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Insights into the physiological role of pig liver esterase: Isoenzymes show differences in the demethylation of prenylated proteins

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ABSTRACT

The possible physiological role of PLE (E.C. 3.1.1.1) located in the endoplasmic reticulum (ER) of pig liver cells in the conversion of endogenous compounds was investigated as it was reported, that PLE acts as prenylated methylated protein methyl esterase (PMPMEase) hydrolysing methylesters of prenylated proteins. Using the specific PMPMEase substrate benzoyl–glycyl–farnesyl–cysteine methyl ester (BzGFCM), six different PLE isoenzymes expressed recombinantly in the yeast *Pichia pastoris* were found active. Activities ranged from 1.6–15.6 mU per mg protein and it is suggested that Pro285 has a major influence on high activity. In addition, the role of the C-terminal HAEL retention signal for translocation of pig liver esterase (PLE) in the endoplasmic reticulum (ER) of eukaryotic cells was studied using the γ-isoenzyme of PLE expressed in *Pichia pastoris*. Using truncated versions (HAE, HA, H and without retention signal) of the enzyme it was found that in contrast to earlier reports no influence of the signal peptide on the expression rate of PLE was found. However, higher enzyme activities were obtained in the periplasmatic fraction compared to the supernatant irrespective of the presence or absence of HAEL and the trimeric formation seems to occur in the supernatant of *P. pastoris* X33 enabling an easier transition of monomeric forms through cell membranes.

1. Introduction

Pig liver esterase (PLE, EC 3.1.1.1) belongs to the class of mammalian carboxylesterases comprising a multi-gene family whose gene products are localized in the endoplasmic reticulum (ER) of many tissues. These enzymes efficiently catalyze the hydrolysis of a variety of ester- and amide-containing compounds, for example drugs (including prodrugs), to the corresponding free acids and thus are involved in the hydrolysis of various esters of the pig diet and detoxification of xenobiotics.[1] This broad substrate specificity also made PLE one of the most important hydrolases in organic synthesis.[2]

To ensure a localisation of PLE in the ER of pig liver cells, an ER-retention signal is essential and C-terminal tetrapeptides of the type (H/K)XEL were found to fulfil this function. These signals were investigated in detail[3] and the last two amino acids, glutamate (E) and leucine (L) seem to be necessary to maintain this function.[4] The attachment of different XXEL-tetrapeptides to originally secreted proteins caused retention in the ER,[5] in contrast to the natural C-terminal tetrapeptide TEHT of rat liver esterase for example, which results in secretion of this protein.[6] The retention of PLE in the endoplasmic reticulum is important for the metabolism of substances and includes the advantage of correct folding and disulfide bond formation, because of the oxidative environment in the ER.[7] In line with these findings, Lange et al. reported that recombinant expression of PLE in *Pichia pastoris* was only possible after deletion of the putative ER-retention signal HAEL.[8] In order to find further evidence for the role of the HAEL tetrapeptide, we systematically varied this motive and studied its effect on expression and localization of PLE.

Although the substrate spectrum of PLE for exogenous compounds and especially a broad range of chemicals useful in organic synthesis has been explored in detail in the past decades, little was known about endogenous compounds converted by PLE until recently, when the hydrolysis of methyl esters of prenylated proteins was proposed. This reaction is the only reversible step in the prenylation pathway of proteins facilitating the regulation of this process.

In eukaryotic cells, a specific set of proteins is modified by C-terminal attachment of farnesyl groups (15 carbon atoms) or geranylgeranyl groups (20 carbon atoms) that function both as anchors for fixing proteins to membranes and as molecular handles for facilitating binding of these proteins to other proteins.[9,10]

The addition of a prenyl group typically occurs at the sulfhydryl group of a cysteine residue in a C-terminal CAAX sequence of the target protein, where C is cysteine, A can be any aliphatic and X can be any amino acid. The result is a thioether-linked farnesyl or geranylgeranyl group. Once the prenylation reaction has occurred, a specific protease cleaves the three C-terminal residues,

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and the carboxyl group of the now terminal cysteine is methylated by prenylated protein methyl transferases (PPMTases) to produce an ester, which can be hydrolysed by prenylated methylated protein methyl esterases (PMPMEases). All of these modifications appear to be important for subsequent activity of the prenyl-anchored protein. Proteins anchored to membranes via prenyl groups include yeast mating factors, the p21ras protein (the protein product of the ras oncogene), and the nuclear lamins, structural components of the lamina of the inner nuclear membrane.[9–11]

Oboh and Lamango purified the PMPMEase from pig liver and identified the 57 kDa protein as *Sus scrofa* carboxylesterase (pig liver esterase) after proteome analysis and Mascot database search.[12] The specific PMPMEase substrate, benzoyl–glycyl–farnesyl–cysteine methyl ester (BzGFCM) was synthesized[13] and it could be shown that this can be hydrolysed by the purified PMPMEase from pig liver.[12] As our group has succeeded in the past to identify, clone and functionally express several isoenzymes[14,15] of PLE, we report here also on the activity of the distinct isoenzymes in the hydrolysis of BzGFCM.

2. Results and discussion

2.1. Influence of the ER-retention signal HAEL on PLE-expression

It is known from literature that recombinant expression of γPLE in *P. pastoris* can only occur after deletion of the tetrapeptide HAEL, functioning as ER-retention signal in pig liver cells. To examine the influence of the amino acids encoding this tetrapeptide, different variants were constructed using the QuikChange™ method. The resulting γPLE constructs pPICZα-PLE-HAEL (without retention signal), pPICZα-PLE+HAEL (with retention signal), pPICZα-PLE_H, pPICZα-PLE_HA and pPICZα-PLE_HAE were cloned in *Escherichia coli* DH5α and expressed in *P. pastoris* X33. The different cell compartments were isolated and analysed to gain insights into the role of this signal for the secretion of PLE in the eukaryotic host *P. pastoris*. No significant differences in expression level and localisation could be obtained using γPLE construct with or without ER-retention signal HAEL compared to the results from literature, describing an expression of PLE only without HAEL.[8] Furthermore, γPLE seems to be mainly localized in the periplasmatic fraction of *P. pastoris* when expressed with an α-factor signal sequence as a twofold higher enzyme activity was obtained in the periplasm compared to the supernatant (Fig. 1).

Hardly any enzyme activity was determined in the cytosolic fraction, which can be caused either by inclusion body formation or total secretion of the enzyme. The expression levels were checked via native PAGE and esterase activity staining (Fig. 2). As no γPLE band could be detected in the cytosolic fraction, it can be assumed that the enzyme is completely secreted into the periplasm or supernatant. Note that the activity-stained bands of γPLE are more intensive in the supernatant compared to the periplasmatic fraction, because of a concentration step of the supernatant samples.

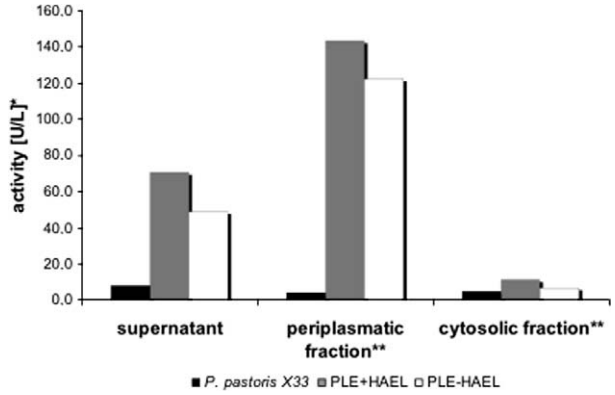


Figure 1. Comparison of PLE activities, containing ER-retention signal HAEL or not, determined in the supernatant, periplasmatic and cytosolic fraction of *P. pastoris* X33. *per L cultivation; **per $OD_{600\ nm}$ of 25.

PLE isoenzymes form rather stable, trimeric quarternary structures of approximately 180 kDa,[8,16] which is clearly larger than proteins usually secreted by *P. pastoris*.[17] That could be an explanation why main enzyme activity was obtained in the periplasm of *P. pastoris*. Hermann et al. obtained similar results expressing and analysing another PLE isoenzyme (APLE).[18] However, they only detected esterase activity in the periplasm, irrespective of the existence of an ER-retention signal.

Furthermore, three esterase bands were stained in the periplasmatic fraction belonging presumably to the monomeric, dimeric and trimeric form of PLE. Interestingly, the main esterase band, which was visible in the supernatant, belongs to the trimeric PLE. Thus, the monomeric or dimeric PLE forms can cross the cell wall and the trimer formation seems to occur in the extracellular medium mainly.

2.2. PLE acts as prenylated methylated protein methyl esterase (PMPMEase)

As mentioned above, Oboh and Lamango isolated the prenylated methylated protein methyl esterase (PMPMEase) from pig liver and identified this enzyme as carboxylesterase from *Sus scrofa*.[12] To elucidate the possible physiological role of the PLE isoenzymes discovered earlier in our group, these were expressed together with the chaperones GroEL/ES in *E. coli* Origami. After cell disruption and lyophilisation, the enzymes were used for the conversion of benzoyl–glycyl–farnesyl–cysteine methyl ester (BzGFCM). This substance was used as substrate analogue for the detection of specific PMPMEase activity, because various choline esterases did not hydrolyse BzGFCM.[13]

The investigations were performed using PLE isoenzymes 1–6 (with γPLE being identical to PLE-1), commercially available PLE from Fluka and APLE.[18] Conversion was determined at different times using RP-HPLC.

It is obvious from Figure 3, that substantial differences in the rate of BzGFCM hydrolysis occured using the PLE isoenzymes (Fig. 3, Table 1). PLE-1 (γPLE) and PLE-2 converted the substrate with similar activities (1.6 and 1.7 $mU/mg_{protein}$). Both isoenzymes differ only in three amino acids explaining similar substrate specificities and properties.[15] In comparison, PLE-3 and PLE-4 hydrolysed BzGFCM very fast resulting in specific activities of 12.0 and 15.6 $mU/mg_{protein}$. After incubation for one hour almost all BzGFCM was converted into BzGFC. These isoenzymes differ from PLE-1 in 20 amino acids (Table 1).

Only PLE-3 and PLE-4 contain a proline at position 285 compared to all other isoenzymes bearing a phenylalanine at the same position. This may have caused the increased conversion rate of BzGFCM. Analysis of the location of this residue in the 3D structure of the isoenzymes was used to explain these results. A homology model of PLE using human carboxylesterase I (pdb: 1MX9[19]) as template was constructed and structural conformation of the different isoenzymes was calculated using the programm Pymol and AMBER 10 (Fig. 4).

Figure 4 shows the differences in the structure of the active site entrance influencing the substrate specificity and probably enantioselectivity. The amino acid variations of PLE-3 compared to PLE-1 caused a gap at the entrance to the active site. From modelling results we suggest that the amino acid exchanges V76G and E77G af-

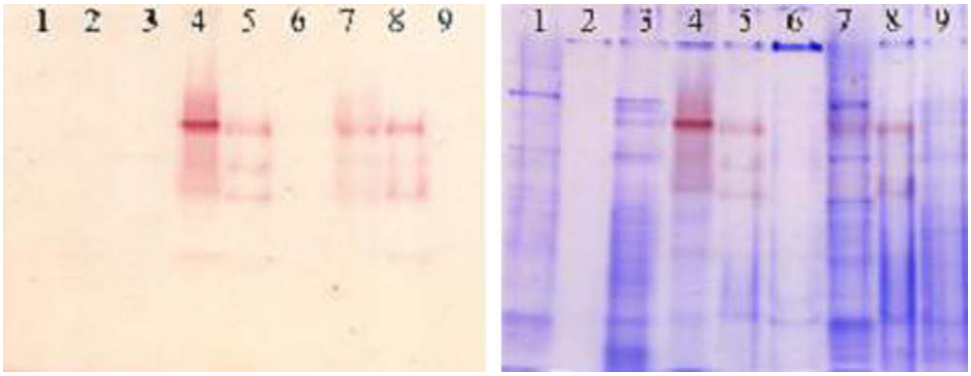


Figure 2. Native PAGE: Fast Red-activity staining (left); Coomassie staining (right); *P. pastoris* WT (1-3); γPLE with HAEL (4–6); γPLE without HAEL (7–9); secreted, concentrated supernatant (1, 4, 7); periplasmatic fraction (2, 5, 8); cytosolic fraction (3, 6, 9).

fected a partly uncoiled α-helix compared to PLE-1 and PLE-2 facilitating the access of the rather bulky substrate BzGFCM to the active site. It is already known that glycine is a strong α-helix breaker.[20,21] However, PLE-6 and APLE contain the same amino acid exchanges resulting only in a moderate activity towards BzGFCM. The α-helix P74-T85 is correctly formed in PLE-6 and APLE, which might be caused by a stabilisation effect of other amino acids, probably at positions 285–287, differing in PLE-3. The amino acids at positions 285–287 situated also at the active site entrance differ extremely in amino acid sequence. PLE-1 and -2 contain F285/L286/T287; PLE-3 and -4 contain P285/L286/T287 and PLE-5, -6 and APLE the amino acids F285/F286/A287. These amino acids and also others (maximal 10 of 25 possible amino acid exchanges) are within a distance of 5 Å from α-helix P74-T85 and have a direct influence.

Furthermore, PLE-4, showing together with PLE-3 highest enzyme activities, comprises V76A and E77G compared to PLE-1 and only a small gap is visible in the structure, caused by an intact α-helix P74-T85. Alanine is a strong helix creator[21] and seems to sustain this helix. Accordingly, the exchanges into these small amino acids are not the only reason for the high activity towards BzGFCM. As discussed before, Pro285 may also affect the structure of the active site entrance of PLE-3 and -4, whereby a sterically hindrance is decreased, which enables the large substrate BzGFCM an easier entrance to the active site (Fig. 4). Proline acts as a structural disruptor of secondary structure elements such as α-helices and β-sheets. The distinctive cyclic structure of proline's side chain locks its φ backbone dihedral angle, giving proline an exceptional conformational rigidity compared to other amino acids. However, proline is commonly found as the first residue of an α-helix and also in the edge strands of β-sheets.[22] Thus, it is likely that this residue is the key amino acid determining the high activity of two isoenzymes towards the PMPMEase substrate.

Hence, pig liver esterase acts as prenylated methylated protein methyl esterase (PMPMEase) hydrolysing methylesters of prenylated proteins. The methylation and demethylation of the terminal carboxylic acid group removes and adds a negative charge, respectively, causing possible allosteric changes. Due to this, the isoprenyl moiety can vary into a sterically hindered state and into a state, in which it is available for interaction with binding sites on other proteins, influencing cell proliferation, differentiation, cell signalling and apoptosis.[9,11,23]

2.3. Conclusion

In conclusion, PLE is originally localized in the endoplasmic reticulum of pig liver cells and contains the ER-retention signal HAEL. In literature it was reported, that recombinant expression of the γ-isoenzyme (PLE-1) in the eukaryotic host *P. pastoris* was only achieved after deletion of this signal peptide.[8] Our results did not confirm this. Soluble expression of PLE was possible with or without HAEL or any truncated version. Nevertheless, highest enzyme yield could be obtained after isolation of the periplasmatic fraction. In addition, trimer formation of PLE seems to occur in the supernatant of *P. pastoris* enabling an easier transition of monomeric forms through cell membranes.

Furthermore, the successful conversion of BzGFCM, a specific substrate analogue of PMPMEase, confirms the results of Oboh and Lamango.[12] They obtained esterase activity of 4.51 $mU/mg_{protein}$ towards BzGFCM from purified enzyme from pig liver containing an undefined composition of isoenzymes similar to commercial PLE from Fluka, which is a crude extract. This activity corresponds to the average of the PLE isoenzyme activity. Consequently, the different isoenzymes showed different activities towards BzGFCM caused by differences in the amino acid sequence.

Thus, pig liver esterase has an important physiological role to balance the level of methylated and unmethylated prenylated proteins. One advantage of PLE is the existence of different isoenzymes facilitating the adaption and fine tuning of metabolic processes.

3. Experimental

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 GATC (Konstanz, Germany). BzGFCM was synthesized as described[12] and kindly provided by Dr. N. Lamango (Tallahassee, USA).

3.1. Microorganisms, plasmids and growth condition

Microorganisms, plasmids and growth condition were used according to Lange et al.[8] and Böttcher et al.[14]

3.2. Site-directed mutagenesis

To introduce specific mutations to alter the HAEL tetrapeptide, the QuikChange™ Site-Directed Mutagenesis method (Stratagene, La Jolla, CA, USA) was applied. As template, the plasmid pPICZα-mPLE* (5171 bp) harbouring the gene encoding the γ-isoenzyme of PLE without the natural signal sequence and without the terminal tetrapeptide HAEL (γPLE-HAEL) was used.[8] The ER-retention signal encoding amino acids were introduced stepwise into the gene encoding γPLE yielding the truncated variants and the protein with the HAEL-signal (PLE_H; PLE_HA; PLE_HAE and PLE+HAEL). The mutated plasmids were transformed into *E. coli* DH5α and after sequence confirmation into *P. pastoris* X33.

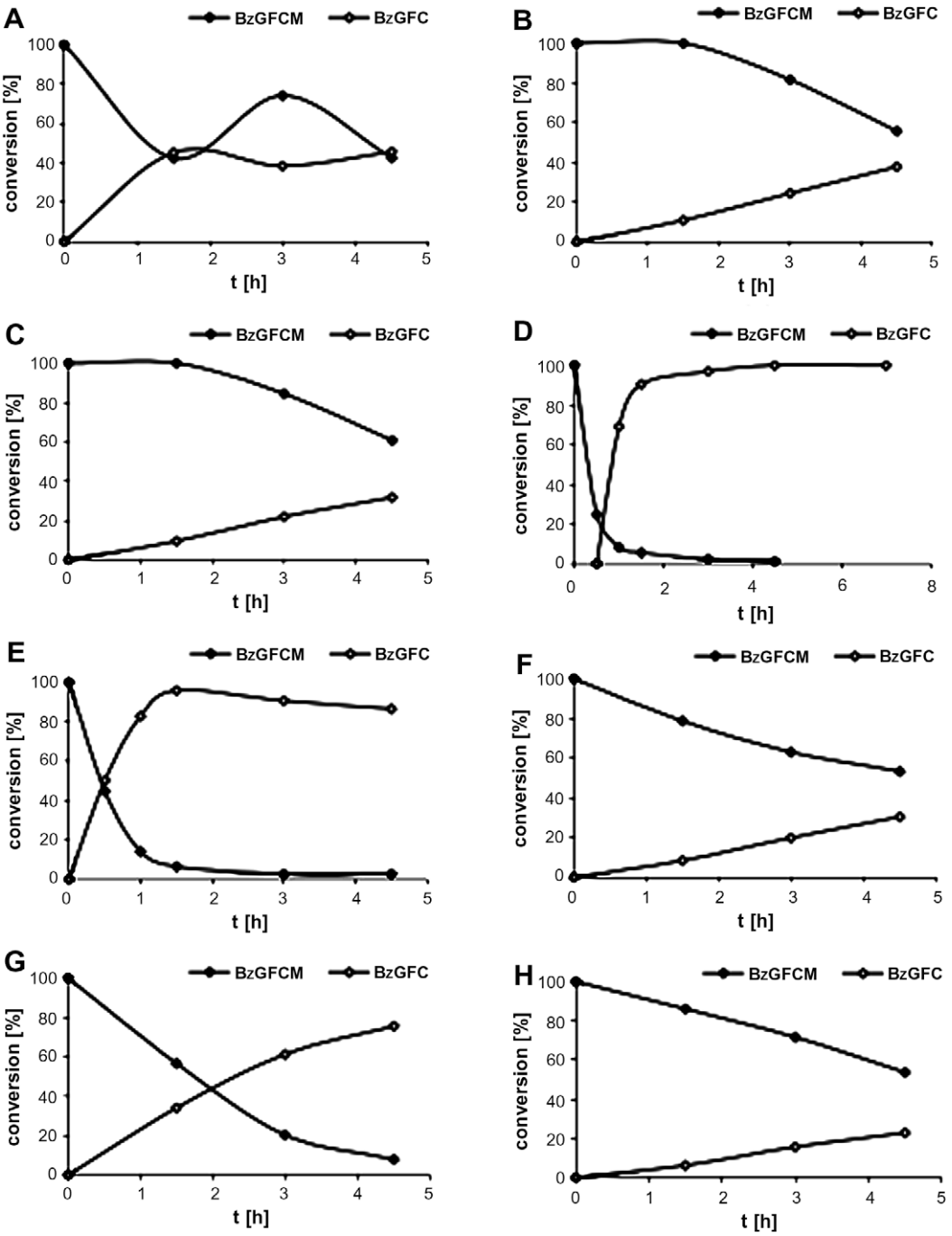


Figure 3. Time-dependent hydrolysis of BzGFCM with commercially available PLE from Fluka (A), PLE-1–6 (B–G) and APLE (F).

3.3. Expression of PLE in *P. pastoris* X33

The expression of γPLE in the yeast *Pichia pastoris* X33 was performed according to Lange et al.[8] The supernatant was concentrated by ultracentrifugation using 20 mL Centricons (NMWL 30,000, Ultracel-PL Membrane, Millipore, USA). The cell pellet (standardized to $OD_{600\ nm}$ of 25) was used for spheroplasting to isolate the periplasmatic fraction. The pellets were resuspended in 20 mL SED-buffer (19 mL 1 M sorbitol and 25 mM EDTA; pH 8 + 1 mL 1 M DTT), centrifuged (5 min; 4000*g*) and resuspended in 20 mL STE (1 M sorbitol, 1 mM EDTA, 20 mM Tris; pH 7.0). Spheroplasting was carried out by adding 800 μg/mL cellsuspension zymolyase (from *Arthrobacter luteus*; Seikagaku Biobusiness, Japan) and incubation for 1.5 h at 30 °C. After centrifugation at 4000*g* for 15 min the supernatant contained the periplasmatic proteins. The following cell lysis was performed by use of glass beads (acid-washed; 425–600 μm; Sigma). After washing with 25 mL breaking buffer (50 mM sodium phosphate buffer, pH 7.4; 1 mM EDTA; 5% glycerol; 1 mM protease inhibitor PMSF), the cells were centrifuged for 10 min at 3000*g* and resuspended in 2 mL breaking buffer. The disruption was carried out by adding the same volume of glass beads and eight cycles of 30 s vortexing, 30 s on ice and centrifugation for 15 min at 8000*g*.

3.4. Expression of PLE in *E. coli* Origami (DE3)

The expression of the enzymes was carried out by coexpression of the chaperones GroEL/ GroES in *E. coli* Origami (DE3) according

Table 1
Different amino acid sequences of PLE isoenzymes (amino acids differ from PLE-1/γPLE are bold) and specific activities towards BzGFCM

Amino acid #	PLE-1	PLE-2	PLE-3	PLE-4	PLE-5	PLE-6	APLE
73	D	D	**E**	D	D	**E**	**E**
75	V	V	**I**	V	V	**I**	**I**
76	V	V	**G**	**A**	**A**	**G**	**G**
77	E	E	**G**	**G**	**G**	**G**	**G**
80	T	T	**L**	T	T	**L**	**L**
87	G	G	**R**	**R**	**R**	**R**	**R**
92	T	T	**I**	**I**	**I**	**I**	**I**
93	L	L	**P**	**P**	**P**	**P**	**P**
129	L	L	**V**	**V**	**V**	**V**	**V**
133	P	P	**S**	**S**	**S**	**S**	**S**
134	M	M	**T**	**T**	**T**	**T**	**T**
138	V	V	**L**	**L**	**L**	**L**	**L**
139	V	V	**A**	**A**	**A**	**A**	**A**
234	L	L	**F**	**F**	**F**	**F**	**F**
236	V	V	**A**	**A**	**A**	**A**	**A**
237	A	A	**G**	**G**	**G**	**G**	**G**
285	F	F	**P**	**P**	F	F	F
286	L	L	L	L	**F**	**F**	**F**
287	T	T	T	T	**A**	**A**	**A**
290	F	F	**L**	**L**	**L**	**L**	**L**
294	Q	Q	**P**	**P**	**P**	**P**	**P**
302	P	P	**T**	**T**	**T**	**T**	**T**
459	F	**A**	F	**A**	**A**	**A**	F
461	L	**F**	L	**F**	**F**	**F**	L
463	K	**R**	K	**R**	**R**	**R**	K
Total # of mutations		3	20	20	21	24	21
(U $mg^{-1}_{protein}$)	1.7	1.6	12.0	15.6	4.4	7.1	2.7

to Böttcher et al.[14] using 0.5 mg/mL L-arabinose for the induction of the chaperone-expression and 40 μM IPTG for the induction of the PLE-expression. The cells were lyophilised after harvesting and disruption by sonification.

3.5. 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 ($\epsilon = 15 \times 10^3\ M^{-1}\ cm^{-1}$) 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.[24]

3.6. Native polyacrylamide gel electrophoresis

The recombinant pig liver esterase solutions (15 μL) were mixed with sample buffer (5 μL) and incubated for 2 min. Samples were separated on polyacrylamide gels (7.5%) with a stacking gel (4.5%). For esterase activity staining, the gel was incubated in a mixture of freshly prepared solutions of α-naphthyl acetate and Fast Red (1:1). In the presence of hydrolytic activity, released α-naphthol forms a red complex with Fast Red.[24] Then the gels were stained with Coomassie Brilliant Blue.

3.7. PMPMEase assays

Ten microlitres of BzGFCM (10 mM in DMSO, final concentration 1 mM) were added to 90 μL enzyme solution in 100 mM Tris–HCl, pH 7.4 and incubated at 37 °C and 750 rpm. The reaction was stopped at different times by adding 100 μL methanol, incubation on ice for 3 min and centrifugation for 3 min at 16000*g*. The supernatant containing BzGFCM and BzGFC (Scheme 1) was analysed by RP-HPLC (PRP-1 column; 60 × 4 mm; 5 μm; CS-Chromatographie Service GmbH, Langerwehe, Germany) at 214 nm using the following conditions:

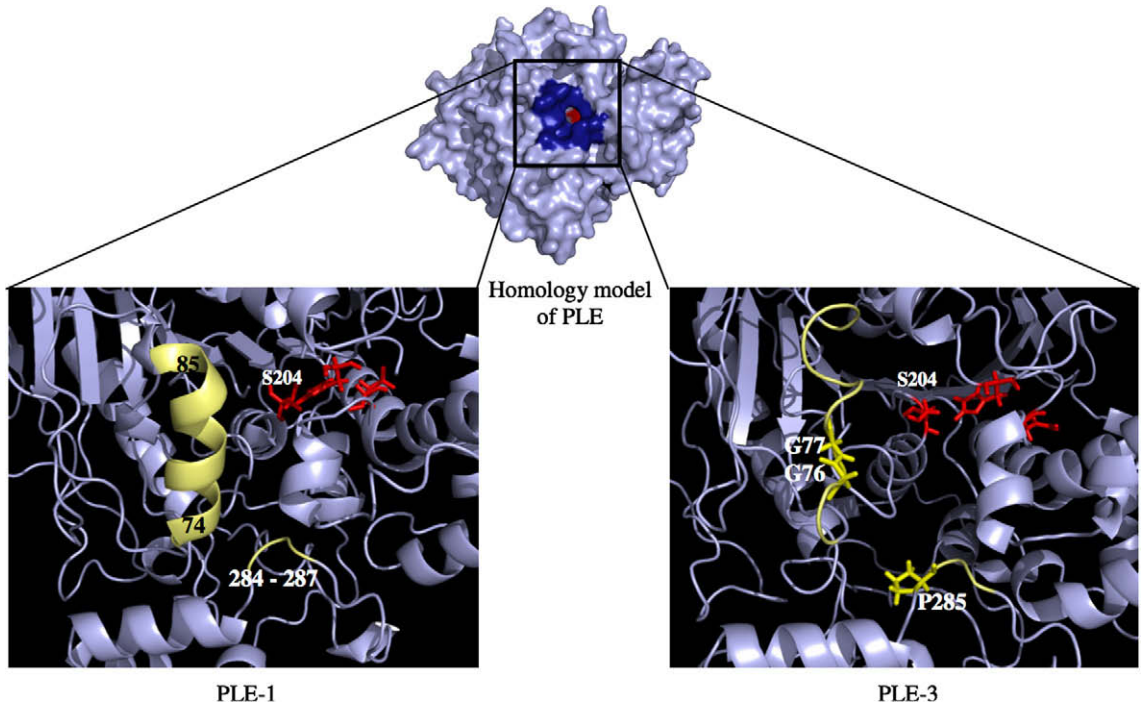
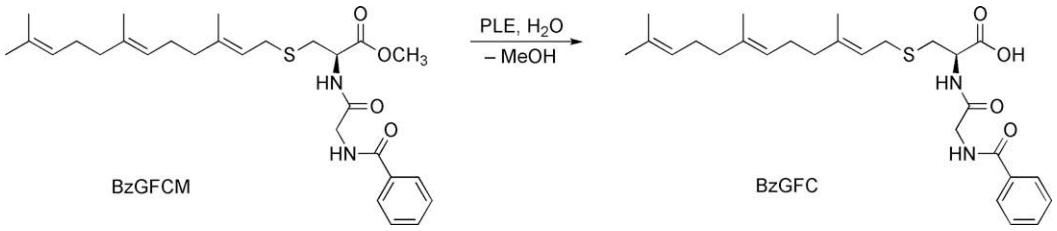


Figure 4. Structures of PLE-1 and PLE-3 based on a homology model of human carboxylesterase I (pdb: 1MX9): active site (red), amino acids at the active site entrance (yellow).



Scheme 1. PLE-catalysed hydrolysis of benzoyl–glycyl–farnesyl–cysteine methyl ester (BzGFCM).

Time	Acetonitrile + 0.1% TFA (%)	A. bidest + 0.1% TFA (%)
0.0	30	70
1.0	95	5
3.0	95	5
4.0	30	70
6.0	30	70

The retention times for BzGFCM and BzGFC were 2.4 min and 2.1 min, respectively.

3.8. Creation of homology models

The 3D structure of PLE was modelled based on the known structure of human liver carboxylesterase I (PDB entry: 1MX9A[25]) using Pymol, an automated protein structure homology-modelling server available at http://pymol.sourceforge.net/ and AMBER 10.[26]

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