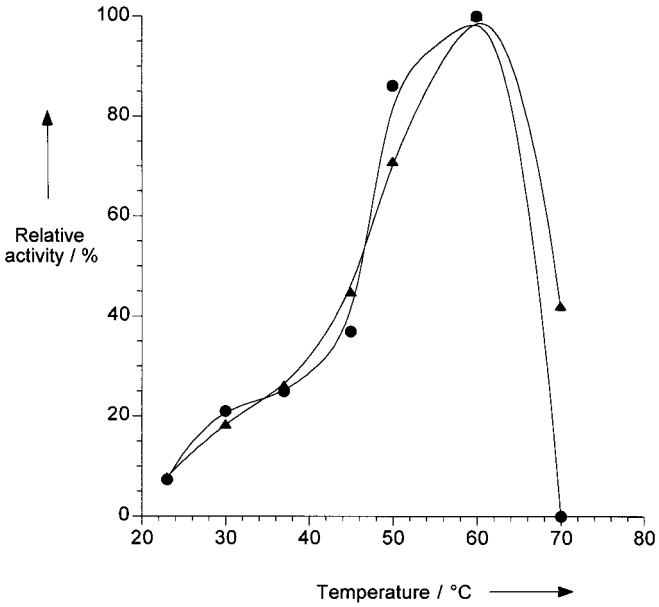

Figure 4. *Temperature profiles for recombinant PLE (●) and the PLE preparation from Fluka (▲), as determined with ethyl caprylate in the pH-stat assay. 100% activity refers to 512 U mg^{-1} (rPLE) and 1323 U mg^{-1} (Fluka PLE), respectively.*

commercial PLE from Sigma as determined with the pNPA assay (Figure 5). However, pH 9.0 was best for the PLE preparation from Fluka, which can be related to a different pattern of subunit "mixtures" or the presence of other hydrolases.

To clarify which isoenzyme was cloned, the substrate specificities of recombinant and commercial PLEs towards proline-β-naphthylamide and methyl butyrate were investigated. Figure 6

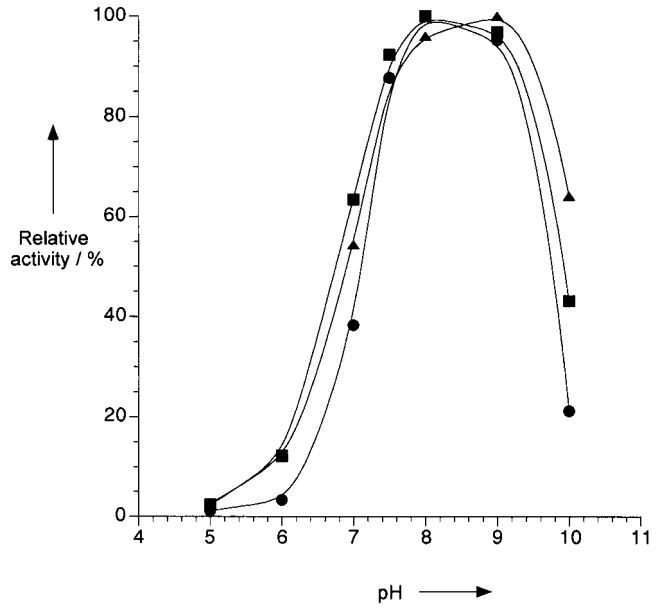

Figure 5. *pH profile of recombinant PLE (●) and the PLE preparations from Fluka (▲) and Sigma (■), as determined using the pNPA assay. 100% activity refers to 595 U mg^{-1} (rPLE), 296 U mg^{-1} (Fluka PLE) and 218 U mg^{-1} (Sigma PLE), respectively.*

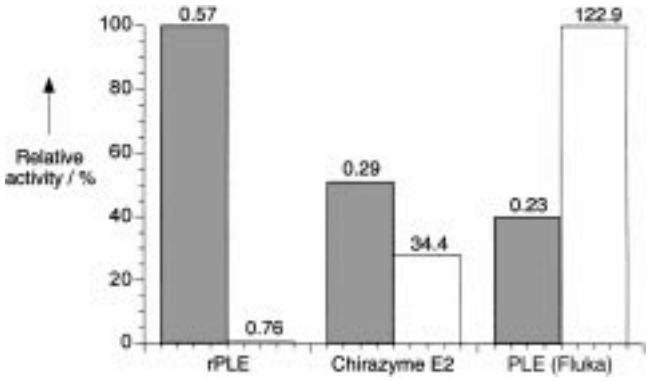

Figure 6. *Relative activity of recombinant and commercial PLE preparations towards proline-β-naphthylamide (grey columns) and methyl butyrate (white columns). Activity of rPLE towards proline-β-naphthylamide and of Fluka PLE towards methyl butyrate was set to 100%, and the activity of the other PLEs was calculated accordingly. Specific activities in U mg^{-1} are shown above the columns.*

clearly shows that recombinant PLE highly prefers PNA and exhibits very low activity towards methyl butyrate, in strong contrast to commercial PLE from Fluka and Chirazyme E2.

Discussion

As already outlined in the introduction, two groups reported the cloning of putative pig liver[4] or intestinal[6, 12] esterase genes, but they did not report the functional expression of active enzymes. Initially, we also failed in functional expression of PLE in either *E. coli* or *P. pastoris*, despite the use of various plasmid constructs and replacement of the 18 amino acid N-terminal leader sequence of PLE with the ompA signal sequence or the α-factor leader sequence, respectively. However, the key to successful functional expression of PLE in *P. pastoris* cells was the deletion of the tetrapeptide His-Ala-Glu-Leu at the C-terminus of the PLE gene, which seems to be an ER retention signal sequence, similar to those previously reported within the carboxylesterase family.[7] In particular, the three (out of four) tetrapeptides His-Val-Glu-Leu, His-Asn-Glu-Leu, and His-Thr-Glu-Leu identified in rat liver carboxylesterase were shown to be involved in retention, as secretion of active protein in COS-1 cells required their deletion. Thus, the existence of similar retention signal sequences in an esterase from pig liver appeared likely.

In order to identify, which PLE isoenzyme was produced by *P. pastoris*, we first subjected the protein to SDS-PAGE, from which the molecular weight was determined to be approximately 61 – 62 kDa (calculated: 62.419 kDa). Without the tags and including the C-terminal His-Ala-Glu-Leu, the molecular weight would be 60.098 kDa, which seems to be closer to the molecular weight reported for the β subunit (59.7 kDa) than to the γ subunit (61.4 kDa).[2, 3] However, this difference could be due to a different glycosylation pattern of pig liver produced esterase compared to that produced in *P. pastoris*. To provide further evidence that it was indeed the γ subunit that was cloned and expressed, recombinant PLE was subjected to hydrolysis reactions with methyl butyrate and proline-β-naphthylamide as substrates. According to the literature,[5] the γ subunit isoenzyme

should exhibit high activity towards proline-β-naphthylamide but little activity towards methyl butyrate. Indeed, we found that the recombinant enzyme highly prefers proline-β-naphthylamide in comparison with commercial PLE preparations (Figure 6), which contain a mixture of different isoenzymes. Native PAGE confirmed that mature PLE (recombinant and the crude extract from pig liver) is active as a trimer, which is in accordance to the observations reported for a proline-β-naphthylamidase.[13]

The active site of lipases and esterases consists of a catalytic triad containing the amino acids Ser-His-Asp. In addition, the consensus sequence motif Gly-Xxx-Ser-Xxx-Gly around the active site serine is found in all of these hydrolases.[1a] Sequence comparison with the data reported by David et al.[6] identified Ser_{203}, His_{448}, and Asp_{97}, as the active site residues of mature rPLE; the consensus sequence motif is Gly-Glu-Ser-Ala-Gly.

The functional expression of the pig liver esterase γ subunit isoenzyme also has a strong impact on biocatalysis. As pig liver esterase represents an enzyme widely used for the synthesis of optically pure products, it is of upmost importance, that the substrate selectivity and, especially, the stereoselectivity of the biocatalyst preparation is related to only one protein. Otherwise, one hydrolase (or isoenzyme) might show lower or even opposite stereoselectivity to others, which in turn will lead to lower overall selectivity. Furthermore, the (iso)enzyme composition of the pig liver extract may vary from batch to batch. It is obvious from the activity stainings in Figures 2 and 3 that crude commercial PLE preparations indeed contain various enzymes capable of cleaving α-naphthyl acetate. Besides the PLE isoenzymes ($\alpha-\gamma$ subunits), other hydrolases—presumably lipases—are also present. This is further supported by the observation that commercial PLE samples but not rPLE act on triolein,[11] which is a true lipase substrate. Thus, the functional expression of rPLE will also offer new opportunities for a reproducible application of this hydrolase in organic synthesis. First results have already shown that recombinant PLE exhibits substantially higher enantioselectivity in the kinetic resolution of acetates of secondary alcohols: In one case inversed stereopreference compared to commercial PLE preparations was also observed.[11]

Our studies might also encourage the cloning and functional expression of other PLE isoenzymes. Special focus should be given to whether similar or related tetrapeptides functioning as retention sequences can be also identified in these proteins.

Experimental Section

Materials: All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, USA), and Merck (Darmstadt, Germany) unless stated otherwise, at the highest purity available. Oligonucleotides were purchased from Interactiva (Ulm, Germany). Commercial PLE samples were obtained from Fluka, Aldrich, Sigma, and Roche Diagnostics (Chirazyme E1 and Chirazyme E2; Penzberg, Germany).

Microorganisms, plasmids, and growth conditions: *E. coli* DH5α [F^- *end*A1 *hsd*R17(rk^-, mk^+) *sup*E44 *thi*-1 λ^- *gyr*A96 *rel*A1 Δ(*arg*F-*lac*zya)U169] was used for the cloning and expression of PLE. Cells were cultivated in low salt Luria Bertani (LB) medium (yeast extract (10 g L^{-1}), peptone (10 g L^{-1}), NaCl (5 g L^{-1})) supplemented with nalidixic acid (50 mg L^{-1}) and, if required, with ampicillin (100 mg L^{-1}) or zeocin (25 mg L^{-1}) (Invitrogen, Carlsbad, CA, USA) at 37 °C.

Pichia pastoris X33 (Invitrogen) was used for expression of recombinant PLE. The following media were employed in the cultivation of *Pichia* cells under different conditions: YPD medium (yeast extract (1 %), peptone (2 %), and glucose (2 %)); YPDS medium (YPD medium supplemented with 1 M sorbitol); BMGY medium (yeast extract (1 %), peptone (2 %), potassium phosphate buffer (pH 6.0, 100 mM), and glycerol (1 %)); BMMY medium (BMGY, but using filter-sterilized methanol (0.5 %) instead of glycerol). The media were supplemented with zeocin (100 mg L^{-1}).

The *E. coli* cloning vector pUC19[14] was used for initial cloning of the PLE cDNA. The *E. coli* vectors pT – BTL2 and pT – ompA – BTL2[15] were used for expression of the PLE gene under the control of the strong, temperature-inducible λP_L promoter. The *E. coli*/*P. pastoris* shuttle vector pPICZαA (Invitrogen) was used for expression of PLE under the control of the alcohol oxidase (AOX1) promoter in *P. pastoris*.

Recombinant DNA technologies: Unless stated otherwise, standard DNA technologies were used.[16] The QIAprep Spin Miniprep kit, the Plasmid Midi kit, and the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) were used for plasmid DNA and DNA gel extractions, respectively. Restriction enzymes and other DNA modifying enzymes were used as specified from the suppliers (New England BioLabs, Beverly, MA, USA; Roche Diagnostics, Penzberg, Germany; GIBCO-BRL Life Technologies, Eggenstein, Germany; MBI Fermentas, St. Leon-Rot, Germany).

DNA-sequencing reactions were carried out on both strands of double-stranded templates using the Taq Ready Reaction Dye Deoxy Terminator Cycle Sequencing kit (Applied Biosystems, Weiterstadt, Germany). The sequencing products were analyzed on a 373 DNA sequencer (Applied Biosystems).

Standard protocols were used for the preparation and transformation of competent *E. coli* cells.[17] Plasmids were transformed into *P. pastoris* by electroporation according to the supplier's instructions.

mRNA isolation, cDNA synthesis and cloning of PLE cDNA: Tissue from fresh pig liver (0.8 g) was homogenized (Polytron PT 3000, Kinematica AG, Littau, Switzerland) and poly(A) RNA was isolated using the Fast Track 2.0 kit (Invitrogen) according to the procedure given in the manual.

First-strand cDNA synthesis by RT-PCR was performed using the cDNA Cycle kit (Invitrogen) with an oligo(dT) primer following the protocol of the kit. RT-PCR products were used as templates for the amplification of the complete PLE cDNA using two gene-specific primers (primer 1: 5′-gatat*cccgggcatatg*tggcttctcccgctggt-3′, with italicized restriction sites *Sma*I, and *Nde*I; primer 2: 5′-gcat*cccgg-gaattct*cacagctcagcatgcttta-3′, italicized restriction sites *Sma*I and *Eco*RI; underlined sections are start and stop codons. Both primers were designed based on the cDNA sequence of porcine liver proline-β-naphthylamidase[4] and contained restriction sites for subsequent cloning of the PCR product. PCR was performed in a thermocycler (Robocycle Gradient 40, Stratagene, La Jolla, CA, USA). After an initial denaturation step (95 °C for 5 min), the following temperature program was used: 95 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, for a total of 28 cycles.

The purified PCR products were initially cloned into the *Sma*I site of the pUC19 vector. The resulting plasmid pUC19 – PLE (Figure 1, **1**) was used for sequence verification and subsequent subcloning of the isolated PLE gene.

Construction of expression vectors: *E. coli*: Plasmid pUC19 – PLE was cleaved with *Nde*I and *Eco*RI, and the resulting 1705 bp fragment encoding the complete PLE sequence, including the native leader

sequence, was inserted into the respective *Nde*I/*Eco*RI site of pT1–BTL2,[9] resulting in pCYTEX–PLE (6652 bps) (Figure 1, **2**). In a second construct, pCYTEX–ompA–mPLE (6682 bps), the native leader sequence of PLE was replaced with the *E. coli* ompA leader sequence to facilitate efficient periplasmic protein secretion in *E. coli*. Therefore, the mature PLE gene without N-terminal leader sequence was amplified and ligated blunt-end into the vector backbone pCYTEX–ompA obtained after PCR amplification of the vector pT–ompA–BTL2 (Figure 1, **3**).

P. pastoris: For expression in *P. pastoris*, the sequence encoding the mature PLE was cloned in-frame downstream of the yeast α-factor secretion signal sequence in the *E. coli*/*P. pastoris* shuttle vector pPICZαA. For this, the gene was amplified with two primers introducing an *Eco*RI site (5′-aagct*gaattc*gggcagccagcctcgccgcct-3′) and an *Xba*I site (5′-gtcag*tctaga*tcacagctcagcatgctttatc-3′, stop codon underlined) at its 5′-end and 3′-end, respectively. *Eco*RI and *Xba*I digestion of the resulting amplification product and ligation into the likewise-cut plasmid pPICZαA obtained expression vector pPICZα–mPLE (5183 bps) (Figure 1, **4**). Expression vectors pPICZα–mPLE* (5171 bps) and pPICZα–mPLE*–tag (5171 bps) (Figure 1, **5**, **6**) containing a truncated mature PLE gene lacking the four C-terminal amino acids His-Ala-Glu-Leu were created in essentially the same manner as pPICZα–mPLE. The deletion was introduced with accordingly modified 3′-primers which either introduced a stop codon (primer: 5′-acc*tctaga*tcactttatcttgggtggcttc-3′, stop codon underlined) to generate pPICZα–mPLE* or allowed C-terminal fusion of the truncated gene in-frame to the His and myc tag sequences provided by pPICZαA (primer: 5′-acc*tctaga*tacttatcttgggtggcttc-3′) to give pPICZα–mPLE*–tag.

The created expression vectors were linearized with *Pme*I and transformed into *P. pastoris* using the Invitrogen electroporation method.

Expression of recombinant PLE in *E. coli* DH5α: Recombinant *E. coli* DH5α harboring pCYTEX–PLE or pCYTEX–ompA–mPLE were grown at 37 °C and 200 rpm in LB medium until an OD_{578} value of 0.8–1.0 was reached. Recombinant protein expression was induced by an increase of temperature to 42 °C. Samples were taken every hour. After 3–4 hours of induction, cells were harvested and stored at −20 °C or used immediately for SDS-PAGE or activity assays.

Shaking-flask cultivation of *P. pastoris* and secreted expression of the pig liver esterase: Recombinant clones selected on zeocin plates were picked and grown in YPDS medium at 30 °C and 200 rpm until the OD_{600} value was approximately 15. This preculture (200 μL) was used to inoculate BMGY medium (25 mL), which was then incubated overnight at 30 °C. The yeast cells were collected by centrifugation (5 min, 3000 *g*, 4 °C) and transferred to the BMMY induction medium until an OD_{600} value of 1.0 was reached. Induction was performed by daily addition of methanol (0.5% (v/v)). After a 96-hour induction, cells were harvested by centrifugation. Supernatants containing recombinant enzyme were concentrated using Centricons (20 mL, NMWL 30 000, Ultracel-PL membrane, Millipore) for 15 min at 4000 *g* and 4 °C. Activity during cultivation, after cell harvesting and in concentrated enzyme solution was determined by the pNPA assay (see below). Proteins were then analyzed by gel electrophoresis (see below). Due to the presence of disturbing peptides in the media (from yeast extract and peptone), protein concentrations were determined by densitometry using known concentrations of bovine serum albumin as a reference protein. For this, the National Institutes of Health (NIH) imager (available at: http://rsb.info.nih.gov/nih-image/download.html) in combination with a special macro (Macintosh version, available from Dr. T. Seebacher, email: thomas.seebacher@uni-tuebingen.de) for molecular mass and protein content determination was used. For better comparison, the protein concentration of commercial PLEs was determined in a similar manner.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE): Commercial pig liver esterase solution (20 μL, 100 U mL^{-1}, based on the pNPA assay) or tenfold-concentrated *P. pastoris* culture supernatants (20 μL) were mixed with SDS–sample (10 μL) buffer. After heating to 95 °C for 5 min, samples were separated on polyacrylamide gels (12.5%) with a stacking gel (4%). Gels were stained for protein detection with Coomassie Brilliant Blue. For esterase-activity staining, proteins were first renaturated by a 12 h incubation in a Triton X-100 solution (0.5% in 0.1 M tris(hydroxymethyl)aminomethane (Tris)/HCl (pH 7.5)). Next, the gel was incubated in a mixture of freshly prepared solutions A and B (1:1) for 2 h (A: α-naphthyl acetate (20 mg) dissolved in acetone (5 mL), followed by addition of Tris/HCl (50 mL, 0.1 M; pH 7.5); B: Fast Red TR salt (50 mg) dissolved in Tris/HCl (50 mL, 0.1 M; pH 7.5)). In the presence of hydrolytic (lipase or esterase) activity, released α-naphthol forms a red complex with Fast Red.[18]

Native polyacrylamide gel electrophoresis: Commercial pig liver esterase solution (10 μL, ≈0.1 U) and concentrated *P. pastoris* culture supernatants (5–10 μL, ≈0.05–0.1 U) were mixed with sample buffer (10 μL). Samples were separated on polyacrylamide gels (7.5%) with a stacking gel (4.5%). Gels were activity stained as described above, followed by staining with Coomassie Brilliant Blue.

Isoelectric focussing: Concentrated *P. pastoris* culture supernatants (1–5 μL, ≈0.02–0.1 U) were mixed with sample buffer (10 μL). Samples were separated on polyacrylamide gels (5%) containing carrier ampholyte (2.4%; pH 3–10; Serva). Gels were activity stained as described above, then the bands were fixed with trichloroacetic acid solution (10% (w/v) for 10 min, then 1% (w/v) overnight) followed by staining with Coomassie Brilliant Blue.

***N*-terminal protein sequencing:** Prior to N-terminal sequence analysis, the commercial PLE preparation from Aldrich—which was the purest product available—was separated on SDS-PAGE as described above. The band at approximately 60 kDa was electroblotted onto a PVDF membrane according to Matsudeira.[19] Sequencing was carried out with a gas-phase sequencer (491 Protein sequencer, Applied Biosystems, Weiterstadt, Germany).

Esterase activity: Esterase activity was determined photometrically in sodium phosphate buffer (50 mM) with *p*-nitrophenyl acetate (10 mM dissolved in dimethyl sulfoxide) as the substrate. The amount of *p*-nitrophenol released was routinely determined at 410 nm ($\varepsilon = 12.36 \times 10^3\,\text{M}^{-1}\,\text{cm}^{-1}$) at room temperature and pH 7.5. In addition, activity measurements were performed at different pH values for pH-optima determination. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per min under the assay conditions. Substrate specificity of PLE was measured by means of a pH-stat assay. A known amount of esterase was added to an emulsion (30 mL) containing ester (5% (v/v); methyl butyrate, ethyl caprylate, ethyl acetate, triolein, or tributyrin) and gum arabic (2% (w/v)) at 37 °C. Liberated acid was titrated automatically in a pH-stat (Schott, Mainz, Germany) with 0.01 N NaOH in order to maintain a constant pH value of 7.5. One unit of activity was defined as the amount of enzyme releasing 1 μmol acid per min under assay conditions. Activity measurements at different temperatures were performed using ethyl caprylate as substrate at pH 7.5.

Proline-β-naphthylamidase activity: Proline-β-naphthylamidase activity was determined photometrically with proline-β-naphthylamide (0.2 mM in dimethyl sulfoxide) as the substrate. The reaction mixture (500 μL) containing Tris/HCl (0.1 M; pH 8.0), substrate solution (50 μL),

and recombinant or commercial PLE preparations (0.4 U (based on the pNPA assay)) was incubated at 37 °C for 30 min. The reaction was terminated by addition of the coupling reagent FastGarnet (Sigma; 1.5 mL) prepared according to Barret.[20] The amount of β-naphthylamine released was determined at 520 nm ($\varepsilon = 24.03 \times 10^3\,\text{M}^{-1}\,\text{cm}^{-1}$). One unit (U) of amidase activity was defined as the amount of enzyme releasing 1 μmol β-naphthylamine per min under the assay conditions.

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