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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Functional expression of the γ-isoenzyme of pig liver carboxyl esterase in *Escherichia coli*

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Abstract The previously reported functional expression of the γ-isoenzyme of pig liver carboxylesterase (γ-rPLE) in *Pichia pastoris* is hampered by the small amount of active enzyme formed. Earlier attempts for expression in *Escherichia coli* failed completely and not even inactive protein was detected. The lack of glycosylation ability of *E. coli* was ruled out as a possible reason, as it could be shown in this work that deglycosylated PLE also is active. Expression of γ-rPLE was studied using a range of *E. coli* strains with careful design of the constructs used and control of the cultivation conditions. Indeed, expression in *E. coli* strains Rosetta, Origami and Rosetta-gami was successful, but the majority of enzymes was present as inclusion bodies and only little soluble but inactive protein was detected. Denaturation and refolding of inclusion bodies failed. However, with the *E. coli* strain Origami, coexpressing the molecular chaperones GroEL und GroES, a functional expression of γ-rPLE was possible. The recombinant enzyme was released by cell disruption and subjected to His-tag purification. The purified esterase had a specific activity of 92 U mg^{-1} protein and a V_{max}/K_m value of 10.8×10^{-3} min^{-1} towards *p*-nitrophenyl acetate. Activity staining of native polyacrylamide gels gave a single band at 175 kDa with esterolytic activity indicating a trimeric form of γ-rPLE (~60 kDa per monomer). γ-rPLE was biochemically characterized and its properties were compared to the enzyme previously expressed in *P. pastoris*. pH and temperature profiles were identical and highest activity was found at pH 8–8.5 and 60 °C, respectively. In the kinetic resolution of (*R*,*S*)-1-phenyl-2-butyl acetate with esterase from both expression hosts, similar enantioselectivities (*E*=50) were found.

Keywords Enzyme catalysis · Hydrolases · Inclusion bodies · Pig liver esterase

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Introduction

Pig liver esterase (PLE, EC 3.1.1.1) is a serine-type esterase and belongs to the class of carboxylester hydrolases. It currently represents the most useful esterase for organic synthesis, and numerous reports have shown its efficient application in kinetic resolutions and desymmetrizations of a wide range of organic compounds (Bornscheuer and Kazlauskas 2005; de Maria et al. 2005; Faber 2000; Jones 1990; Jones et al. 1985; Lam et al. 1986). PLE obtained from pig liver tissue by extraction with organic solvents consists of various isoenzymes, and ααα-, βββ- and γγγ-trimers are the three major components. We could overcome the undesired presence of different PLE isoenzymes in commercial preparations and the interfering influence of other hydrolases by functional overexpression of a specific isoenzyme (γ-rPLE) in the yeast *Pichia pastoris* (Lange et al. 2001). It could also be proven that the recombinant enzyme shows, in contrast to commercial preparations, higher enantioselectivities and partially inversed stereopreference in the kinetic resolution of acetates of secondary alcohols (Musidlowska et al. 2001; Musidlowska-Persson and Bornscheuer 2002). Unfortunately, the productivity in

P. pastoris is unsatisfactory low and alternative expression systems were investigated.

The *E. coli* expression system has many advantages for the high-level production of recombinant proteins like fast growth and protein production and the extensive knowledge on its genetics and molecular biology (Makrides 1996). However, not every gene can be expressed efficiently in this organism due to the unique and subtle structural features of the gene sequence, the stability and translational efficiency of mRNA, the degradation of the protein by host cell proteases and major differences in codon usage between the foreign gene and native *E. coli* (Snehasis and Deb 2005). The overexpression of recombinant proteins of eukaryotic origin in prokaryotic systems has a major drawback. The expression in bacterial hosts often yields inappropriate folded proteins because of the inability to perform posttranslational modifications like glycosylations, the lack of a secretion mechanism for the efficient release of protein into the culture medium and the limited ability to perform disulfide bonds. The application of specialized *E. coli* strains optimized for expression of proteins from hosts with a different codon usage (Novy et al. 2001) or proteins containing disulfide bonds (Besette et al. 1999; Prinz et al. 1997), and the coexpression of molecular chaperones (Nishihara et al. 1998) are strategies of choice to improve the yield of soluble protein.

So far, the expression of PLE in *E. coli* has not been reported. Earlier expression attempts in *E. coli* failed completely and not even inactive protein was detected (Lange et al. 2001). We now describe for the first time the functional expression of a PLE isoenzyme in *E. coli.* The recombinant enzyme was purified via a fused His-tag, was biochemically characterized, and its properties were compared to the recombinant pig liver esterase isoenzyme (γ-rPLE) expressed in *P. pastoris*.

Materials and methods

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany) at the highest purity available, unless stated otherwise. Oligonucleotides were obtained from MWG Biotech (Ebersberg, Germany). (*R*,*S*)-1-phenyl-2-butyl acetate (**1**) was synthesized from commercially available alcohol (**1a**) using standard procedures as described (Musidlowska-Persson and Bornscheuer 2002). Gas chromatographic analysis was conducted using a Heptakis-(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrin column (Hydrodex-β-3*P*, 25 m×0.25 mm; Macherey-Nagel, Düren, Germany); carrier gas: H_2; flame ionisation detector. Retention times: **1** (110 °C isothermal): (*S*)-**1**, 11.1 min; (*R*)-**1**, 11.8 min; (*S*)-**1a**, 12.9 min; and (*R*)-**1a**, 13.7 min. Absolute configurations were assigned as already described (Musidlowska-Persson and Bornscheuer 2002).

Microorganisms, plasmids 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 DH5α [supE44ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1relA1] were used for the cloning experiments. *E. coli* strains Rosetta(DE3)[F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm (DE3) $pRARE^2$ (Cam^R)], Origami(DE3)[Δ(ara-leu) 7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL F'[lac^+ $lacI^q$ pro] (DE3) gor522Tn10 trxB (Kan^R, Str^R, Tet^R)4], Rosetta-gami B(DE3) [F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm lacY1 aphC (DE3) gor522::Tn10 trxB $pRARE^2$ (Cam^R, Kan^R, Tet^R)], all from Novagen (Madison, WI, USA), and BL21 Star(DE3) (Invitrogen) [F^- ompT $hsdS_B(r_B^- m_B^-)$ gal dcm rne131(DE3) were used for expression experiments.

For coexpression experiments, the Chaperone Plasmid Set (TAKARA BIO, Inc., Otsu, Shiga, Japan) containing the plasmids pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16 was used.

The *E. coli* cloning vector pET101/D-TOPO from TOPO directional cloning kit (Invitrogen) was used for the blunt end ligation of all PCR products to simplify the later cohesive end generation and sticky end ligation into the expression vector. The *E. coli* expression vectors pET22b and pET15b were obtained from Novagen. 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 different temperatures (20–37 °C).

Recombinant DNA technologies

All routine DNA technologies like subcloning and ligation were performed according to standard protocols (Sambrook and Russel 2001). For cloning, the Champion pET Directional TOPO Expression Kit (Invitrogen) was used. A QIAprep Spin Miniprep kit and a QIAQuick Gel Extraction kit (Qiagen, Hilden, Germany) were used for DNA purification. Restriction enzymes and other DNA-modifying enzymes were used as specified by the suppliers (New England Biolabs, Beverly, MA, USA; Promega, Madison, WI, USA). DNA-sequencing reactions were carried out at MWG-Biotech. Standard protocols were used for the preparation and transformation of competent *E. coli* cells (Chung et al. 1989).

Construction of expression vectors

Plasmid pCYTEX-ompA-mPLE (Lange et al. 2001) was cleaved with *Nde*I and *Eco*RI, and the resulting 1,710-bp fragment encoding the complete PLE sequence, including the *E. coli* OmpA leader sequence to facilitate efficient periplasmic protein secretion in *E. coli* and the putative C-terminal ER retention signal HAEL, was inserted into the respective *Nde*I/*Eco*RI site of pET22b to give pET22b-OmpA-mPLE (7,142 bp) (Fig. 2,**1**). In all following constructs, the C-terminal signal sequence was substituted by a stop codon during amplification. In a second construct, the ompA leader sequence was replaced by the pelB sequence from pET22b. Therefore, mature PLE gene was amplified without N-terminal leader sequence to create an N-terminal *Eco*RI site, a C-terminal *Xho*I site and a stop codon following blunt end ligation into pET101/D-TOPO. After generation of cohesive ends by enzymatic cleavage and purification via gel extraction, the gene was ligated into pET22b with the N-terminal pelB leader sequence for potential periplasmic localization connected by a 32-bp linker. To stay in-frame, one cytosine was added to the 5′-primer resulting in pET22b-pelB-mPLE (7,103 bp) (Fig. 2,**2**).

For cytoplasmic expression in *E. coli*, Origami and Rosetta-gami constructs without N-terminal leader sequences were required. The sequence encoding the mature PLE was amplified without OmpA leader and a new N-terminal *Nde*I restriction site was created. After blunt end ligation into pET101/D-TOPO and digestion with *Nde*I/*Eco*RI, the mPLE gene was ligated into the likewise-cut pET22b to generate pET22b-mPLE (7,039 bp) (Fig. 2,**3**). Cloning into the vector pET15b via *Nde*I/*Xho*I restrictions sites, also introduced by PCR, allowed an N-terminal fusion of the PLE gene to a His-tag sequence and gave pET15b-mPLE-tag (Fig. 2,**4**).

Enzymatic deglycosylation

Fifteen milligram of recombinant γ-PLE preparation from *P. pastoris* (Lange et al. 2001) was incubated for 10 min at 100 °C in 1× glycoprotein denaturation buffer (30 μL). After cooling to room temperature, 3 μL of 10× G7 buffer and 3 μL 10% NP40 solution were added. Finally, the enzymatic reaction was started by addition of 5 μL PNGaseF (New England Biolabs) and was kept for 1 h at 37 °C. The product was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue and activity staining.

Expression of recombinant PLE in *E. coli*

Expression constructs were transformed into different recombinant *E. coli* strains that were used for expression experiments of recombinant PLE. Single colonies were grown overnight in 5 mL of LB medium at 30 °C. The next day, the preculture was diluted into the main culture to an OD_{600} value of 0.05. In general, cells were grown at 30 °C and 200 rpm in 50 mL LB medium until an OD_{600} value of 1 was reached. Recombinant protein expression was induced by addition of 20–500 μM isopropyl β-D-thiogalactopyranoside (IPTG). Samples of 5 mL were taken every 2 h; 24 h after induction, cells were harvested and stored at −20 °C or used immediately after cell disruption for SDS-PAGE, native PAGE or activity assays.

Coexpression of recombinant PLE with chaperones in *E. coli* Origami(DE3)

Initially, *E. coli* Origami(DE3) cells were transformed with one of the five different chaperone plasmids (pG-KJE8, pGro7, pKJE7, pG-Tf2 and pTf16). After the production of chemically competent cells for each Origami-chaperone strain, these cells were transformed with expression construct **4**. The coexpression of the chaperones with PLE was performed in LB medium containing 20 μg mL^{-1} chloramphenicol and 50 μg mL^{-1} ampicillin for plasmid selection. The chaperone expression was immediately initiated by addition of 1 mg mL^{-1} *L*-arabinose. When the OD_{600} reached a value of 0.5, the PLE production starts after induction with 40 μM IPTG. After 24 h, the cells were harvested, resuspended in 10 mL sodium phosphate buffer (50 mM, pH 7.5) and sonified. The protein content and activity of the soluble protein were determined by Bradford assay and *p*-nitrophenyl acetate (pNPA) assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Samples from cultivation after sonification were divided into soluble and insoluble fractions (10 μL) and were analysed by SDS-PAGE on polyacrylamide gels (12.5% or 8%) with a stacking gel (4%). The proteins in the low-molecular-weight standard mixture obtained from Sigma or Roth were used as reference. Gels were stained for protein detection with Coomassie brilliant blue. For esterase-activity staining, proteins were first renatured by incubation for 1 h in Triton X-100 solution (0.5% in 0.05 M phosphate buffer, pH 7.5). Next, the gel was incubated in a mixture of freshly prepared solutions of α-naphthyl acetate and Fast Red. In the presence of hydrolytic activity, released α-naphthol forms a red complex with Fast Red (Krebsfänger et al. 1998).

Western blotting

Western blot analysis was done on nitrocellulose membrane against the His-tag using Ni–nitrilotriacetic acid (NTA)

conjugate solution and nitroblue tetrazolium (NBT)/bromochloroindolyl phosphate (BCIP) as substrate following the instructions of the manual (QIAexpress Detection and Assay Handbook, 1999).

Native polyacrylamide gel electrophoresis

Recombinant PLE solution (5–15 μL, corresponding to 0.05–0.15 U from pNPA assay) was mixed with sample buffer (5–10 μL). Samples were separated on polyacrylamide gels (7.5%) with a stacking gel (4.5%). Gels were activity stained as described above, followed by Coomassie brilliant blue staining.

Molecular weight determination by Ferguson analysis

Recombinant PLE samples were characterized on a set of native gels with various polyacrylamide concentrations (4.5, 5, 5.5, 6, 7, 8, 9, 10%) with a 4.5% stacking gel. The proteins in the high-molecular-weight standard mixture obtained from Sigma were used as reference. Gels were activity stained as described above, followed by staining with Coomassie brilliant blue. The electrophoretic mobility (R_f) of the protein was determined in each gel relative to the tracking dye; $100\times\log(R_f\times100)$ is plotted against the percent polyacrylamide concentration for each protein. The slope of such a plot is the retardation coefficient (K_R). From these plots, individual slopes are determined for each standard protein and the logarithm of the molecular weight of a standard protein. This gives a linear plot from which the molecular weight of the unknown protein was calculated (Sigma Tech. Bulletin No. MKR-137, 1986).

Esterase activity

Esterase activity was determined spectrophotometrically in sodium phosphate buffer (50 mM) with *p*-nitrophenyl acetate (10 mM dissolved in dimethyl sulfoxide) as substrate. The amount of *p*-nitrophenol released was determined at 410 nm ($\varepsilon=15\times10^3$ M^{-1} cm^{-1}) at room temperature and pH 7.5. One unit (U) of esterase activity was defined as the amount of enzyme releasing 1 μM of *p*-nitrophenol per minute under assay conditions (Krebsfänger et al. 1998). Substrate specificity of PLE was measured in a pH-stat assay. A known amount of esterase was added to an emulsion (20 mL) containing ester substrate [5% (v/v); tributyrin, ethyl caprylate or methylbutyrate] 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 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 minute under assay conditions. Activity measurements for temperature profile were performed using tributyrin as substrate at pH 7.5.

Esterase-catalysed kinetic resolution of (*R*,*S*)-1-phenyl-2-butyl acetate (**1**)

(*R*,*S*)-1-phenyl-2-butyl acetate was 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, Hamburg, Germany) at 37 °C. For each reaction, 0.2-U esterase (based on the pNPA assay) was used. Reactions were stopped 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 the literature (Chen et al. 1982).

Results

Expression of recombinant PLE

For the expression of a protein of eukaryotic origin in a prokaryotic host, it had to be elucidated first, if a non-glycosylated PLE can still be active, because native PLE has been shown to be a glycoprotein (Heymann and Junge 1979) and glycosidic chains were already reported to influence enzyme activity and stability (Brocca et al. 2000; Yoshimasu et al. 2004). Therefore, a sample of recombinant PLE from a *P. pastoris* culture (γ-rPLE) was treated with PNGaseF and deglycosylated enzymatically. The subsequent SDS-PAGE analysis and activity staining showed two active protein bands at ~63 and ~58 kDa representing glycosylated and non-glycosylated γ-rPLE (Fig. 1). Thus, glycosylation is no prerequisite, and therefore, the protein should be in principle expressable in active form in *E. coli* (Hirschberg et al. 2001).

Four different expression systems were constructed (Fig. 2) and investigated to produce the pig liver esterase isoenzyme in specialized *E. coli* strains.

Constructs **1** and **2** were designed for periplasmic expression of PLE in *E. coli* strains Rosetta(DE3), BL21 Star(DE3). For cytoplasmic expression in Origami(DE3) and Rosetta-gami B(DE3), the leader signal-free constructs **3** and **4** were created. In all four systems, the majority of the protein was insoluble and present as inclusion bodies in the cytoplasm (data not shown). Furthermore, we could not find in our studies an influence of the HAEL sequence encoding for an endoplasmic reticulum retention signal for the expression in the prokaryotic host *E. coli*.

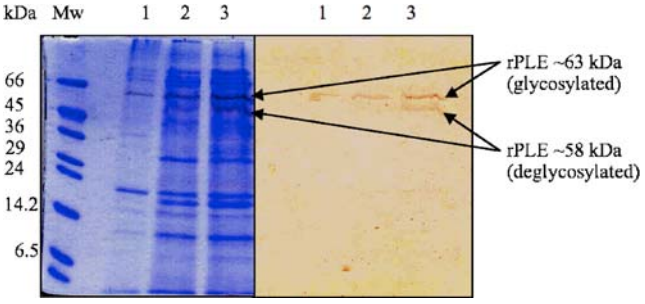

Fig. 1 SDS-PAGE analysis of *P. pastoris* PLE samples: activity stained (*right part*) and Coomassie stained (*left part*). *Mw* molecular weight standard (66 kDa, bovine serum albumin; 45 kDa, chicken egg ovalbumin; 36 kDa, rabbit muscle glyceraldehyde-3-phosphat dehydrogenase; 29 kDa, bovine erythrocytes carbonic anhydrase; 24 kDa, bovine pancreas trypsinogen; 20 kDa soy bean trypsin inhibitor; 14.2 kDa, bovine milk α-lactalbumin; 6.5 kDa bovine lung aprotinin). *Lane 1*: 0.2 U γ-PLE (control); *lanes 2 and 3*: 0.1 U+0.2 U rec. PLE, PNGaseF treated. Units are based on the pNPA assay

Attempts to increase the solubility of the protein by using lower growth temperatures, additives like ethanol (Thomas and Baneyx 1997) or sucrose and different inducer concentrations were unsuccessful. Additionally, renaturation of the protein using urea or guanidine and the attempts for subsequent refolding under various conditions failed because of reaggregation of the protein. However, expression of the PLE gene in the *E. coli* strain Origami (DE3) and coexpression of the chaperones GroEL and GroES lead to a considerable amount of soluble and functional pig liver esterase γ-isoenzyme.

Characterization of recombinant mPLE

After a 24-h cultivation, *E. coli* Origami(DE3) cells were harvested by centrifugation. The cell pellet was washed twice with phosphate buffer and disrupted by sonification. Supernatant and insoluble fractions were collected separately for different analysis methods. His-tag purification of the recombinant PLE yielded a specific activity of

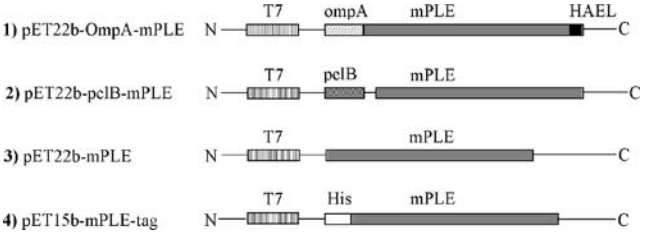

Fig. 2 Expression vectors constructed: Expression cassettes containing the promotor, the leader sequence, the PLE gene and the N-terminal tags are given. Vectors **1** and **2** were used for periplasmic expression, vectors **3** and **4** for cytoplasmic expression. *T7* IPTG-inducible promoter, *ompA* outer membrane protein A secretion signal, *pelB* pectate lyase B signal sequence for periplasmic translocation, *HAEL* C-terminal endoplasmic reticulum retention signal, *His* sixfold His-tag

92 U mg^{-1} protein (based on the pNPA assay). SDS-PAGE analysis (Fig. 3) of crude cells extract showed one major protein band with a molecular weight of 61 kDa in the soluble and also in the insoluble fraction. This value closely matches the theoretical calculated size of 61.5 kDa. The protein band at 60 kDa in the inclusion body fraction is mainly correlated to highly overexpressed chaperone GroEL that has nearly the same size as γ-PLE. On native PAGE, only one protein band showed hydrolytic activity upon α-naphthyl acetate/Fast Red staining. The native molecular weight determined by Ferguson analysis was approx. 175 kDa, confirming the presence of a PLE trimer as the most active form (Fig. 4, left part). To confirm the expression of recombinant γ-PLE, a Western blot against the His-tag was performed. Indeed the detection was successful and a thin red band appeared on the nitrocellulose membrane (Fig. 4, right part).

The V_{max}/K_m value of purified recombinant PLE isoenzyme was 10.8×10^{-3} min^{-1} with pNPA as substrate, which was lower than the corresponding value of 109×10^{-3} min^{-1} for PLE expressed in *P. pastoris*. This difference in kinetic values is mostly due to the difficulties in the determination of protein content in the culture media of *P. pastoris*, which—in contrast to the *E. coli*-derived product—could not be performed with a Bradford assay but by densitometric estimation only.

For comparison of PLE substrate specificity from both hosts, different substrates were used in pH-stat assays (Fig. 5). There were only slight differences in the hydrolytic activities between the two PLE preparations from different

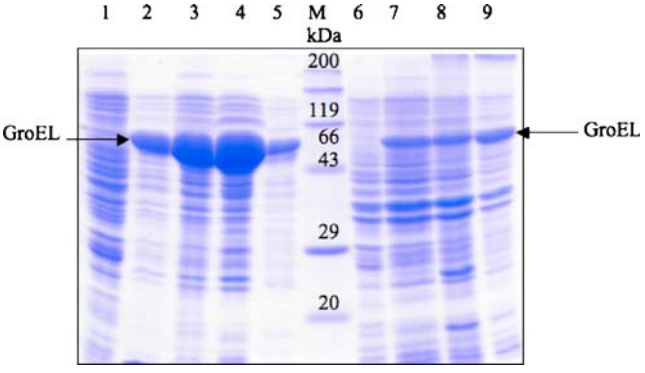

Fig. 3 SDS-PAGE analysis of soluble (*left gel*) and insoluble (*right gel*) fractions from *E. coli* Origami cell lysate from construct **4** transformed with chaperone pGro7. The gel was stained with Coomassie brilliant blue. *Lane 1*: control 1 (*E. coli* Origami WT); *lane 2*: control 2 (*E. coli* Origami-pGro7); *lane 3*: *E. coli* Origami-pGro7-pET15b-γPLE (37 °C cultivation); *lane 4*: *E. coli* Origami-pGro7-pET15b-γPLE (30 °C cultivation); *lane 5*: His-tag-purified PLE; *M* molecular weight standard (200 kDa, bovine myosine; 119 kDa, rec. *E. coli* β-galactosidase; 66 kDa, bovine serum albumin; 43 kDa, ovalbumin; 29 kDa, bovine erythrocytes carbonic anhydrase; 20 kDa, soy bean trypsin inhibitor; 14.5 kDa, chicken lysozyme); *lanes 6–9*: inclusion body fractions of samples from *lanes 1–4*

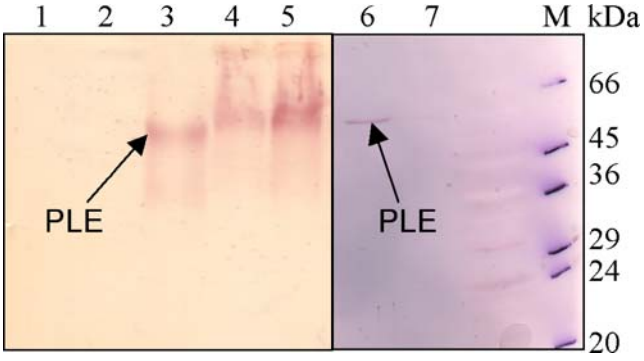

Fig. 4 *Left part*: Native PAGE analysis of recombinant PLE from *E. coli* Origami(DE3) coexpressing the chaperones GroEL und GroES. Gels were activity stained only. *Lane 1*: *E. coli* Origami WT; *lane 2*: *E. coli* Origami-pGro7; *lane 3*: His-tag-purified PLE; *lane 4*: *E. coli* Origami-pGro7-pET15b-γ -PLE (37 °C cultivation); *lane 5*: *E. coli* Origami-pGro7-pET15b-γ-PLE (30 °C cultivation). *Right part*: Western blot of recombinant γ-PLE produced in *E. coli* Origami (DE3) coexpressing the chaperones GroEL/GroES. *Lane 6*: His-tag-purified γ-PLE; *lane 7*: *E. coli* Origami WT cell extract; *M* molecular weight standard (66 kDa, bovine serum albumin; 45 kDa, chicken egg ovalbumin; 36 kDa, rabbit muscle glyceraldehyde-3-phosphat dehydrogenase; 29 kDa bovine erythrocytes carbonic anhydrase; 24 kDa, bovine pancreas trypsinogen; 14 kDa, bovine milk α-lactalbumin). The marker proteins are only valid for the right gel

origin. Recombinant PLE from *E. coli* hydrolysed ethyl caprylate and tributyrin similar to PLE from *P. pastoris* with almost equal activity, whereas tributyrin was preferred as a typical esterase substrate, and methyl acetate was cleaved only with low activity.

Activity measurements of recombinant PLE from *E. coli* and *P. pastoris* from 20 to 70 °C showed a comparable temperature behaviour in the hydrolysis of tributyrin. The highest activity was observed at 60 °C (Fig. 6), but in contrast to earlier studies (Lange et al. 2001), the recombinant PLE from both hosts was still active at 70 °C.

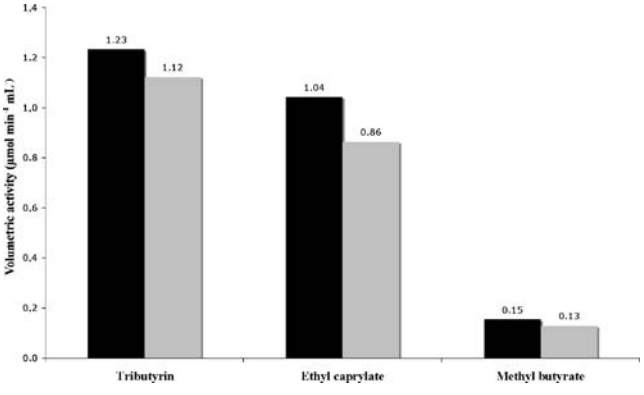

Fig. 5 Volumetric activities of recombinant PLE from *E. coli* pET15b-mPLE-tag (*black columns*) and *P. pastoris* (*grey columns*) towards tributyrin, ethyl caprylate and methyl butyrate as determined in the pH-stat assay

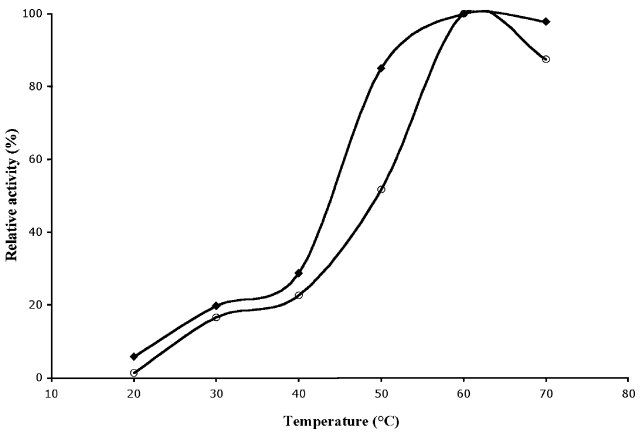

Fig. 6 Temperature profiles for recombinant PLE from *E. coli* (construct **4**) and *P. pastoris* as determined with tributyrin in the pH-stat assay. 100% activity refers to 5.44 U mL^{-1} for *E. coli* PLE (♦) and 3.9 U mL^{-1} for *P. pastoris* PLE (●), respectively

For the determination of the pH-profile, the pNPA assay was used. Because no universal buffer works over the pH range 5–10, the pH profile was determined with three different buffers for optimal buffering conditions. Citrate buffer at a concentration of 10 mM was chosen for a pH range from 5 to 7, 10 mM phosphate buffer for pH 7 to 8 and 10 mM borax buffer for pH 8 to 10. To investigate the influence of different buffers on enzyme activity, the pNPA assay at pH 7.0 was performed twice with citrate and phosphate puffer, at pH 7.5 with all three buffers and at pH 8.0 with phosphate and borax buffers. At pH 7.0 and 7.5, the activity values for all buffers were relatively close together for both enzyme samples. However, at pH 8, there was a difference in the activity of the enzyme using either

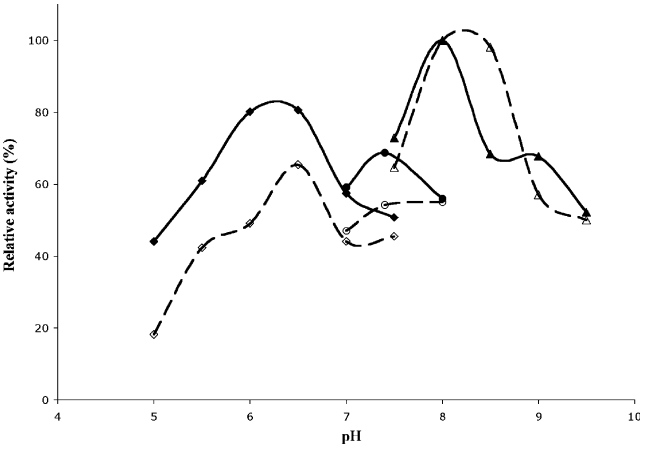

Fig. 7 pH profiles for recombinant PLE from *E. coli* (construct **4**) and *P. pastoris* in citrate buffer (♦; ◊), phosphate buffer (●; ○) and borate buffer (▲; △) as determined using the pNPA assay. 100% activity refers to the highest activity measured in all buffers, which were 1.67 U mL^{-1} for *E. coli* PLE (*filled symbols* and *solid line*) and 1.42 U mL^{-1} for *P. pastoris* PLE (*empty symbols* and *broken line*), respectively

phosphate or borax buffer, which we attributed to the properties of the different buffers and their effect on esterase activity. Both the pH profiles for PLE from *P. pastoris* and from *E. coli* showed the same two maxima at pH 6.5 in citrate buffer and pH 8-8.5 in borax buffer (Fig. 7).

Recombinant γ-isoenzyme of pig liver esterase expressed in an eukaryotic host was already shown to hydrolyze (*R*,*S*)-1-phenyl-2-butyl acetate with higher enantioselectivity (*E*=50) compared to commercial PLE samples (*E*=1–4), and the (*S*)-alcohol **1a** was obtained in all cases (Musidlowska-Persson and Bornscheuer 2003). For the *E. coli*-expressed γ-PLE, we could confirm these values as with both enzymes similar enantioselectivities, reaction times and only slightly smaller conversion were observed.

Discussion

The high-level expression of functional recombinant γ-isoenzyme of PLE in *E. coli* was the aim of these investigations because the already reported expression in *P. pastoris* yielded only very small amounts of enzyme (0.5 U mL^{-1} culture supernatant) after a 96-h cultivation (Lange et al. 2001). Compared to the yeast expression system, the expression in *E. coli* is easier, inexpensive and less time-consuming because of rapid growth and protein production. Another major advantage is the substantial physiological and genetic knowledge and advanced molecular tools that allow straightforward manipulations.

First expression attempts in the *E. coli* DH5α strain failed most likely due to the different prokaryotic codon usage. With the strain Rosetta(DE3) that supplies codons rarely used in *E. coli*, high levels of recombinant protein were formed, but unfortunately, these were present as inclusion bodies. Different strategies to increase the level of soluble protein failed, and the inclusion bodies seem to result from the inability of *E. coli* to form disulfide bridges in the cytoplasm because under physiological conditions, it is maintained in a reduced state that strongly disfavours the formation of stable disulfide bonds. The Origami strain (Novagen) has mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes that enhance the disulfide bond formation in the cytoplasm (Besette et al. 1999). However, the expression of leader-sequence-free PLE construct in our investigations did not yield soluble protein. The denaturation and refolding of PLE in the presence of the glutathione redox system also failed because of repeated reaggregation.

Key to success was the use of *E. coli* strain Origami (DE3) coexpressing the molecular chaperones GroEL/GroES and harbouring the IPTG-inducible commercial vector pET15b with a leader-sequence-free His-tagged PLE (construct **4**). The cultivation temperature also had a considerable influence on the amount of functional enzyme formed. After protein expression at 37 °C, only very low esterase activity was found, but cultivation at 30 °C resulted in soluble and active γ-PLE.

The successful functional expression of this PLE isoenzyme in the prokaryotic host *E. coli* now enables the production of large amounts of active enzyme. Methods for directed evolution to alter enzyme features are now applicable, and also the improvement of the enzyme activity by site-directed mutagenesis is now substantially facilitated. The expression of recombinant PLE in *E. coli* shows some important advantages like uncomplicated enzyme recovery, optional high cell density fermentation with cheap cultivation media and the absence of other disturbing esterases. Already, the productivity of the *E. coli* cultivations is significantly higher (27 U L^{-1} h^{-1}) compared to *P. pastoris* (4 U L^{-1} h^{-1}).

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