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(54) **Title**: IMPROVED PIG LIVER ESTERASES

(57) **Abstract**: The invention relates to an isolated polypeptide having esterase activity comprising an amino acid sequence shown in any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 or a homologue thereof, comprising an amino acid substitution or deletion of one or more amino acids as shown in said SEQ ID NO's and resulting in a mutant polypeptide having an increased concentration of the fraction of the mutant polypeptide being present as an active and soluble protein in cleared lysate of the mutant polypeptide expressed in E.coli relative to the concentration of the fraction of the polypeptide without the mutation being present as an active and soluble protein in cleared lysate of the polypeptide without the one or more deletion or substitution expressed in E.coli under the same conditions. The invention also relates to nucleic acid encoding the polypeptides according to the invention, and the use of the polypeptides.

IMPROVED PIG LIVER ESTERASES

The invention relates to isolated mutant polypeptides having esterase activity and having an increased concentration of the fraction of the polypeptide being present as an active and soluble protein in cleared lysate of the polypeptide expressed in E.coli relative to the polypeptide without certain mutations in its amino acid sequence. The invention also relates to isolated nucleic acid sequences encoding the mutant pig liver esterases and to use of the mutant polypeptides according to the invention.

Pig liver esterases (PLEs) are known as a very useful class of hydrolases, for example they are very useful in the enantioselective hydrolysis of esters. The fact that they are deemed useful is quite astonishing as there have been severe drawbacks for the use of PLEs, a crude lysate isolated from pig liver. (i) There are various iso-enzymes having different properties and enantioselectivity can, therefore, change from batch to batch and can also change with reaction time due to differences in operational stability. (ii) The risk of viral or prion contamination of crude pig liver extract is a major concern for the pharmaceutical industry. (iii) In addition, products made with the help of PLEs might not be considered kosher or halal.

Because of these limitations, various efforts are known to recombinantly produce pig liver iso-enzymes. While initially the methylotrophic yeast Pichia pastoris seemed to be a good expression system for pig liver esterases, in the end Escherichia coli proved to be a better host after improvement of specific host, gene and expression systems contributing to correct enzyme folding.

International patent application WO 2009/004093 describes the expression of pig liver esterase in E.coli, and is hereby incorporated by reference.

Since PLEs are such useful enzymes, there remains a need to further improve the activity levels that can be achieved with the enzyme.

Surprisingly, it has now been found that expression levels and therefore activity levels of pig liver esterase iso-form mutants and homologous esterases expressed in E. coli can significantly be improved by substituting one or more residues in its amino acid sequence, namely one or more of the amino acids responsible for multimer formation of the pig liver esterase, by an amino acid that does not result in or reduces the tendency for multimer formation, thereby changing the quaternary structure of PLE.

The finding was triggered by the preparation of a mutation on position 788 of the Open Reading Frame encoding APLE (SEQ ID NO 1), which is corresponding to nucleotide position 5541 in SEQ ID NO 1, whereby the replacement was T-> A, leading to the replacement of the hydrophobic valine in the APLE enzyme by a negatively charged aspartic acid: V263D. A computer model indicated that this mutation was located on a helix at the very outside of the enzyme, thus, remote from the active site cavity of the enzyme, while it was also found that the total activity of this mutated enzyme towards (4E)-5-chloro-2-isopropylpent-4-enoic-acid methyl ester increased from 6.5 to 11.6 Units/mg total soluble protein. In case of dimethyl-3-(3,4-dichlorphenyl)-glutarate, activity increased from 36 to 42 mU/mg of total soluble protein and in case of para-nitrophenyl acetate activity raised from 15.4 to 24.3 U/mg total soluble protein. At first, there was no explanation why a mutation in an outer region of APLE would have such a strong effect on various conversions. However, analysis of the structure of the human homolog hCE1 identified valine on position 263 of APLE as being potentially important for multimerization. Upon introducing a charged aspartic acid instead, the formerly existing hydrophobic interaction, stabilizing interaction between two subunits, is impaired. A negatively charged amino acid may repel the other subunit instead of binding it through hydrophobic interaction.

Next, the hypothesis of multimer formation being disrupted by a change introduced by aspartic acid on position 263 disrupting hydrophobic interactions and, therefore, leading to the formation of monomers was tested. Analyzing a computer model of APLE it was concluded that valine on position 263 of one monomer may interact with leucine on position 43 of another monomer. It was postulated that trimer formation is at least partly due to alternating hydrophobic interaction of L43 and V263 between three sub-units in total. Thus, regardless of which amino acid is replaced by aspartic acid the interaction should be interrupted. Testing the hypothesis, leucine on position 43 was replaced by aspartic acid, which resulted in monomerisation.

This mutation also resulted in monomer formation, thus, this proved that the replacement of amino acids at certain positions of a PLE monomer that without the replacement can form a multimer increases the amount of enzyme present in the monomeric form, and although the L43D variant yielded less soluble protein than the V263D variant, it is clear that the activity found for a certain amount of cleared lysate comprising enzymes mutated at a position involved in multimer formation and used for a certain conversion increased relative to the same amount of cleared lysate comprising enzymes without mutations used for the same conversion. The same

mutation was introduced in all polypeptides according to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14, and resulted in the same effect.

Thus, the invention relates to an isolated polypeptide having esterase activity comprising an amino acid sequence shown in any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 or a homologue thereof, comprising an amino acid substitution or deletion of one or more amino acids as shown in said SEQ ID NO's and resulting in a mutant polypeptide having an increased concentration of the fraction of the mutant polypeptide being present as an active and soluble protein in cleared lysate of the mutant polypeptide expressed in E. coli relative to the concentration of the fraction of the polypeptide without the mutation being present as an active and soluble protein in cleared lysate of the polypeptide without the one or more deletion or substitution expressed in E.coli under the same conditions.

It is preferred to substitute one or more of the amino acids rather than to delete one or more amino acids.

In this text, relative esterase activity is a comparison of activities of wild-type (i.e. the non mutagenized parent enzyme) and the respective mutant enzyme which were prepared (see at the Materials and methods section under the header "Expression and cell harvest"; where preparation of cleared lysate is described and where under "Quantification of PLE activity" the methods for measuring activity are described) under the same conditions using the same amount of the soluble protein preparation (cleared lysate). The relative activity (r [%]) is calculated by the release of para-nitrophenol per minute of the mutant lysate ([p-NPAmut]) divided by the release of para-nitrophenol per minute of the corresponding wild-type lysate ([p-NPAwt]) multiplied by 100% according to the formula r = ([p-NPAmut]/[p-NPAwt])x100%.

In one embodiment, the invention relates to an isolated polypeptide according to the invention, which polypeptide shows an increase of 10% in esterase activity compared to the esterase activity of the corresponding wild-type polypeptide without deletion or substitution of amino acids.

Enzymes have a primary (amino acid sequence), secondary (mainly alpha-helix and beta-sheet) and tertiary (structure of one peptide chain) structure. In addition, some enzymes form quaternary structures which are conglomerates (multimers) of the same (homo) or different (hetero) subunits (peptide chains). Pig liver esterases are homo trimers. The quaternary structure of the enzymes and mutants can be tested by glycerol density gradient centrifugation (see Example 3 for a description of Glycerol density gradient centrifugation) and native gel electrophoresis (see in the

Materials and Methods section under the header “Native Gel Electrophoresis” for a description of the method).

Multimer formation is usually caused by multiple intermolecular attractive forces of different amino acids such as hydrophobic/hydrophobic or ionic (positive/negative) interactions of the different subunits (monomers). Such interactions are often stabilizing enzymes and are, therefore, often beneficial for enzymes. In the present invention it was surprisingly found that destruction of only a few of such attractive forces lead to a changed quaternary structure and higher relative activities of PLE and homologous enzymes in cleared lysates.

Thus, the invention relates to an isolated polypeptide having esterase activity comprising an amino acid sequence shown in any one of SEQ ID NO’s 2, 4, 6, 8, 10, 12 or 14 or a homologue thereof, comprising an amino acid substitution or deletion of one or more amino acids as shown in said SEQ ID NO’s, wherein at least one amino acid substitution or deletion has taken place at an amino acid position which is located at a point of interaction of monomers when the monomers are forming multimers and which destroys that point of interaction between the monomers, and resulting in a mutant polypeptide having an increased concentration of the fraction of the mutant polypeptide being present as an active and soluble protein in cleared lysate of the mutant polypeptide expressed in E.coli relative to the concentration of the fraction of the polypeptide without the mutation being present as an active and soluble protein in cleared lysate of the polypeptide without the one or more deletion or substitution expressed in E.coli under the same conditions. Preferably, the invention relates to an isolated polypeptide having esterase activity comprising an amino acid sequence according to any one of SEQ ID NO’s 2, 4, 6, 8, 10, 12 or 14, and isolated polypeptides having an amino acid identity of at least more than 90%, preferably more than 95% identity to any one of SEQ ID NO’s 2, 4, 6, 8, 10, 12 or 14, and where at least one amino acid substitution or deletion has taken place at an amino acid position which is located at a point of interaction of monomers when the monomers are forming multimers and which destroys that point of interaction between the monomers. Preferably the substitution or deletion has been carried out at one or more positions selected from the group of amino acid positions 43, 260, 263, 266 or 270, or positions corresponding thereto. Preferred substitutions are L43D, T260P, T260A, V263D and V263G or positions corresponding thereto.

In particular, the invention relates to isolated polypeptides having esterase activity said polypeptide comprising an amino acid sequence shown in any

one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 or a homologue thereof having an amino acid identity of more than 90%, preferably more than 95%, more preferably more than 97%, most preferably more than 98% identity to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14, comprising one or more amino acid substitutions selected from the group of positions 43, 260, 263 or positions corresponding thereto, preferably those substitutions are selected from the group of substititions L43D, T260P, T260A, V263D, V263G or positions corresponding thereto.

In the framework of this invention percentages identity (or homology) were or may be determined as described in Tatiana A. Tatusova, Thomas L. Madden (1999), "Blast 2 sequences - a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250, using the following standard parameters at http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/wblast2.cgi

- for Protein sequences:
  Matrix: BLOSUM62
  Open gap: 5
  extension gap: 2
  Penalties gap x_dropoff: 11
  Expected: 10
  word size: 11

- for nucleotides:
  Reward for match:1
  Penalty for mismatch:-2
  Open gap: 11
  extension gap: 1
  Penalties gap x_dropoff: 50
  Expected: 10
  word size: 3

Positions that can be targeted for destroying attractive forces of different monomers can be identified by analyzing X-ray structures of PLE or homologous enzymes (e.g. human liver carboxylesterase 1, PDB entry 1MX1 or the rabbit liver carboxylesterase 1, PDB entry 1K4Y) or by random mutagenesis experiments. Preferred amino acid positions for targeting substitutions are positions 43, 260 and 263, and amino acids of other subunits that interact with these amino acids.

After identification of amino acid positions that contribute to the quaternary structure using the known methods, amino acid substitutions are chosen to destroy the attractive forces. Preferred substitutions are exchange of hydrophobic residues, e.g. Alanine (abbreviated as Ala or A), Valine (abbreviated as Val or V), Isoleucine (abbreviated as Ile or I), Leucine (abbreviated as Leu or L), Methionine (abbreviated as Met or M), Phenylalanine (abbreviated as Phe or F), Tryptophane (abbreviated as Trp or W), Cysteine (abbreviated as Cys or C), Proline (abbreviated as Pro or P), with less hydrophobic or hydrophilic amino acids, e.g. Lysine (abbreviated as Lys or K), Arginine (abbreviated as Arg or R), Aspartic acid (abbreviated as Asp or D), Glutamic acid (abbreviated as Glu or E), Serine (abbreviated as Ser or S), Tyrosine (abbreviated as Tyr or Y), Threonine (abbreviated as Thr or T), Glycine (abbreviated as Gly or G), Histidine (abbreviated as His or H), Glutamine (abbreviated as Gln or Q), Asparagine (abbreviated as Asn or N) and vice versa. Other preferred amino acid substitutions target destruction of ionic forces by substituting positively charged amino acids (e.g. Lysine, Arginine or Histidine) with negatively charged amino acids (e.g. Aspartic acid or Glutamic acids) or vice versa.

Thus, a preferred mutation is the replacement of L43 with any amino acid chosen from the group of K, R, D, E, S, Y, T, G, H, Q and N. Another preferred mutation is the replacement of T260 with any amino acid chosen from the group of A, V, I, L, M, F, W, C and P. Another preferred mutation is H266 with any amino acid chosen from the group of A, V, I, L, M, F, W, C and P and D and E. Another preferred mutation is the replacement of Q270 with any amino acid chosen from the group of A, V, I, L, M, F, W, C and P. All mutations are described relative to any one of the amino acid sequences according to SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14.

Particularly preferred are the following mutations, based on the amino acid sequence of any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14: V263D, L43D, T260P and T260A.

It will be understood by a person skilled in the art that one or more combinations of the described mutations are also possible, as long as the replacements do not result in an amino acid sequence that allows increased multimer formation relative to the amount of multimer formation before the mutation to occur.

As is known, the numbering of amino acids is dependent on the species the protein originates from. The numbering can also change as the result of deletions or insertions. It is known, however, to a skilled person how to align sequences. Thus, in this text the phrase "or corresponding thereto" is used to describe

amino acid positions that except for the number, are the same as the positions 43, 260 and 263 in SEQ ID NO 1.

The isolated polypeptides of the invention may in addition to mutations that decrease multimer formation comprise one or more further mutation, that improve the selectivity and/or activity towards a desired substrate.

Thus, in a preferred embodiment the invention relates to an isolated polypeptide having esterase activity, said polypeptide comprising an amino acid sequence shown in any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 or a homologue thereof having an amino acid identity of at least more than 90%, preferably more than 95% identity to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14, comprising one or more amino acid substitutions at positions 43, 260 and 263, or positions corresponding thereto, and one or more amino acid substitution selected from the group of F234S and L238V. Even more preferred in this embodiment, the amino acid substitutions are one or more substitutions on one or more positions selected from the group of position comprising position 43, position 260 and position 263, or more specifically selected from the group of L43D, T260P, T260A, V263D and V263G.

The F234S mutation was the result of the replacement of T on position 701 of the gene encoding for APLE by C. The L238V mutation was the result of the replacement of T by A on position 712 of the gene encoding for APLE.

Independent of the mutations that prevent multimer formation, mutants of any one of the polypeptides comprising an amino acid sequence according to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 comprising at least one of these two mutations are very useful for the conversion of para-Nitrophenyl acetate, and/or dimethyl-3-(3,4-dichlorphenyl)-glutarate and/or for the resolution of racemic (4e)-5-chloro-2-isopropylpent-4-enoic acid methyl ester. Thus, the invention also relates to such polypeptides.

Thus, the invention also relates to an isolated polypeptide having esterase activity, said polypeptide comprising an amino acid sequence shown in any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 or a homologue thereof having an amino acid identity of at least 95% identity, preferably 97%, more preferably 98% to any one of SEQ ID's NO's 2, 4, 6, 8, 10, 12 or 14, comprising one or more amino acid substitutions selected from the group of L238V and F234S. Preferably, in addition to the L238V and/or F234S mutation being present, the following positions have the following amino acid residues: at positions 129, 133, 134, 138 and 139 the residues are V, S, T, L and A, respectively. The invention also relates to nucleic acids encoding the

polypeptides according to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 having at least one mutation chosen from L238V and F234S. Also the use of the polypeptides according to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 having at least one mutation chosen from L238V and F234S for the production of an acid, ester or alcohol, or more in particular for the conversion of para-Nitrophenyl acetate, or the resolution of racemic (4e)-5-chloro-2-isopropylpent-4-enoic acid methyl ester or the conversion of dimethyl-3-(3,4-dichlorphenyl)-glutarate is part of the invention.

The invention also relates to nucleic acids encoding the isolated polypeptides according to the invention. In particular, the invention relates to nucleic acids which are the coding sequences in SEQ ID NO's 1, 3, 5, 7, 9, 11 or 13 and homologues thereof, preferably with more than 80% identity, more preferably more than 90%, even more preferred more than 95%, most preferably more than 98% identity to nucleic acids which are the coding sequences in SEQ ID NO's 1, 3, 5, 7, 9, 11 or 13.

In an embodiment, the invention relates to a process for the manufacture of acids, esters or alcohols, wherein an isolated peptide according to the invention is applied. Also, the invention relates to such process wherein α-alkylated acids and/or esters are manufactured, more specifically a process wherein optically pure α-alkylated acids selected from these α-alkylated acids are reduced to their corresponding alcohols. In a further embodiment, these alcohols are further applied as building blocks for dipeptide mimetics, dipeptide mimetics being synthetic copies of natural dipeptides. Furthermore, the invention relates to the application of these building blocks in blood pressure lowering agents.

The invention further relates to all possible combinations of different embodiments and/or preferred features according to the isolated peptide, the nucleic acid sequence, the use of the polypeptide and the process according to the invention as described herein.

In addition, the invention relates to all embodiments in combination with SEQ ID NO's 1, 3, 5, 7, 9, 11 and 13, wherein the polypeptides are encoded by the Open Reading Frames indicated in these sequences.

Materials and Methods

General techniques to prepare polypeptides and mutants thereof are known in the art, and may be found in e.g. Sambrook et al. Molecular Cloning: A

Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory , Cold Spring Harbor, NY, (2001) .

Preparation of recombinant E. coli expressing APLE, γPLE, PICE, PLE2, PLE3, PLE4, PLE5 and corresponding mutants.

Synthetic genes γPLE, PICE, PLE2, PLE3, PLE4, PLE5 (see SEQ ID NO's 4, 6, 8, 10, 12 or 14) were cut with NdeI and HindIII and cloned into the NdeI and HindIII restriction sites of pCm470_DsbC_APLE-C8P which is coding for APLE (see SEQ ID NO 2 and Figure 1) using standard molecular biology techniques as described in Sambrook, J., Fritsch, E.F. and Manniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press.

pCm470_DsbC_APLE-C8P and the plasmids derived above coding for APLE, γPLE, PICE, PLE2, PLE3, PLE4, PLE5 were transformed into E. coli Origami B (DE3).

Mutants of APLE, γPLE, PICE, PLE2, PLE3, PLE4, PLE5 were prepared by site-saturation mutagenesis using the QuikChange® Site-Directed Mutagenesis Kit (Catalog #200518) of Stratagene (Stratagene, 11011 North Torrey Pines Road La Jolla, CA 92037) according to the provided INSTRUCTION MANUAL using the above described plasmids coding for APLE, γPLE, PICE, PLE2, PLE3, PLE4, PLE5 as templates.

Examples for mutagenesis primers for site directed mutagenesis are described in SEQ ID NO's 15-24. The resulting mutant plasmids of APLE, γPLE, PICE, PLE2, PLE3, PLE4, PLE5 were transformed into E. coli Origami B (DE3).

SeqID 15: Primer L43D_F
5' GTCCCTTTTGCTAAGCCACCTGACGGATCTTTGAGGTTTGC 3'
SeqID 16: Primer L43D_R
5' GCAAACCTCAAAGATCCGTCAGGTGGCTTAGCAAAAGGGAC 3'
SeqID 17: Primer T260P_F
5' GCAGGATGCAAAACTACTCCTTCGGCAGTCTTCGTGC 3'
SeqID 18: Primer T260P_R
5' GCACGAAGACTGCCGAAGGAGTAGTTTTGCATCCTGC 3'
SeqID 19: Primer T260A_F
5' GCAGGATGCAAAACTACTGCTTCGGCAGTCTTCGTGC 3'
SeqID 20: Primer T260A_R
5' GCACGAAGACTGCCGAAGCAGTAGTTTTGCATCCTGC 3'

SeqID 21: Primer V263D_F
5' CTACTACTTCGGCAGACTTCGTGCATTGTTTGC 3'
SeqID 22: Primer V263D_R
5' GCAAACAATGCACGAAGTCTGCCGAAGTAGTAG 3'
SeqID 23: Primer V263G_F
5' CAAAACTACTACTTCGGCAGGGTTCGTGCATTGTTTGCGTC 3'
SeqID 24: Primer V263G_R
5' GACGCAAACAATGCACGAACCCTGCCGAAGTAGTAGTTTTG 3'

Expression and cell harvest.

All media components and antibiotics were bought from Roth GmbH & Co. KG (Karlsruhe, Germany). Culturing conditions were as follows: 20 ml of pre-culture medium LB medium (Lennox), containing 10 µg/ml chloramphenicol were inoculated with colonies of recombinant E.coli expressing APLE, γPLE, PICE, PLE2, PLE3, PLE4, PLE5 and corresponding mutants (preparation see above) and incubated in 100 ml Erlenmeyer flasks at 28°C and 200 rpm over night (18 h). 10 ml thereof were used to inoculate 500 ml of main culture medium LB medium (Lennox), containing 10 µg/ml chloramphenicol) in 2 l baffled shake flasks. The main culture was incubated at 28°C and 120 rpm and was induced by 0.1 mM IPTG at OD_{600} 0.6 – 0.8 over night (18 h). For harvesting cells, cultures were centrifuged at 4,000 x g for 10 min at 4°C. Pellets were resuspended in 25 ml 20 mM potassium phosphate buffer, pH 8.0. Cells were sonicated in an ice-water cooled pulping beaker for 5 min with 80 % duty cycle and output control level 8 using a Branson Sonfier® 250 (Branson, Danbury, USA). After centrifugation at 75,600 x g and 10°C for 1 h the supernatant containing soluble proteins was sterile filtered (0.2 µm filters) and stored at 4°C. These cleared lysates were used for activity determination using the para-nitrophenyl acetate (p-NPA) (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) assay.

Native Gel Electrophoresis

Blue native gel electrophoresis (BN-PAGE) of bacterial lysates containing APLE was performed according to the method described by Reisinger and Eichacker (2006), "Analysis of membrane protein complexes by blue native PAGE", Proteomics 6 Suppl 2, 6-15, with the following modifications. Aliquots containing 200 µg of soluble proteins were diluted with 10 mM Tris/HCl pH 7.4 to a final volume of 95 µl. Five µl of loading buffer was added to the sample before applying them on an 8 – 16 %

linear gradient gel. Gradient gels (16 x 20 cm) were created with the help of a Bio-Rad Gradient Former model 485 (Bio-Rad Laboratories, Vienna, Austria). Electrophoresis was performed at a constant current of 24 mA per gel at 4°C. After electrophoresis, gels were incubated in 0.1 M potassium phosphate buffer, pH 7.0, for 20 min at room temperature. For the in-gel-detection of esterase activity, the substrate fluorescein diacetate (FDA) was dissolved in acetone [4 mg/ml] and applied on a Biodyne® A membrane (0.45 mm, 16 x 20 cm) (Pall Life Science, Michigan, USA). After evaporation of acetone, the membrane was brought in close contact with the gel which was placed on a glass plate. The gel-membrane sandwich was incubated at 37°C for 30 min before detection of fluorescent bands.

SDS-PAGE, Western Blot analysis and native gel electrophoresis.

SDS-PAGE was based on standard protocols. Prior to loading onto the gels (separation gel: 12.5 %, stacking gel: 4 %) samples containing 80 µg of total proteins were mixed with respective amount of 2x sample buffer and heated at 40°C for 15 min. A TE 22 Mighty Small Transphor Tank Transfer Unit (Amersham Biosciences, Uppsala, Sweden) was used for blotting proteins onto a Hybond-ECL™ nitrocellulose membrane (Amersham Biosciences). The primary antibody was a polyclonal rabbit antibody against porcine liver carboxylesterase (abcam, Cambridge, UK). The secondary antibody was a polyclonal goat-anti-rabbit antibody conjugated with alkaline phosphatase adsorbed against human serum proteins (Leinco Technologies, St. Louis, USA). Detection was either done with BCIP/NBT detection (CALBIOCHEM/EMD, La Jolla, USA) directly on the membrane. Protein standard used was PageRuler™ prestained protein ladder (Fermentas GmbH, St. Leon-Rot, Germany).

Quantification of PLE activity.

Activity towards rac. (4E)-5-chloro-2-isopropylpent-4-enoic-acid methyl ester and dimethyl-3-(3,4-dichlorphenyl)-glutarate was determined by autotitration. Measurements were performed on a Mettler Toledo DL50 GraphiX (Mettler-Toledo GmbH; Giessen, Germany), using 0.1 M and 0.01 M NaOH, respectively, as titrating agent (Roth GmbH & Co. KG, Karlsruhe, Germany). The total reaction mixture of 50 ml consisted of 5 ml substrate i.e. racemic (4E)-5-chloro-2-isopropylpent-4-enoic-acid methyl ester or dimethyl-3-(3,4-dichlorphenyl)-glutarate [100 mg/ml] dissolved in toluene, 5 ml of 10 % Tergitol® NP-9 (Sigma-Aldrich, Vienna,

Austria) and 10 to 40 mg of soluble proteins. Volumes were adapted to 50 ml by 20 mM potassium phosphate buffer, pH 8.0.

The following p-NPA assay is used:

Para-nitrophenyl acetate (p-NPA) (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) assays were performed with cleared lysates at room temperature in 100 mM Tris/HCl buffer, pH 7.0, using 2 mM p-NPA. The release of para-nitrophenol was quantified at 405 nm (ε = 9.5946 ml µmol-1 cm-1) using a BeckmanCoulterDU® 800 Spectrophotometer (Beckman Coulter GmbH, Krefeld, Germany). One unit is defined as the amount of enzyme which releases 1 µmol para-nitrophenol in one minute under the above reaction conditions.

EXAMPLES

The invention will be elucidated with reference to the following examples, without however being restricted by these:

Example 1: Quantification of PLE activity.

The p-NPA assay as described above was used as the amount of enzyme which releases 1 µmol para-nitrophenol in one minute under the above reaction conditions.

Figure 2 shows the increase in total cellular activity towards p-NPA of V263D-mutant PLEs expressed in E. coli compared to the PLE wild-type enzymes without the V263D-mutation expressed in E. coli. As the 100 % level for each PLE is taken the level of the respective PLE wild-type variant.

In the framework of this invention all polypeptides according to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 and homoloques thereof are referred to as PLE. Thus, also the enzyme called Porcine Intestinal Carboxyl Esterase (PICE) is a PLE in the framework of this invention.

It is clear that all PLE's upon inserting the V263D mutation showed an improved activity.

Example 2

The change in the quarternary structure from multimer to monomer in iso-enzymes with V263D mutations was further seen on native protein gel electrophoresis (figure 3).

Esterase activity was visualized herein through staining with fluorescein resulting from hydrolysis of fluorescein diacetate (FDA). All 7 wild-type enzymes are trimers of about the same height on the native gel, whereas the V263D mutants show bands on the lower third of the gel. Multiple bands were excised and analyzed on an SDS-PAGE gel. Multiple bands result in one clear band at the size of about 58 kDa (data not shown). It is assumed that these bands result from different conformations of the same monomer.

Example 3: Determination of quaternary structure

Glycerol density gradient centrifugation.

1 ml fractions ranging from 50 % to 5 % glycerol in 20 mM potassium phosphate buffer, pH 8.0, were carefully layered on top of each other into Ultra-Clear™ centrifuge tubes (14 x 89 mm) (Beckman, Palo Alto, USA). Afterwards, 500 µl lysate containing soluble proteins in the same buffer were carefully layered on top. High speed centrifugation at approx. 200,000 x g was performed in a SW 41 rotor (Beckman, Palo Alto, USA) for 20 h at 4°C. Fractions, i.e. 500 µl of 0 % glycerol and 1 ml of 5 – 50 % glycerol, were carefully collected in 1.5 ml reaction tubes and stored at 4°C.

Filter assay:

Whatman Qualitative Standard Filter Circles – Student Grade/Grade 93 Ø 85 mm (Whatman International Ltd., Maidstone, England) were soaked in assay mix containing 2 mg/ml phenol red, pH 7.5, 1 % (v/v) Tergitol® NP-9 (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany) and 10 % (v/v) racemic (4E)-5-chloro-2-isopropylpent-4-enoic-acid methyl ester (DSM Fine Chemicals Austria GmbH, Linz, Austria) (for the supplemental figures 5-10, the substrate was 10 % (v/v) racemic dimethyl methylsuccinate). If the pH value of the assay mix was too low, increasing volumes of 1 M potassium phosphate buffer, pH 8.0, were added. 2 µl of cell free extracts were spotted onto the filters. Color change from red to yellow, caused by hydrolysis of esters and liberation of respective carboxylic acids, indicated enzyme activity. The quality of the substrate, the amount of additional buffer and the starting pH determined the time necessary for a color change.

The result shows that the main fraction of the wild-type APLE localizes between 25% and 30% glycerol concentration, while the main fraction of the mutant, monomeric APLE localizes between 15 and 20% glycerol, see Figure 4.

Similar experiments were performed for all naturally occuring PLE variants, and they all showed the same effect. (See supplemental figures 5-10). Protein standard used in all supplemental experiments was the PageRuler™ prestained protein ladder.

Example 4

The activity towards para-nitrophenyl acetate (light grey) and dimethyl-3-(3,4-dichlorphenyl)-glutarate (grey), and the activity towards para-nitrophenyl acetate (light grey) and rac. (4E)-5-chloro-2-isopropylpent-4-enoic-acid methyl ester (dark grey) was measured as described above under "The Materials and methods" section, "Quantification of PLE-activity". The results are shown in figure 11a en 11b.

DESCRIPTION OF FIGURES:

Figure 3:

1a. APLE
1b: APLE with V263D mutation
2a: PLE3
2b: PLE3 with V263D mutation
3a:PLE4
3b: PLE4 with V263D mutation
4a: PLE5
4b: PLE5 with V263D mutation
Neg.C: negative control E.coli origami B
5a:γ-PLE
5b: γ-PLE with V263D mutation
6a: PLE2
6b:PLE2 with V263D mutation
7a:PICE
7b:PICE withV263D mutation

Figure 4:

a, b: APLE wild-type
c, d: APLE-V263D
a, c: PH-shift assays
b, d: Western Blotting

Figure 5:

a, b: γPLE wild-type
c, d: γPLE-V263D
a, c: PH-shift assays
b, d: Western Blotting

Figure 6:

a, b: PICE wild-type
c, d: PICE-V263D
a, c: PH-shift assays
b, d: Western Blotting

Figure 7:

a, b: PLE2 wild-type
c, d: PLE2-V263D
a, c: PH-shift assays
b, d: Western Blotting

Figure 8:

a, b: PLE3 wild-type
c, d: PLE3-V263D
a, c: PH-shift assays
b, d: Western Blotting

Figure 9:

a, b	PLE4 wild-type
c, d	PLE4-V263D
a, c	PH-shift assays
b, d	Western Blotting

Figure 10:

a, b	PLE5 wild-type
c, d	PLE5-V263D
a, c	PH-shift assays
b, d	Western Blotting

CLAIMS

1. An isolated polypeptide having esterase activity comprising an amino acid sequence shown in any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 or a homologue thereof, comprising an amino acid substitution or deletion of one or more amino acids as shown in said SEQ ID NO's, wherein at least one amino acid substitution has taken place at an amino acid position which is located at a point of interaction of monomers when the monomers are forming multimers and which destroys that point of interaction between the monomers, and resulting in a mutant polypeptide having an increased concentration of the fraction of the mutant polypeptide being present as an active and soluble protein in cleared lysate of the mutant polypeptide expressed in E.coli relative to the concentration of the fraction of the polypeptide without the mutation being present as an active and soluble protein in cleared lysate of the polypeptide without the one or more deletion or substitution expressed in E.coli under the same conditions.

2. An isolated polypeptide according to claim 1 comprising amino acid sequences with more than 90%, more preferably more than 95% identity to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14.

3. An isolated polypeptide according to any one of claims 1-2, which polypeptide shows an increase of at least 10% in esterase activity compared to the esterase activity of the corresponding wild-type polypeptide without deletion or substitution of amino acids.

4. An isolated polypeptide according to any one of claims 1-3, wherein the substitution or deletion has been carried out at one or more positions selected from the group of amino acid positions 43, 260, 263, 266 or 270, or positions corresponding thereto.

5. An isolated polypeptide having esterase activity, said polypeptide comprising an amino acid sequence shown in any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14 or a homologue thereof having an amino acid identity of more than 90%, preferably more than 95%, more preferably more than 98% identity to any one of SEQ ID NO's 2, 4, 6, 8, 10, 12 or 14, comprising one or more amino acid substitutions at positions 43, 260 and/or 263 or positions corresponding thereto.

6. An isolated polypeptide according to claim 4 or 5, wherein the amino acid substitutions are selected from the group of L43D, T260P, T260A, V263D, V263G or positions corresponding thereto.

7. An isolated polypeptide according to any one of claims 1-6, comprising at least one further mutation selected from the group of L238V and F234S.

8. A nucleic acid sequence encoding the polypeptide according to any one of claims 1-7.

9. Use of a polypeptide according to any one of claims 1- 7 for the production of an acid, ester or alcohol.

10. Process for the manufacture of acids, esters or alcohols, wherein an isolated polypeptide according to any of one claims 1-7 is applied.

11. Process according to claim 10, wherein α-alkylated acids and/or esters are manufactured.

12. Process according to claim 11, wherein optically pure α-alkylated acids are reduced to their corresponding alcohols.

13. Process according to claim 10 or 12, wherein the alcohols are further applied as building blocks for dipeptide mimetics.

14. Process according to claim 13, wherein the building blocks are applied in blood pressure lowering agents.

FIG. 1

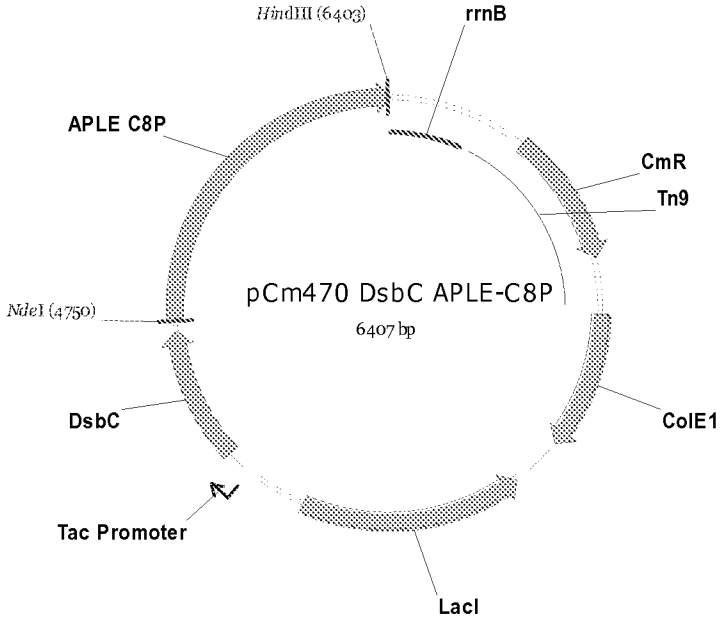

HindIII (6403)
rrnB
APLE C8P
CmR
Tn9
pCm470 DsbC APLE-C8P
6407 bp
NdeI (4750)
DsbC
ColE1
Tac Promoter
LacI

FIG. 2

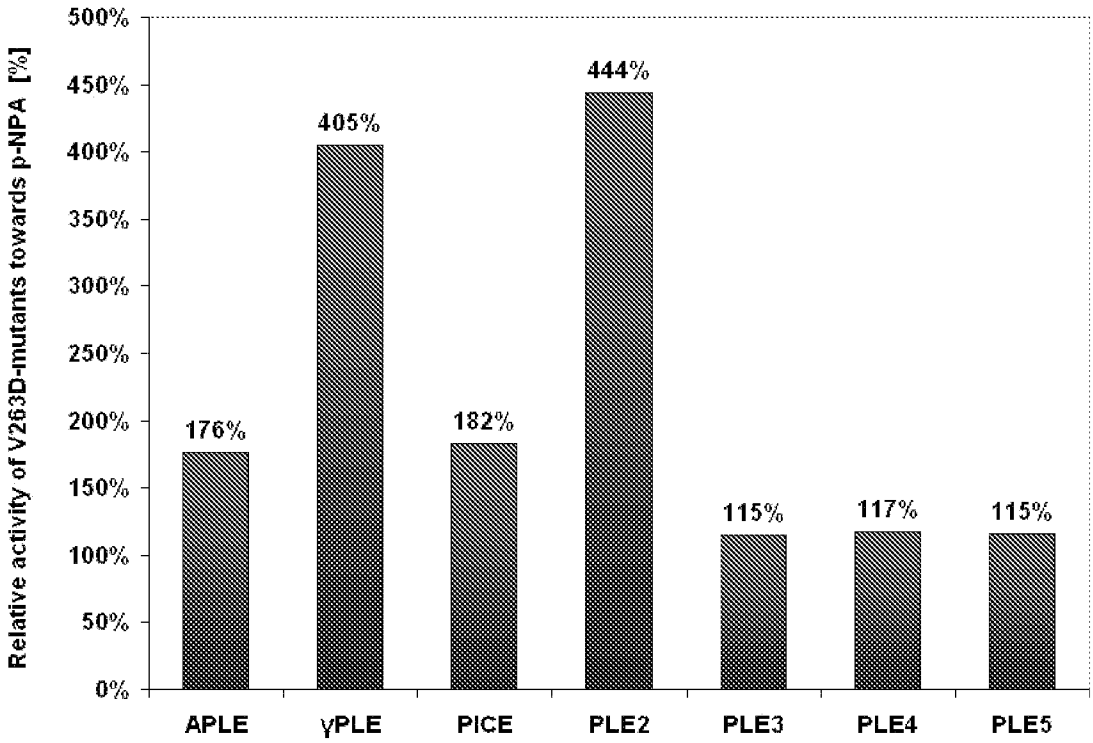

Relative activity of V263D-mutants towards p-NPA [%]
500%
450%
400%
350%
300%
250%
200%
150%
100%
50%
0%
176%
405%
182%
444%
115%
117%
115%
APLE
γPLE
PICE
PLE2
PLE3
PLE4
PLE5

FIG. 3

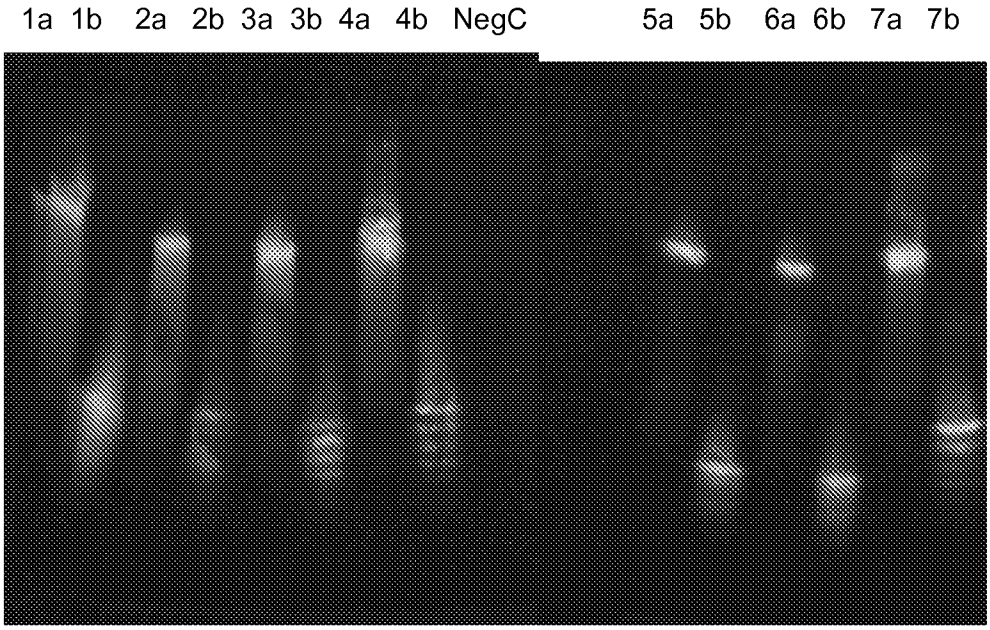

1a 1b 2a 2b 3a 3b 4a 4b NegC 5a 5b 6a 6b 7a 7b

FIG. 4

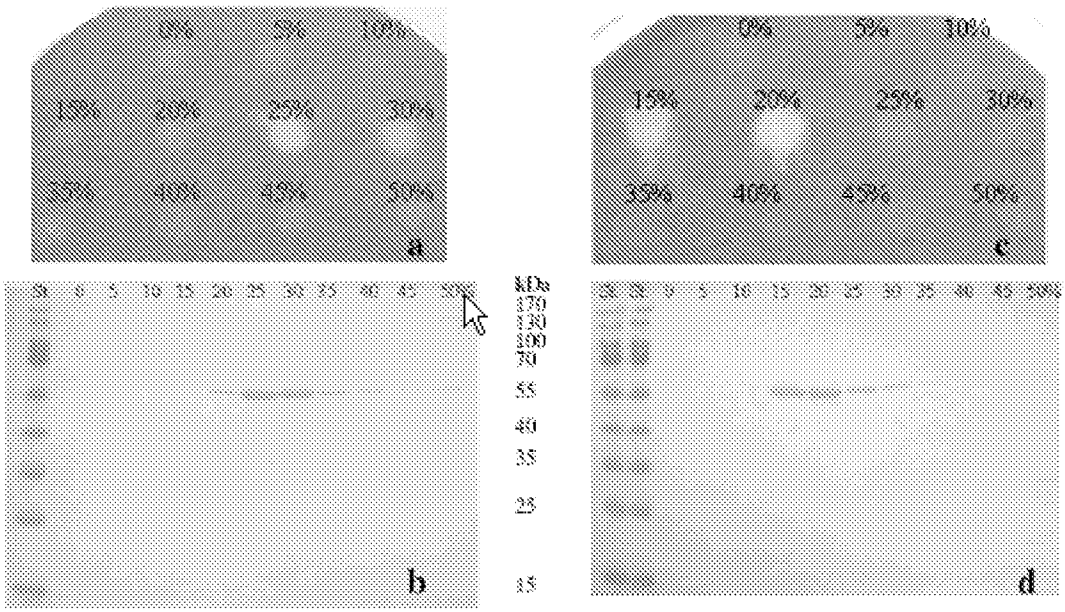

a
b
c
d
kDa
170
130
100
70
55
40
35
25
15

FIG. 5

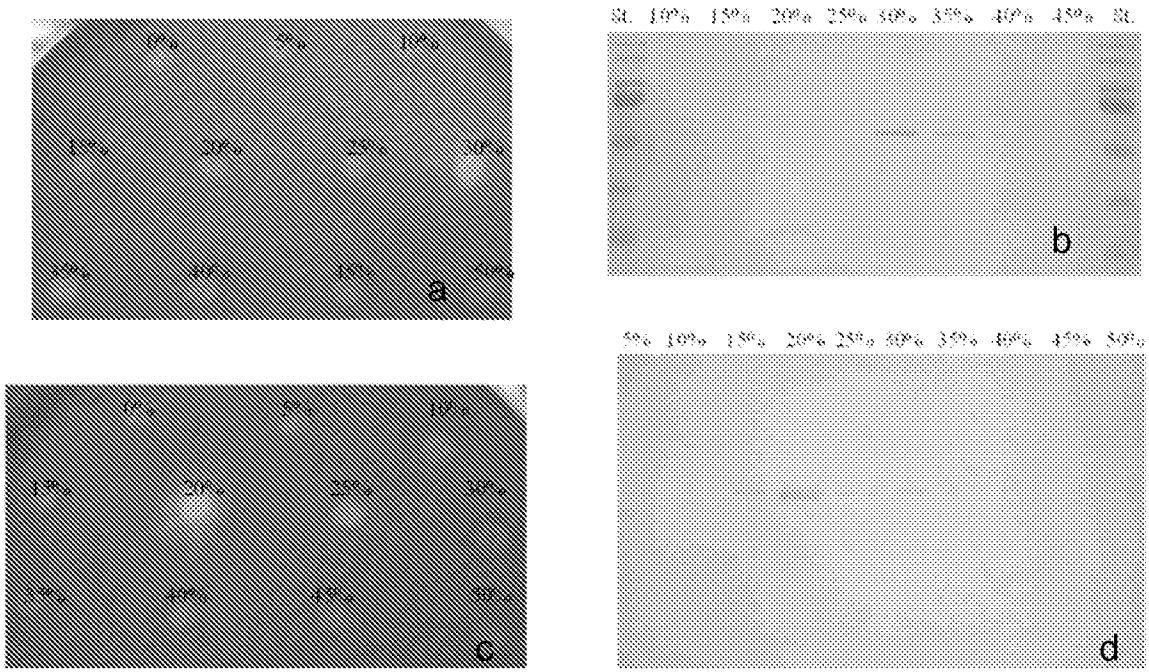


FIG. 6

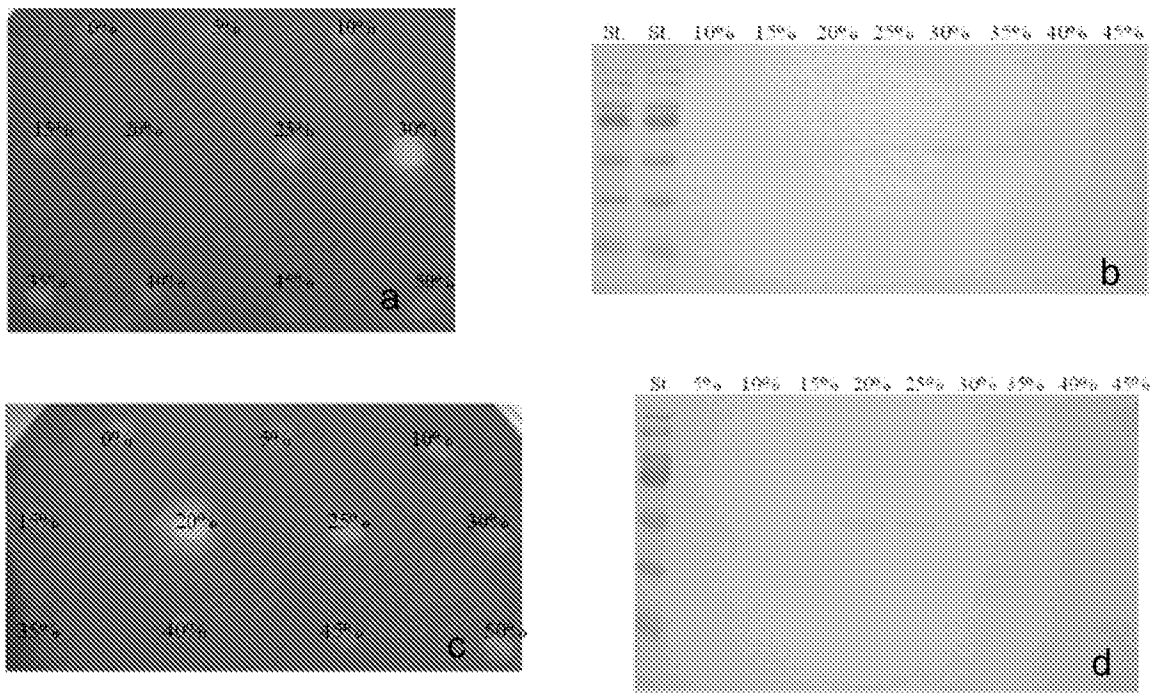


FIG. 7

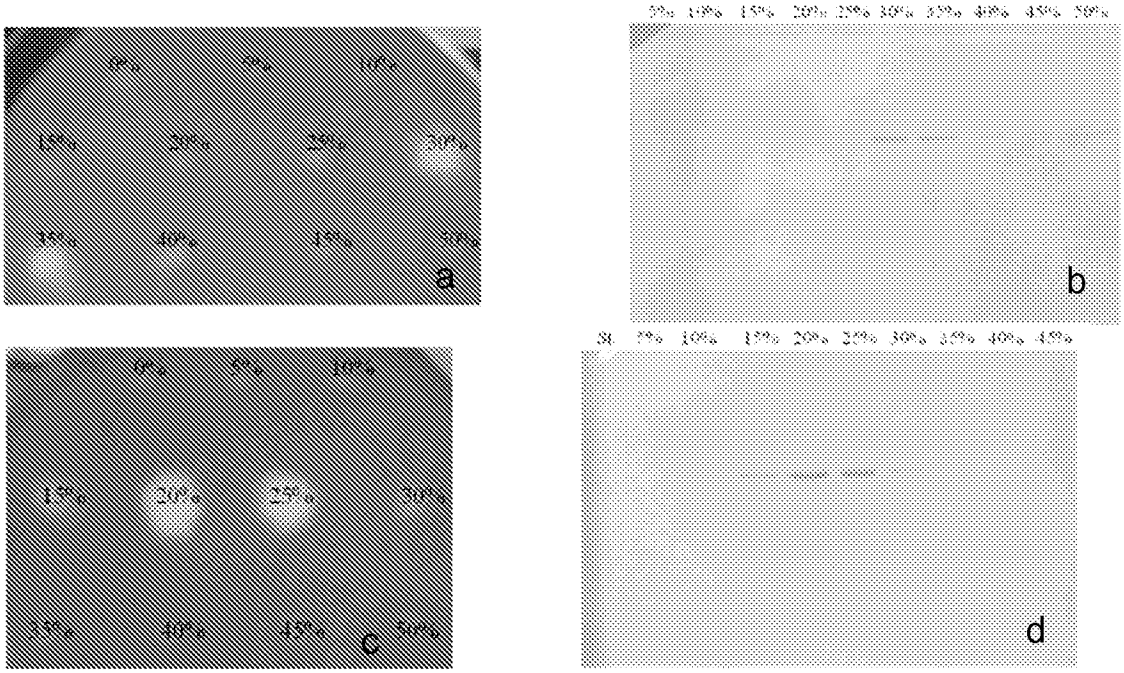


FIG. 8

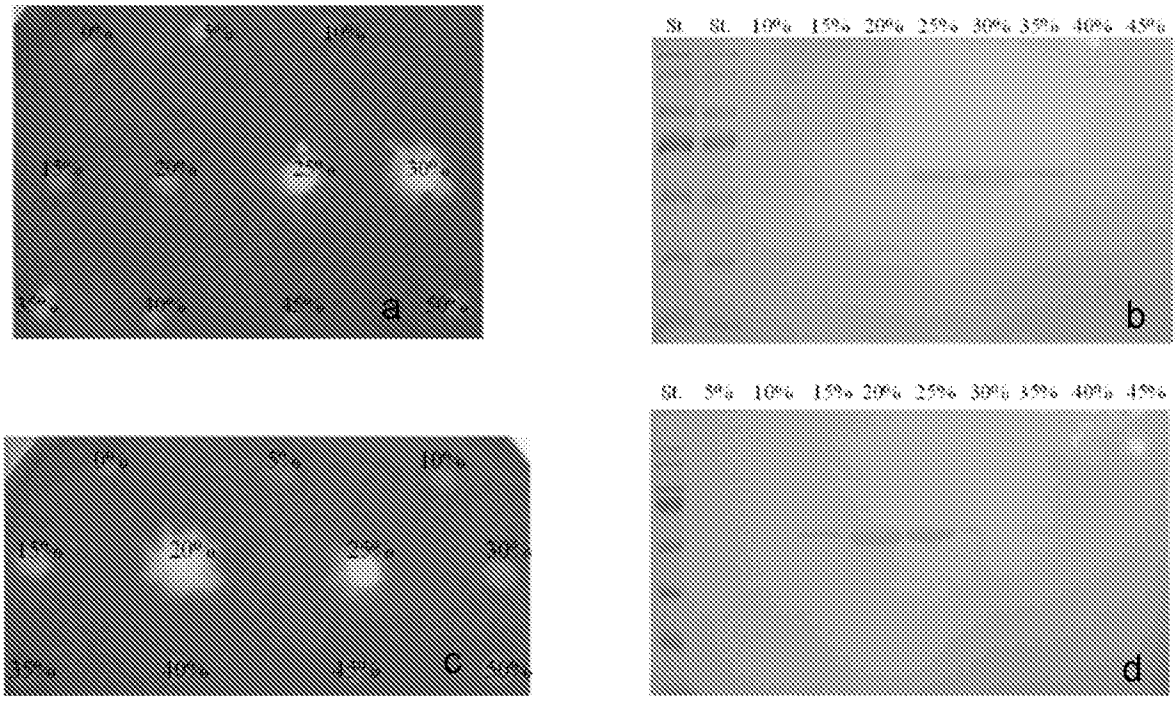


FIG. 9

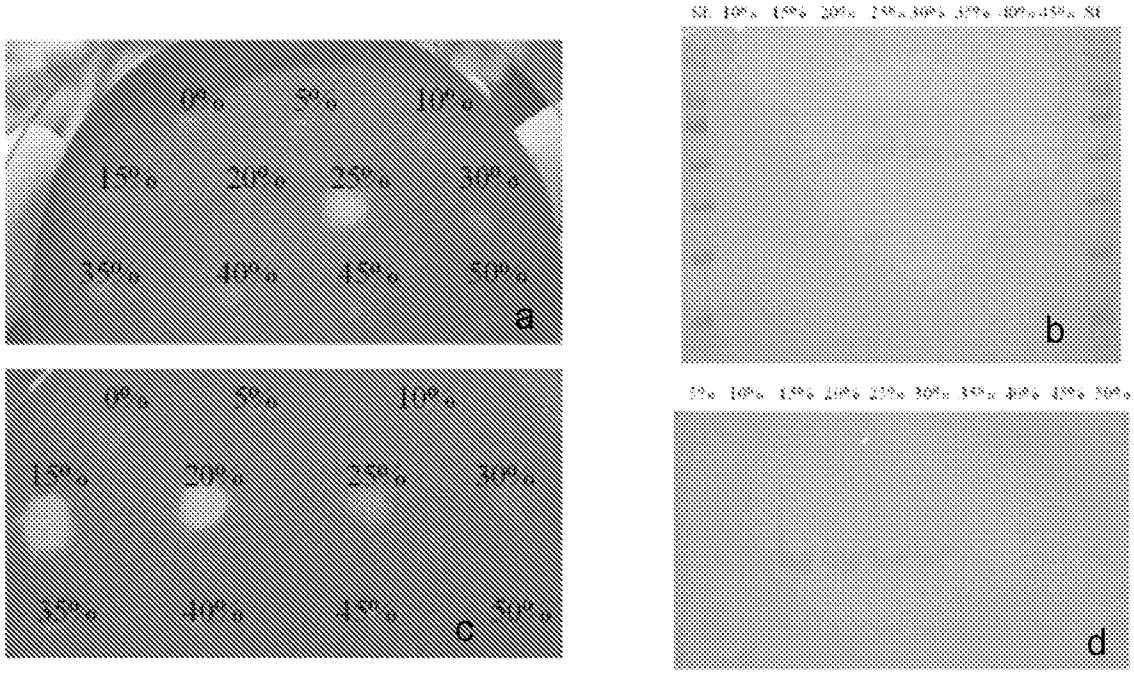


FIG. 10

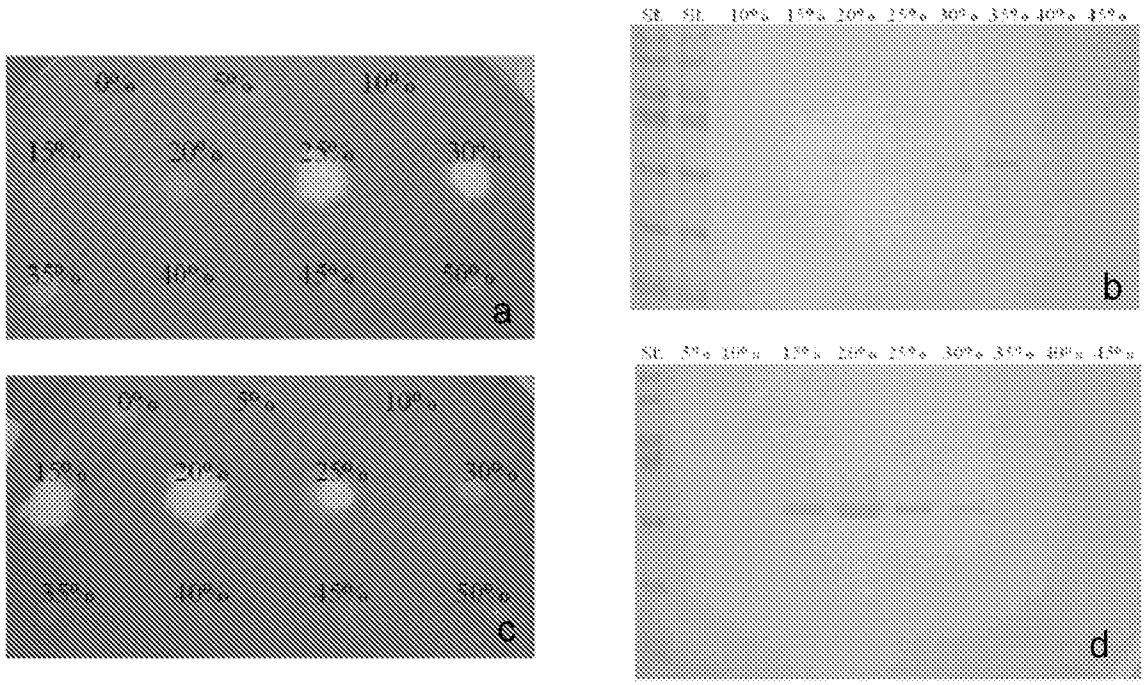


FIG. 11a

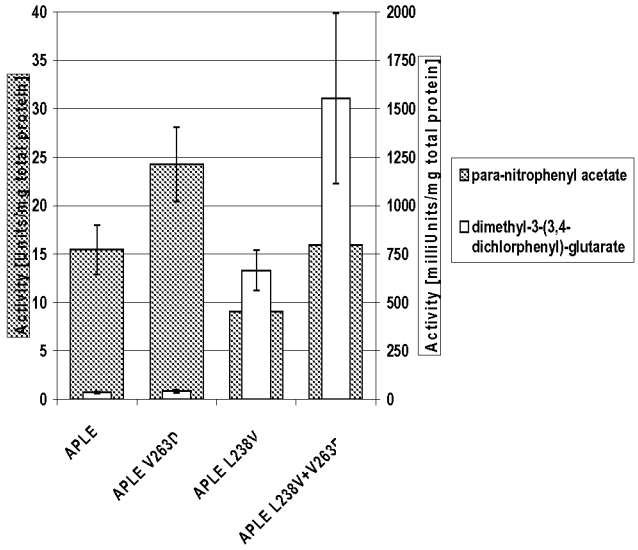
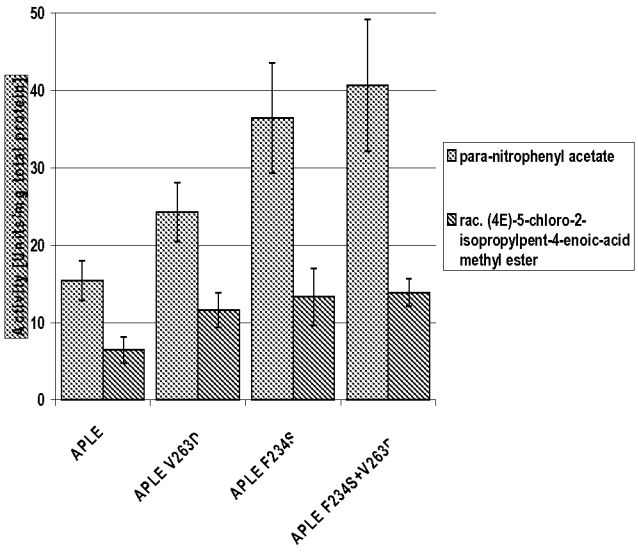


FIG. 11b



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/055564

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, WPI Data, FSTA, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2009/004093 A (DSM IP ASSETS BV [NL]; KIETZMANN MARTIN [AT]; SCHWAB HELMUT [AT]; PICH) 8 January 2009 (2009-01-08) cited in the application page 17; sequences 12,35,37	1,3,8-11
A	WO 2008/116745 A (EVONIK DEGUSSA GMBH [DE]; BORNSCHEUER UWE T [DE]; HUMMEL ANKE [DE]; BO) 2 October 2008 (2008-10-02) page 22; claims 1-7; figure 4; sequences 2,4,10	1,3,8-12
A	WO 2007/073845 A2 (DSM FINE CHEM AUSTRIA GMBH [AT]; STEINBAUER GERHARD [AT]; STANEK MICHA) 5 July 2007 (2007-07-05) page 1	13,14

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 June 2010	18/06/2010

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Deleu, Laurent

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HERMANN ET AL: "Alternative pig liver esterase (APLE) - Cloning, identification and functional expression in Pichia pastoris of a versatile new biocatalyst" JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 133, no. 3, 1 November 2007 (2007-11-01), pages 301-310, XP022402121 ISSN: 0168-1656 page 306 - page 307; figure 1	1-14
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2010/055564

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