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CA 2635380 A1 2007/07/05

(21) 2 635 380

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2006/12/08
(87) Date publication PCT/PCT Publication Date: 2007/07/05
(85) Entrée phase nationale/National Entry: 2008/06/26
(86) N° demande PCT/PCT Application No.: EP 2006/011832
(87) N° publication PCT/PCT Publication No.: 2007/073845
(30) Priorité/Priority: 2005/12/27 (ATA 2081/2005)

(51) Cl.Int./Int.Cl. *C12N 9/18* (2006.01),
C12N 15/52 (2006.01), *C12P 41/00* (2006.01),
C12P 7/02 (2006.01)

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(54) Titre : NOUVEAU POLYPEPTIDE PRESENTANT UNE ACTIVITE D'ESTERASE, ESTERASE RECOMBINEE ET LEUR UTILISATION

(54) Title: POLYPEPTIDE HAVING ESTERASE ACTIVITY AND RECOMBINANT ESTERASE AND USE THEREOF

(57) Abrégé/Abstract:
Polypeptide and recombinant protein having esterase activity which exhibit the amino acid sequence SEQ. ID. No. 1 and the use thereof.

CA 2635380 A1 2007/07/05

(21) 2 635 380

(13) A1

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 July 2007 (05.07.2007)

PCT

(10) International Publication Number
WO 2007/073845 A3

(51) International Patent Classification:
C12N 9/18 (2006.01) *C12P 41/00* (2006.01)
C12N 15/52 (2006.01) *C12P 7/02* (2006.01)

(21) International Application Number:
PCT/EP2006/011832

(22) International Filing Date:
8 December 2006 (08.12.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
A 2061/2005 27 December 2005 (27.12.2005) AT

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report:
2 August 2007

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: POLYPEPTIDE HAVING ESTERASE ACTIVITY AND ECOMBINANT ESTERASE AND USE THEREOF

(57) Abstract: Polypeptide and recombinant protein having esterase activity which exhibit the amino acid sequence SEQ. ID. No. 1 and the use thereof.

WO 2007/073845 A3

Novel polypeptide having esterase activity and recombinant esterase and use thereof

The invention relates to a novel polypeptide having esterase activity, especially having 2-alkyl-5-halopent-4-enecarboxylesterase activity, and to an enzymatically active recombinant protein having esterase activity and to the use thereof for resolving racemates of 2-alkyl-5-halopent-4-enecarboxylic ester enantiomer mixtures.

Enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic acids and their esters are valuable intermediates for preparing pharmaceuticals, such as, for instance, for delta-amino-gamma-hydroxy-omega-arylalkanecarboxamides, which have renin-inhibiting properties and can be used as antihypertensive agents in pharmaceutical preparations.

Esterases are generally employed in the resolution of racemates and asymmetrization.
However, only very few esterases suitable for preparing chiral compounds are commercially available.
The use of esterase extracts from pig liver is known on the preparative scale. Pig liver esterase (PLE) was isolated long ago from natural sources, and its activity has also been known for a long time (Simonds, J.P. (1919) Amer. J. Physiol. 48, 141; Bamann, E. et al. (1934) Hoppe-Seyler Z. 229, 15; Falconer J.S. and Taylor, D.B. (1946) Biochem. J. 40, 831-834).

Various studies have also already been carried out in order to characterize PLE (Heymann, E. and Junge, W. (1979) Eur. J. Biochem. 95, 509-518; Lehner, R. and Verger, T. (1997) Biochemistry 36, 1861-1868).
It has further been possible to show, for example in WO 01/09079, that esterase extracts from pig liver can selectively hydrolyze the (R) enantiomer of methyl 5-chloro-2-(1-methylethyl)-4-pentenoate.

However, the use of such esterase extracts from natural sources, such as pig liver, is associated with disadvantages.

In the first place, the qualities of the different batches vary and thus make it difficult to optimize industrial processes. Secondly, the use of animal resources in the manufacture of pharmaceutical products is undesired because the presence of viruses and prions cannot always be precluded.

For these reasons there is a need to produce recombinant pig liver esterases of standardized quality in microorganisms.

The cloning of putative esterase genes is described for example in FEBS Lett. (1991), 293, 37-41. The first functional expression of an active pig liver esterase enzyme was described for the first time in WO 02/48322. WO 2004/055177 describes the preparation of further recombinant esterases by site directed mutagenesis of the recombinant pig liver esterase of seq. ID No. 1 (rPLE) from WO 02/48322. As is evident from the description of WO 2004/055177 and from the article authored by the same inventors in Protein Engineering, 16, 1139-1145, 2003, the modifications of the rPLE sequence from WO 02/48322 were chosen so that a recombinant intestinal pig esterase (PICE) disclosed in David et al., (1998) Eur. J. Biochem. 257, 142-148, is obtained.

The resolution of racemic 2-alkyl-5-halopent-4-enecarboxylic esters is not described in any of these articles.

However, since the need for esterases which have the desired stereoselective activity for 2-alkyl-5-halopent-4-enecarboxylic esters and which can easily be prepared biotechnologically is not met, it was an object of the present invention to provide a corresponding novel recombinant esterase.

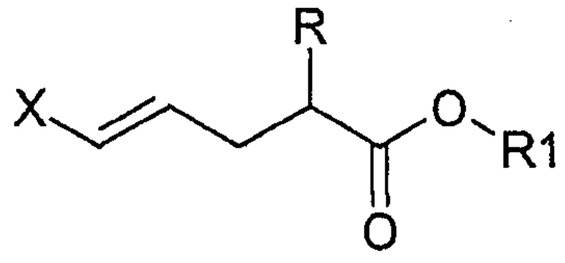
In an attempt to isolate and to clone the gene described in FEBS Lett. (1991), 293, 37-41 and WO 02/48322 for known pig liver esterase (PLE) as cDNA starting from mRNA from pig liver, a second, novel esterase sequence was found in addition to the known PLE sequence. Following expression of the two sequences, in which the corresponding proteins or esterases, namely the known rPLE and a novel, recombinant "alternative" esterase (rAPLE), was prepared, it unexpectedly emerged that only the rAPLE is capable of selective resolution of racemic 2-alkyl-5-halopent-4-enecarboxylic esters.

It was thus possible to achieve the object of the present invention by a novel polypeptide having esterase activity and a novel recombinant esterase (rAPLE), whose amino acid sequence differs in 21 of a total of 548 amino acids from the known PLE sequence. The novel rAPLE differs in the amino acid sequence also from the known pig intestinal carboxylesterase (PICE) in 12 of a total of 548 amino acids. However, PICE is found in the pig intestinal tract.

The present invention accordingly relates to a polypeptide having esterase activity, which comprises the amino acid sequence SEQ. ID. No. 1.

The present invention further relates to a novel recombinant protein having esterase activity, which comprises the amino acid sequence SEQ. ID. No. 1.

The polypeptide and the recombinant rAPLE of the invention have the ability to resolve stereoselectively racemic 2-alkyl-5-halopent-4-enecarboxylic esters of the formula (I)



(I)

in which R is a C_1-C_6-alkyl radical, R_1 is C_1-C_4-alkyl and X is chlorine, bromine or iodine.

The polypeptide of the invention having esterase activity, and the novel recombinant esterase rAPLE differ, as stated above, in 21 of a total of 548 amino acids of the known sequence disclosed in FEBS Lett. (1991), 293, 37-41 and in 12 of a total of 548 amino acids from the known PICE protein disclosed in David et al., (1998) Eur. J. Biochem. 257, 142-148.

The sequence of the protein of the novel rAPLE of the invention differs in the following amino acid positions from the known sequence of the PLE protein:

APLE	Position	PLE
Glu	73	Asp
Ile	75	Val
Gly	76	Val
Gly	77	Glu
Leu	80	Thr
Arg	87	Gly
Ile	92	Thr
Pro	93	Leu
Val	129	Leu
Ser	133	Pro
Thr	134	Met
Leu	138	Val
Ala	139	Val
Phe	234	Leu
Ala	236	Val
Gly	237	Ala

Phe	**286**	**Leu**
Ala	**287**	**Thr**
Leu	**290**	**Phe**
Pro	**294**	**Gln**
Thr	**302**	**Pro**

The protein of the invention and the novel recombinant rAPLE may moreover be in the form of a modified sequence as shown in SEQ ID No 1, which can be obtained for example by usual modifications such as, for instance, exchange, deletion or attachment of amino acid(s) in the sequence at the N or C terminus, such as, for instance, GluAlaGluAla from the α factor signal sequence, or by fusion to other proteins.

The invention also further includes muteins having modifications within the protein sequence of the enzyme of the invention having the appropriate activity, in particular on 2-alkyl-5-halopent-4-enecarboxylic esters. Muteins can be obtained for example by modifications of the DNA which codes for the enzyme of the invention, by known mutagenesis techniques (random mutagenesis, site-directed mutagenesis, directed evolution, gene shuffling etc.) so that the DNA codes for an enzyme which differs at least by one amino acid from the enzyme of the invention, and subsequent expression of the modified DNA in a suitable host cell. The invention thus also includes modified DNA sequences as shown in SEQ ID. No 1, obtained by the mutations, deletions, extensions, fusions described above, and which code for enzymes having the desired esterase activity.

Esterase activity, especially 2-alkyl-5-halopent-4-ene-carboxylesterase activity, is defined in this connection as the ability to resolve racemates of 2-alkyl-5-halopent-4-enecarboxylic esters of the formula (I).

The polypeptide of the invention and the recombinant rAPLE can be prepared as described below:

Firstly, mRNA is isolated from pig liver using a suitable kit, and then the cDNA is generated by reverse transcription based on the mRNA extract.
Subsequently, specific PCR primers based on the sequence of the known pig liver esterase gene of GenBank accesssion No. X63323 (Matsushima et al., 1991) is prepared, followed by amplification and cloning.
These specific primers are:
Primer 1: 5'-CA*GAATTC*ATGGCTATC**GGGCAGCCAGCCTCGC**-3'
Primer 2: 5'-CCG*GAATTC*AGCCTCCCCT**TCACAGCTCAG**-3'

This part of the primers which comprises the appropriate nucleotide sequences coding for the PLE protein and which is obligatorily present in the primers is in bold script.
The other sequence part of the primers comprises for example information for cleavage sites for restriction endonucleases (in italics) or sequence elements which are important for expression. This part may vary in the preparation of the rAPLE of the invention.

Amplification then takes place with primers 1 and 2 by prior art PCR methods.
The PCR product is subsequently used to prepare by prior art methods expression constructs for heterologous expression of the encoded rAPLE protein in suitable host organisms. This preferably entails the PCR product being initially cloned into suitable plasmid vectors.

The recombinant plasmids obtained in this way are then transformed into a suitable host, for example *Escherichia coli*. Inserts of several resulting clones are then sequenced.
Unexpectedly, 2 groups of recombinant clones with different sequences were identified therein, one being

100% identical to the expected sequence for PLE according to Matsushima et al., (1991) FEBS Lett. 293, 37-41, and a novel nucleotide sequence as shown in SEQ. ID. No. 2 (APLE sequence) which leads after expression to the amino acid sequence SEQ. ID. No. 1 of the invention.

The present invention further relates to a nucleic acid or nucleotide sequence which codes for the polypeptide of the invention and the recombinant esterase rAPLE. For example, such a nucleic acid has the nucleotide sequence shown in SEQ. ID. No. 2.
The invention also relates further to nucleotide sequences which include a nucleotide sequence which codes for the polypeptide of the invention and the recombinant esterase rAPLE, or comprises the nucleotide sequence shown in SEQ. ID. No. 2.

A further possibility is to prepare appropriate oligonucleotides corresponding to nucleic acid sequences according to the present invention which code for the esterase of the invention by standardized synthetic techniques, for example with use of automated DNA synthesizers.
The purely synthetic preparation of the nucleic acid sequences which code for the esterase of the invention is particularly advantageous for use in the production of pharmaceuticals or their intermediates, because enzymes are thus not obtained from animal sources.

Expression of the two sequences found (PLE and APLE sequences) then takes place.
The known pig liver esterase (PLE, Swiss-Prot ID Q29550) comprises an N-terminal signal sequence and a C-terminal ER retention signal, the last 4 amino acids HAEL.
In order to express the known PLE and the novel APLE, vectors in which the sequences are introduced into suitable expression systems constructed. These

expression constructs are then transformed into suitable host cells.

Suitable host cells in this connection are for example microorganisms, animal cell lines and plants.

Both prokaryotic and eukaryotic microorganisms can be employed. Preferred prokarytic hosts (bacteria) are *Escherichia coli*, and strains from the genera *Bacillus* (*e.g. B.subtilis, B.licheniformis, B.amyloliquefaciens*), *Pseudomonas* (*e.g. P.fluorescens, P.putida*), *or Streptomyces* (*e.g. S.lividans, S.tendae*) Eukaryotic microorganisms are preferred, and fungi are particularly preferred. Examples thereof are *Saccharomyces cerevisiae, Pichia pastoris, Kluyveromyces lactis or Aspergillus sp.*.

Expression may be secretory or intracellular and both inducible and constitutive.

For bacterial expression a choice of species-specific signals can be obtained, a.o. as commercially available strains and vectors for protein expression (e.g provided by companies like Invitrogen, Novagen, New England Biolabs), that allow inducible or constitutive expression, intracellular and secretory localization of the target protein; in addition, technology to enable or promote the correct folding of proteins in order to result in soluble and active protein may be applied.

The proteins are preferably expressed in a secretory manner, in which case vectors in which the sequences of PLE and APLE are linked N-terminally to the α factor signal sequence of *S. cerevisiae* are preferably constructed.

It is further possible to prepare constructs in which the C-terminal tetrapeptide HAEL, which serves as ER retention signal as described for example in Hardwick et al., (1990) EMBO J. 9, 623-630, is additionally deleted.

A further preferred expression is inducible expression of constructs with or without ER retention signal.

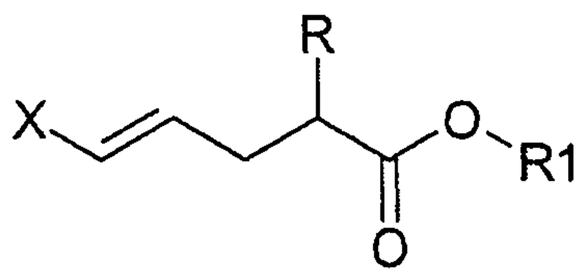
Unexpectedly, constructs having the ER retention signal can also be expressed and lead to an rAPLE which is

capable of selective resolution of racemic 2-alkyl-5-halopent-4-enecarboxylic esters.

The amino acid sequence of the novel esterase rAPLE which is derived from the nucleotide sequence of the APLE gene is depicted in SEQ ID No. 1.

It has unexpectedly been possible to find that the novel polypeptide or the rAPLE protein is able, in contrast to the known rPLE, in each case obtained by expression of the DNA segments coding for APLE and PLE, respectively, for example in *P. pastoris* cells, to resolve racemic 2-alkyl-5-halopent-4-enecarboxylic esters stereoselectively.

The invention accordingly further relates to the use of the polypeptide having esterase activity and of the recombinant esterase (rAPLE) of the invention, which have at least 80% identity to the sequence shown in SEQ ID No. 1, for resolving racemates of 2-alkyl-5-halopent-4-enecarboxylic esters of the formula (I)



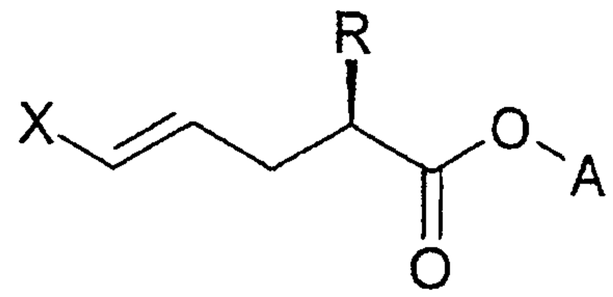
(I)

in which R is a C_1-C_6-alkyl radical, R_1 is C_1-C_4-alkyl and X is chlorine, bromine or iodine.

The polypeptide having esterase activity and the recombinant esterase (rAPLE) of the invention preferably have at least 90%, particularly preferably at least 98%, identity to the sequence of the protein shown in SEQ ID No. 1. It is also possible to employ polypeptide having an esterase activity or the recombinant esterase (rAPLE) of the invention with modified DNA sequences shown in SEQ ID. No. 1, obtained

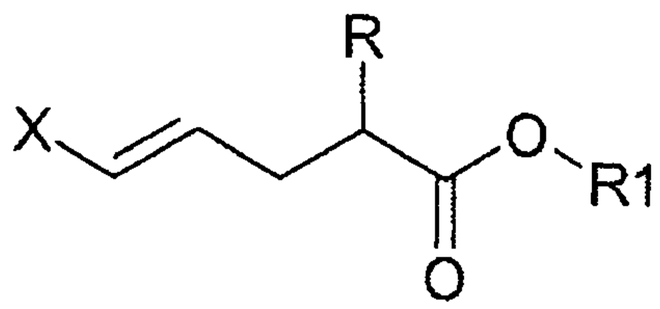
by usual modifications such as, for instance, mutations, deletions, extensions, fusions, which code for enzymes having the desired esterase activity.

In this connection, enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic acids or their esters of the formula (II)



(II)

in which R is a C_1-C_6-alkyl radical, A is equal to H, R_1, where R_1 may be C_1-C_4-alkyl, or R_2, where R_2 is an alkyl group, but is not equal to R_1, and X is chlorine, bromine or iodine, are obtained by an enantiomeric mixture of a 2-alkyl-5-halopent-4-enecarboxylic ester of the formula (I)



(I)

in which R, R_1 and X are as defined above,
being converted by means of the polypeptide of the invention or of the rAPLE of the invention in the presence of water or an alcohol of the formula R_2OH, where R_2 is an alkyl group which is not equal to R_1, as nucleophile, and

a) either the remaining enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic ester of the formula (II) with A equal to R_1 being isolated or
b) if an alcohol is employed as nucleophile, the resulting enantiomerically enriched 2-alkyl-5-

halopent-4-enecarboxylic ester of the formula (II) with A equal to R_2 being isolated, or

c) if water is employed as nucleophile, the resulting 2-alkyl-5-halopent-4-enecarboxylic acid of the formula (II) with A equal to H being isolated.

R in the formula (II) is a C_1-C_6-alkyl radical such as, for instance, methyl, ethyl, n- and i-propyl, n-, i- and t-butyl, pentyl and hexyl.
C_1-C_4-Alkyl radicals are preferred, and the i-propyl radical is particularly preferred.
A is H, R_1, where R_1 is a C_1-C_4-alkyl radical, preferably a C_1-C_2-alkyl radical and particularly preferably a methyl radical, or R_2, where R_2 is an alkyl radical which is not equal to R_1. R_2 is particularly preferably a C_1-C_6-alkyl radical.
X is chlorine, bromine or iodine, preferably chlorine.

Enantiomerically enriched compounds mean in this connection those which exhibit an enantiomeric excess (ee) of >80%, preferably of >90% and particularly preferably of >97%.

The enzyme of the invention can moreover be used in any form. For example as dispersion, as solution, immobilized, as crude enzyme, as enzyme which has been obtained from its source by a combination of known purification methods, as whole cells (where appropriate immobilized and/or permeabilized) which have the required enzymatic activity (naturally or through genetic modifications) or in a lysate of such cells.

The reaction temperature for the conversion of the invention is normally between 0 and 90°C, preferably between 10 and 60°C. The pH of the reaction solution is between 4 and 11, preferably between 6 and 9.

The choice of the solvent depends on the nucleophile employed.

If, for example, water is the nucleophile, solvents which can be employed are water, a mixture of water with a water-miscible solvent, for example with an alcohol such as, for instance, methanol, ethanol, isopropanol, t-butanol, etc., dioxane, tetrahydrofuran, acetone or dimethyl sulfoxide or a two-phase system of water and of a water-immiscible solvent, for example an aromatic compound such as, for instance, toluene, xylene, etc., an alkane such as, for instance, hexane, heptane, cyclohexane, etc., ether such as, for instance, diisopropyl ether, methyl t-butyl ether, etc. If the nucleophile is an alcohol, the solvent preferably employed is the alcohol R_2OH where R_2 is an alkyl group which is not, however, equal to R_1. However, it is also possible to use mixtures of the alcohol with an organic solvent such as, for instance, tetrahydrofuran, heptane, toluene, hexane, CH_3CN, methyl t-butyl ether etc.

After the enzymatically catalyzed racemate resolution has taken place, the desired final product is isolated. This may be either the remaining enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic ester of the formula (II) with A equal to R_1, or if water is the nucleophile the enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic acid of the formula (II) with A equal to H which has formed, or if the alcohol is the nucleophile the enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic ester of the formula (II) with A equal to R_2.

The isolation can take place for example by conventional methods such as, for instance, extraction, crystallization, column chromatography, distillation, etc.

The method of the invention results in the corresponding acids or esters of the formula (I) in theoretical yields of up to 98% yield and with an e.e. of up to >99%.

Example 1: mRNA isolation and generation of cDNA

0.7 g of liver from a freshly slaughtered pig, obtained from a local abattoir, was frozen in liquid nitrogen and homogenized with a mortar, and the liberated mRNA was isolated or extracted using the Fast Track mRNA extraction kit 2.0 (Invitrogen, Carlsbad, Calif., USA) in accordance with the statements made by the manufacturer (Fast Track 2.0 kit manual; version J; 082301; 25-0099). The extraction afforded a total amount of 12.9 µg of mRNA.

0.26 µg of this mRNA was then used as template for generating cDNA using the SuperScript III First-Strand synthesis system for RT-PCR according to the manufacturer's statements.

Example 2: Amplification and cloning of cDNA fragments from pig liver

Specific primers based on the sequence of the pig liver esterase gene of GenBank accession No. X63323 (Matsushima et al., 1991) were prepared:

Primer 1: 5'-CA*GAATTC*ATGGCTATC**GGGCAGCCAGCCTCGC**-3' (SEQ ID. No. 3)

Primer 2: 5'-CCG*GAATTC*AGCCTCCCCT**TCACAGCTCAG** -3' (SEQ ID No. 4)

Bases homologous to the known PLE sequence are in bold script. Recognition sequences for restriction endonucleases are italicized for emphasis.

The amplification took place in a 50 µl mixture with 1 U of Phusion DNA polymerase (Finnzymes, Espoo, Finland), with 500 ng of cDNA as template, 20 µmol each of primer 1 and 2, 5 µl of a dNTP mix (2 mM each), all in 1×Phusion HF buffer in accordance with 'Phusion High-Fidelity DNA Polymerase' manual (Finnzymes), starting with a 30 second denaturation step at 98°C, followed by 30 cycles (10 sec 98°C, 20 sec 68°C, 1 min 72°C) for amplification and a final incubation at 70°C for 8 min to prepare complete products.

This PCR resulted in a DNA fragment with a size of 1.8 kb (found by agarose gel electrophoresis).

This PCR product was then purified using the Qiaquick kit (Qiagen, Hilden, Germany) in accordance with the manual included.

About 0.1 µg of the purified PCR product was cut with the restriction endonuclease *Eco*RI and cloned into the plasmid vectors pHILZ and pHIL-D2 via the *Eco*RI cleavage sites.

The vectors were then transformed into TOP10 electrocompetent cells prepared in accordance with 'Current Protocols in Molecular Biology'.

Inserts of several resulting clones were sequenced using the 'Dye Deoxy Terminator Cycle Sequencing' kit (Applied Biosystems Inc., Forster City, Calif., USA).

Two sequences were identified thereby, one corresponding 100% to the expected sequence published by Matsushima et al., (1991) FEBS Lett. 293, 37-41, and the other sequence corresponding to SEQ ID No. 2.

Example 3: Introduction of the α factor signal sequence and variations of the C-terminal end.

In order to enable secretory expression of the known protein PLE and the protein rAPLE of the invention, vectors in which the sequence of PLE and APLE was connected N-terminally to the α factor start sequence of the cloning vector pPICZ α (Invitrogen) were constructed. In addition, constructs in which the C-terminal tetrapeptide HAEL was deleted were prepared.

PCR I: The EcoRIalpha1/alphaPLE2 primer pair was used to amplify the α factor signal sequence of the cloning vector pPICZ α (Invitrogen). The PCR was carried out in a 50 µl mixture (2 ng of template, 0.5 µM of each primer, 0.2 mM dNTPs, 1 U of the Phusion DNA polymerase (Finnzymes) all in 1×Phusion HF buffer in accordance with the 'Phusion High-Fidelity DNA Polymerase' manual (Finnzymes)).

Denaturation at 95°C for 3 minutes was followed by amplification in 30 cycles (30 sec 95°C, 30 sec 57°C, 15 sec 72°C) and a final step at 72°C for 7 min.

PCR II: The PLE and APLE sequences were amplified from pHILZ plasmids using either the PLEalpha1/EcoRIPLE+ER2 primer pair or the PLEalpha1/EcoRIPLE2 (deletion of the C-terminal HAEL tetrapeptide) primer pair.
These PCRs were again carried out in 50 µl mixtures (2 ng of template, 0.5 µM of each primer, 0.2 mM dNTPs, 1 U of the Phusion DNA polymerase (Finnzymes) all in 1×Phusion HF buffer in accordance with the 'Phusion High-Fidelity DNA Polymerase' Manual (Finnzymes)).
A denaturation at 95°C for 3 minutes was followed by amplification in 30 cycles (30 sec 95°C, 30 sec 57°C, 15 sec 72°C) and a final step at 72°C for 7 min.

PCR III: 3 µl of the products from PCR 1 and PCR II were used to combine these two products by primerless PCR.
The extension was carried out in a 45 µl mixture with 0.2 mM dNTPs, 1 U of the Phusion DNA polymerase (Finnzymes) all in 1×Phusion HF buffer.
The reaction mixture was heated at 95°C for 3 minutes and then 10 cycles with 30 sec at 95°C and 45 sec at 72°C were carried out. To amplify these overlapping extension products, 5 µl of primer mix (3 µl of water, 1 µl of 5 µM EcoRIalpha1 primer and 1 µl of 5 µM EcoRIPLE+ER2 or EcoRIPLE2 primer) were added. The products were amplified with 20 PCR cycles (30 sec 95°C, 30 sec 57°C, 1 min 72°C) and a single temperature stop at 72°C for 7 min.

Primer sequences:

EcoRIalpha1: 5'-TCTTCGAA*GAATTC*ACG**ATGAGATTTCCTTCAATTTTTACTGC**-3'

(SEQ ID No. 5)

alphaPLE2: 5'-GAGGCTGGCTGCCC**AGCTTCAGCCTCTCTTTTCTCG**-3'

(SEQ ID No. 6)

PLEalpha1: 5'-AGAGAGGCTGAAGCT**GGGCAGCCAGCCTCGCCG**-3'

(SEQ ID No. 7)

EcoRIPLE+ER2: 5'-AT*GGTACCGAATTC***TCACAGCTCAGCATGCTTTATCTTG**-3'

(SEQ ID No. 8)

EcoRIPLE2: 5'-AT*GGTACCGAATTC*TCA**CTTTATCTTGGGTGGCTTCTTTG**-3'

(SEQ ID No. 9)

Regions with homology to the templates in bold, recognition sequences for restriction endonucleases in italics.

Example 4: Construction of expression constructs for the heterologous expression of pig liver esterases in *Pichia pastoris*

The overlapping extension PCR products from example 3 were purified using the Qiaquick kit (Qiagen, Hilden, Germany) in accordance with the manual included. About 0.1 µg of the purified PCR products was cut using the EcoRI restriction endonuclease and cloned via the EcoRI cleavage site into the plasmid vector pGAPZ A (Invitrogen).

Correct orientation of the insert in relation to the promoters was checked with the aid of control cleavages, for example with NcoI.

In each case, a clone with correctly oriented insert was selected, sequenced and preserved.

The corresponding plasmids were named as follows:

Plasmids which contained the known PLE sequence as disclosed in Matsushima et al., (1991) FEBS Lett. 293, 37-41, were called pGAPZ A PLE-ER (the HAEL tetrapeptide was deleted) and pGAPZ A PLE+ER (HAEL tetrapeptide still present).

Plasmids derived from the novel APLE sequence were called pGAPZ A APLE-ER (the HAEL tetrapeptide was

deleted) and pGAPZ A APLE-ER (HAEL tetrapeptide still present).

Example 5: Constitutive expression of pig liver esterases in *Pichia pastoris*

The plasmids pGAPZ A PLE-ER, pGAPZ A PLE+ER, pGAPZ A APLE-ER and pGAPZ A APLE+ER were transformed into *P. pastoris* X-33. The transformation took place in accordance with the instructions of the protocol for the Pichia Expressions kit from Invitrogen. The transformants were selected on YPD plates (1% yeast extract, 2% peptone, 2% D-glucose, 2% agar) which contained 100 mg/l zeocin. 52 zeocin-resistant clones were streaked onto YPD plates with 100 mg/l zeocin and preserved in 15% glycerol.

Example 6: Qualitative analysis of the esterase activity

P. pastoris transformants were cultured on YPD plates with 100 mg/l zeocin at 30°C for 48 h. The cells were lifted onto Whatman 541 hardened ashless 70 mm∅ filters and air-dried. The filters were incubated with a solution of 6 mg of α-naphthyl acetate (Sigma, dissolved in 500 µl of acetone), 2.5 mg of tetrazotized o-dianisidine (Fast Blue Salt BN, Sigma, dissolved in 125 µl of water) and 5 ml of 0.1 M potassium phosphate buffer, pH 7, in order to visualize the esterase activity by a color reaction.
Activities were detected in all transformants which had integrated one of the 4 plasmids pGAPZ A PLE-ER, pGAPZ A PLE+ER, pGAPZ A APLE-ER and pGAPZ A APLE+ER. This proves the expression of functional proteins having esterase activity. As a check, a clone with integrated empty vector was also tested in the same way. In this case, no significant esterase activity was visible in the comparable reaction period.

Example 7: Stereoselective esterase activity in relation to methyl 5-chloro-2-(1-methylethyl)-4-pentenoate

P. pastoris transformants as described in example 6 were cultured on YPD plates with 100 mg/l zeocin at 30°C for 48 h. The cells were lifted onto Whatman 541 hardened ashless 70 mm∅ filters and air-dried. The filters were incubated either with substrate solution A (100 µl of racemic methyl 5-chloro-2-(1-methylethyl)-4-pentenoate, 200 µl of 0.1 M potassium phosphate buffer, pH 8; 150 µl of 10 mg/ml phenol red; 450 µl of DMSO; 650 µl of H_2O) or with substrate solution B (identical to solution A but employing methyl (2S,4E)-5-chloro-2-(1-methylethyl)-4-pentenoate instead of the racemate). Owing to the specific esterase activity on the substrates, the liberation of acid on hydrolysis of the ester substrate results in a pH decrease which in turn leads to a change in the color of the phenol red indicator to yellow.

It emerged from this that transformants containing the plasmid pGAPZ A APLE-ER gave signals (yellow coloration around the colony) after incubation for 3 to 4 hours if they were tested on substrate solution A, whereas no significant conversion could be found with substrate solution B (figure 1).

Transformants obtained with the plasmids pGAPZ A PLE-ER or pGAPZ A PLE+ER showed no reaction with substrate solutions A or B under the same conditions.

This showed that the recombinant rAPLE has a substrate specificity different from recombinant rPLE and that hydrolysis of methyl 5-chloro-2-(1-methylethyl)-4-pentenoate using rAPLE takes place stereoselectively for the (R) enantiomer.

Example 8: SDS polyacrylamide gel electrophoresis

10 µl of a 2× SDS sample buffer (125 mM Tris-HCl, pH 6.8; 4% SDS, 20% glycerol, 5% β-mercaptoethanol, 0.05%

bromophenol blue) were added to 10 µl of commercially available pig liver esterase or 10 µl of the 60-fold concentrated (Centricon Ultrafiltrations-Spin Columns, from Sartorius) supernatants of the *P. pastoris* cultures (72 h at 100 rpm and 28°C in 250 ml of YPD medium in 2 l Erlenmeyer flasks with baffles) which contained either the plasmid pGAPZ A APLE-ER or an empty pGAPZ A plasmid (control strain).

After the samples had been heated at 95°C for 5 minutes, the proteins were separated on a 12.5% polyacrylamide gel (4% stacking gel) and stained with Coomassie Brilliant Blue R250 for detection. The SDS-PAGE shows a protein band with the expected size for rAPLE (~60 kDa) in the yeast strain having the pGAPZ A APLE plasmid, but not in the control strain (figure 2). The commercially available pig liver esterase which was also analyzed for comparison showed two protein bands in the same size range (arrow in figure 2).

Example 9: Induced expression of pig liver esterases with the AOX1 promoter

The plasmids pGAPZ A PLE-ER, pGAPZ A PLE+ER, pGAPZ A APLE-ER and pGAPZ A APLE+ER were cut with the restriction endonuclease XhoI, and the respective fragments coding for APLE and PLE proteins, with or without ER retention signal, were cloned via the XhoI cleavage site into the pPIC9 vector (Invitrogen). Correct orientation of the fragments in relation to the *AOX1* promoter was checked by means of control cuts with the restriction endonuclease NcoI. The vectors having the *AOX1* promoter were named, in analogy to the plasmids named in example 4, pPIC9 PLE-ER, pPIC9 PLE+ER, pPIC9 APLE-ER and pPIC9 APLE+ER, linearized with SalI and transformed into *P. pastoris* KM71. Transformation and selection for His prototrophy took place in accordance with the instructions of the Pichia expression kit from Invitrogen. Selected transformants and the KM71 strain were cultured on complete medium in

accordance with the Pichia expression kit from Invitrogen overnight and induced with 1% methanol for 48 h. The resulting cultures were analyzed by means of the qualitative pH-shift assay described in example 7, testing 2 µl of the cultures in the mixtures in each case. Expression under the control of the inducible AOX1 promoter led to very much higher, by comparison with the situation described for constitutive expression in example 7, rAPLE enzymic activities in relation to racemic methyl 5-chloro-2-(1-methylethyl)-4-pentenoate. The phenyl red color change (red to yellow) was detectable after only a few minutes (figure 3). Unexpectedly, the rAPLE activity was independent of the presence of the ER retention signal HAEL at the C terminus, i.e. even cells which expressed rAPLE with ER retention signal showed activity. By contrast, yeast strains for producing rPLE had no activity in relation to racemic methyl 5-chloro-2-(1-methylethyl)-4-pentenoate.

SEQUENCE LISTING

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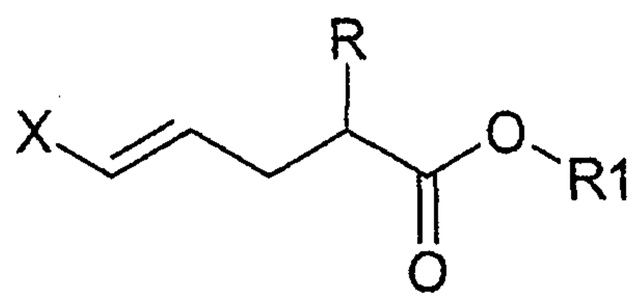
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Claims

1. A polypeptide having esterase activity and exhibiting the amino acid sequence SEQ. ID. No. 1.

2. A recombinant protein having esterase activity and exhibiting the amino acid sequence SEQ. ID. No. 1.

3. A polypeptide or recombinant protein which exhibits at least 80% identity to the amino acid sequence shown in SEQ ID No. 1 and has activity for racemate resolution of 2-alkyl-5-halopent-4-enecarboxylic esters of the formula (I)



(I)

in which R is a C_1-C_6-alkyl radical, R_1 is C_1-C_4-alkyl and X is chlorine, bromine or iodine.

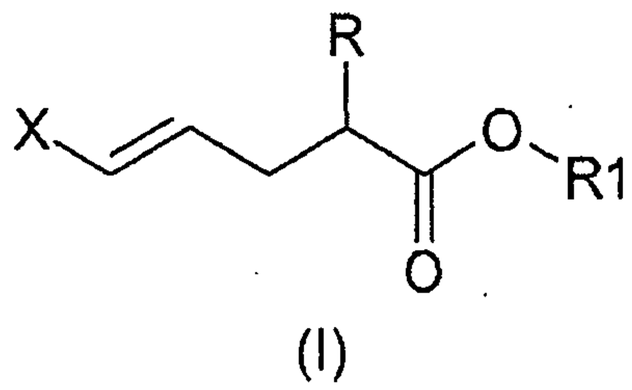
4. A polypeptide or recombinant protein having esterase activity as claimed in any of claims 1-3, which exhibits an amino acid sequence modified by usual modifications from the group of mutation, deletion, insertion, extension and/or fusion.

5. A nucleotide sequence which codes for a polypeptide as claimed in claim 1 or a recombinant protein as claimed in claim 2.

6. A nucleotide sequence which has the sequence shown in SEQ ID No. 2.

7. A nucleotide sequence which comprises a nucleotide sequence as claimed in claim 5 or 6.

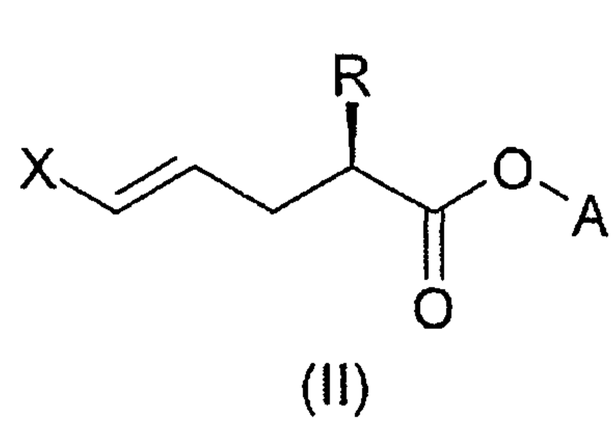
8. The use of a polypeptide or of a recombinant protein as claimed in claim 1, 2, 3 or 4 for racemic resolution of 2-alkyl-5-halopent-4-ene-carboxylic esters of the formula (I)



(I)

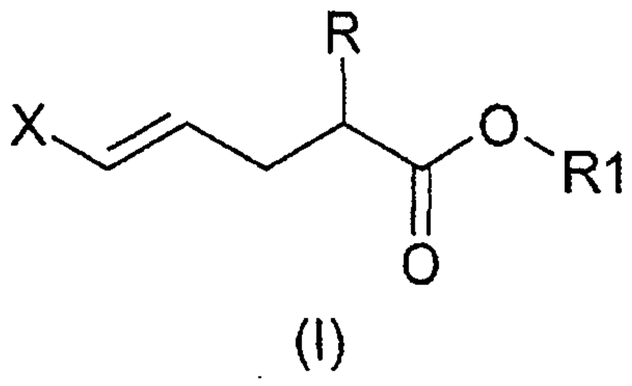
in which R is a C_1-C_6-alkyl radical, R_1 is C_1-C_4-alkyl and X is chlorine, bromine or iodine.

9. A method for preparing enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic acids or esters thereof of the formula (II)



(II)

in which R is a C_1-C_6-alkyl radical, A is equal to H, R_1, where R_1 may be C_1-C_4-alkyl, or R_2, where R_2 is an alkyl group, but is not equal to R_1, and X is chlorine, bromine or iodine, which comprises a mixture of enantiomers of a 2-alkyl-5-halopent-4-enecarboxylic ester of the formula (I)



(I)

in which R, R_1 and X are as defined above,
being converted by means of a polypeptide or a recombinant esterase as claimed in claim 1, 2, 3 or 4 in the presence of water or an alcohol of the formula R_2OH, where R_2 is an alkyl group which is not equal to R_1, as nucleophile, and

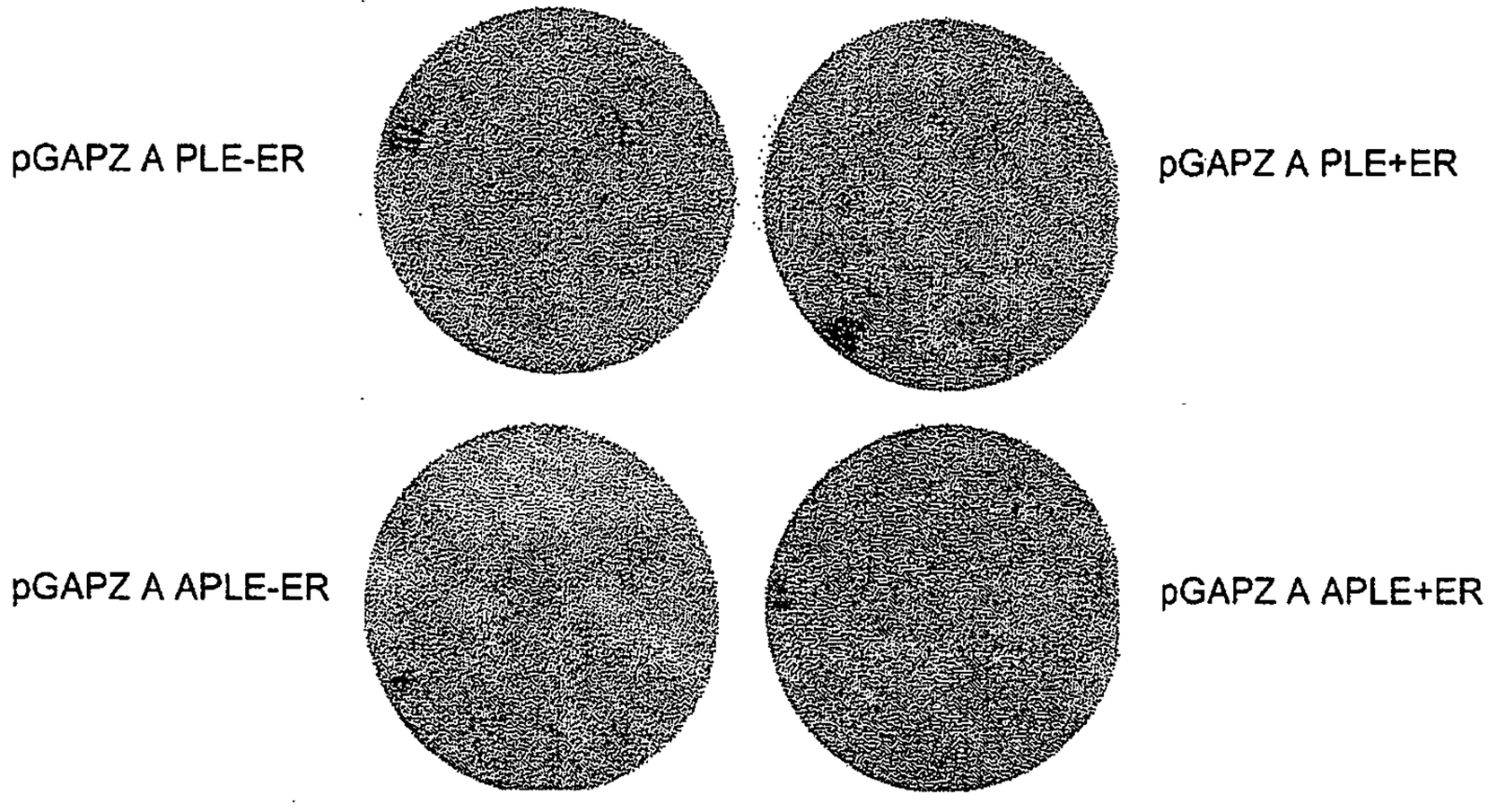
a) either the remaining enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic ester of the formula (II) with A equal to R_1 being isolated or

b) if an alcohol is employed as nucleophile, the resulting enantiomerically enriched 2-alkyl-5-halopent-4-enecarboxylic ester of the formula (II) with A equal to R_2 being isolated, or

c) if water is employed as nucleophile, the resulting 2-alkyl-5-halopent-4-enecarboxylic acid of the formula (II) with A equal to H being isolated.

Figure 1: Stereoselective esterase activity on methyl 5-chloro-2-(1-methylethyl)-4-pentenoate

A: Activity on the racemic substrate: *P. pastoris* X-33 transformants



B: Activity on the racemic substrate vs. (S) enantiomer

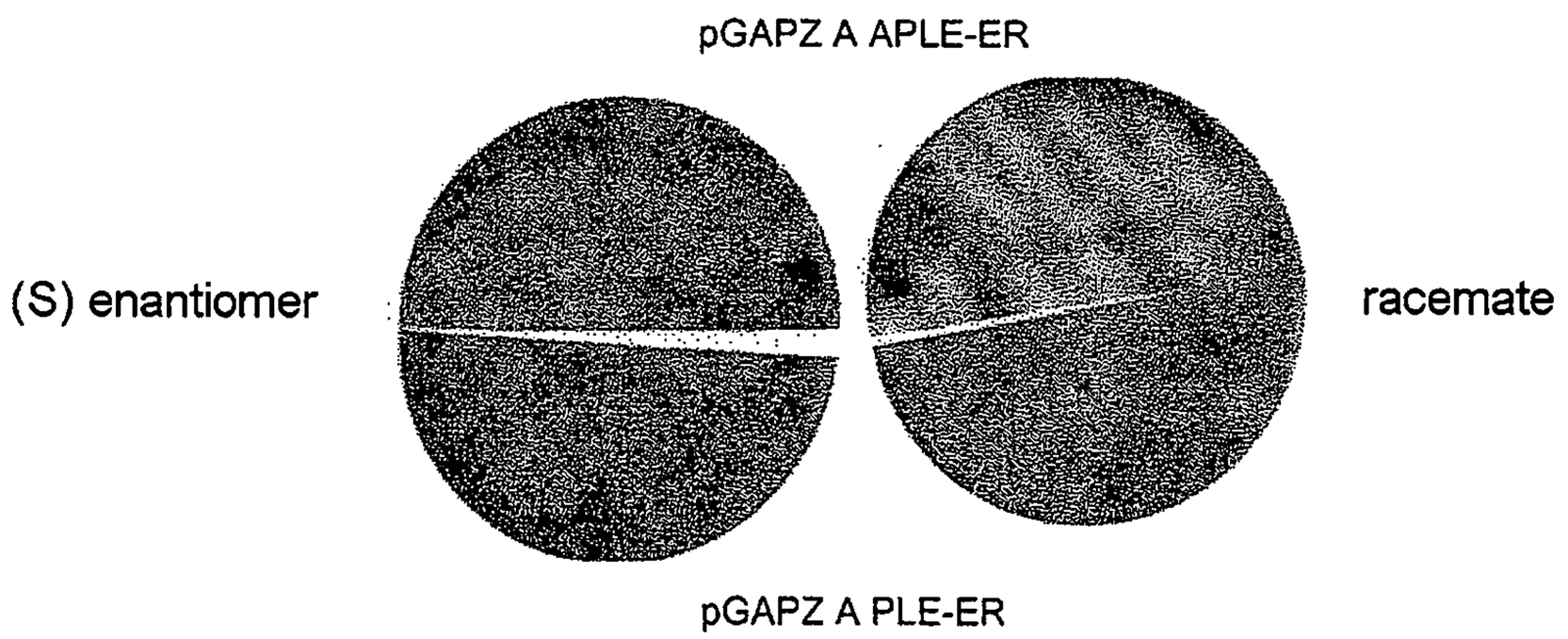
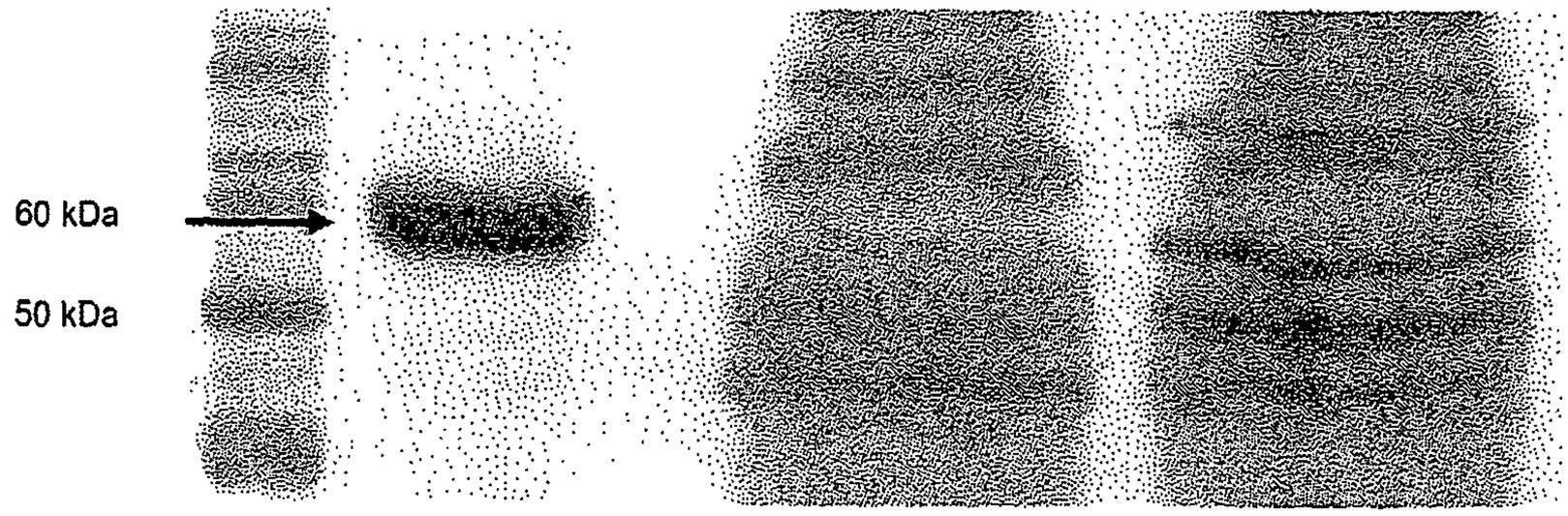


Figure 2: SDS-PAGE of recombinant APLE



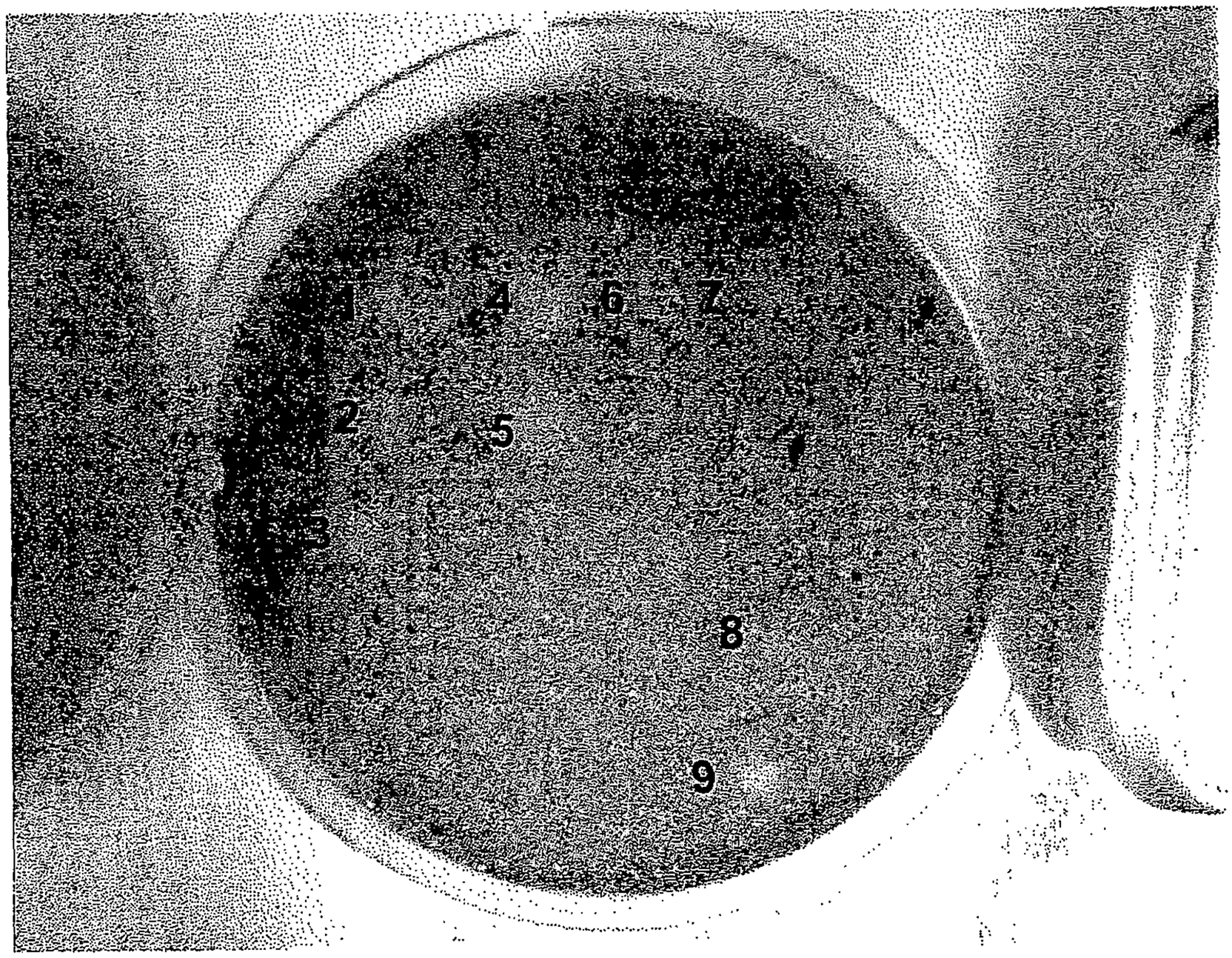
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2: Commercially available PLE

3: *P. pastoris* X-33 with integrated pGAPZ A (control strain)

4: *P. pastoris* X-33 with integrated pGAPZ A APLE-ER

Figure 3: Induced expression of pig liver esterases with the AOX1 promoter



1: *P. pastoris* KM71 with integrated pPIC9 APLE-ER
2: *P.* pastoris KM71 with integrated pPIC9 APLE-ER
3: P. pastoris KM71 with integrated pPIC9 APLE-ER
4: P. pastoris KM71 with integrated pPIC9 APLE+ER
5: P. pastoris KM71 with integrated pPIC9 APLE+ER
6: P. pastoris KM71 with integrated pPIC9 PLE-ER
7: P. pastoris KM71 with integrated pPIC9 PLE+ER
8: P. pastoris KM71
9: Commercially available PLE

SEQ ID No 1

Protein sequence of rAPLE

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SEQ ID No 2:

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tgggccaact ttgctcgcag tgggaacccc aatggggagg ggctgcccca ttggccgatg
tacgaccagg aagaagggta ccttcagatc ggcgtcaaca cccaggcagc caagaggctg
aaaggtgaag aagtggcctt ctggaacgat ctcctgtcca aggaggcagc aaagaagcca
cccaagataa agcatgctga gctgtga

SEQ ID. No. 3: Primer 1

5'-CA*GAATTC*ATGGCTATC**GGGCAGCCAGCCTCGC**-3'

SEQ ID No. 4: Primer 2

5'-CCG*GAATTC*AGCCTCCCCTT**CACAGCTCAG** -3'

SEQ ID No. 5: EcoRIalpha1

5'-TCTTCGAA*GAATTC*ACG**ATGAGATTTCCTTCAATTTTTACTGC**-3'

SEQ ID No. 6: alphaPLE2

5'-GAGGCTGGCTGCCC**AGCTTCAGCCTCTCTTTTCTCG**-3'

SEQ ID No. 7: PLEalpha1

5'-AGAGAGGCTGAAGCT**GGGCAGCCAGCCTCGCCG**-3'

SEQ ID No. 8: EcoRIPLE+ER2

5'-AT*GGTACCGAATTC***TCACAGCTCAGCATGCTTTATCTTG**-3'

SEQ ID No. 9: EcoRIPLE2

5'-AT*GGTACCGAATTC*TCA**CTTTATCTTGGGTGGCTTCTTTG**-3'