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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 June 2016 (09.06.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/087496 A1

(51) International Patent Classification:
C12N 9/20 (2006.01) *C12R 1/84* (2006.01)
A61K 38/46 (2006.01)

(21) International Application Number:
PCT/EP2015/078317

(22) International Filing Date:
2 December 2015 (02.12.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1421343.3 2 December 2014 (02.12.2014) GB

(71) Applicants: **VIB VZW** [BE/BE]; Rijvisschestraat 120, 9052 Gent (BE). **UNIVERSITEIT GENT** [BE/BE]; Sint-Pietersnieuwstraat 25, 9000 Gent (BE).

(72) Inventors: **CALLEWAERT, Nico**; Begijnhoflaan 15, 9850 Nevele (BE). **DE VISSCHER, Charlotte**; Poeldendries 9, 9850 Landegem (BE).

(74) Common Representative: **VIB VZW**; Rijvisschestraat 120, 9052 Gent (BE).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, 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, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2016/087496 A1

(54) Title: IMPROVED PRODUCTION OF LIPASE IN YEAST

(57) Abstract: The present invention relates to the recombinant production of lipases in yeast. The present invention provides means and methods for improving the production of lipases in yeast.

IMPROVED PRODUCTION OF LIPASE IN YEAST

Field of the invention

The present invention relates to the recombinant production of lipases in yeast. The present invention provides means and methods for improving the production of lipases in yeast.

Introduction to the invention

Human lysosomal acid lipase (hLAL) is a lipase involved in metabolism of cholesteryl esters (CE) and triacylglycerides (TG). Consequently, hLAL plays a crucial role in cholesterol homeostasis. CE together with TG are transported in low-density-lipoprotein (LDL) particles to be taken up by the cell through binding to a LDL-receptor. Once taken up, the CE and TG contained in these particles are hydrolysed into free cholesterol and fatty acids. This free cholesterol can either be used for membrane formation or can be converted again into CE by the action of Acyl CoA:Cholesterol acyl transferase (ACAT) for storage in cholesteryl ester droplets. When high concentrations of free cholesterol are present in the cell, the biosynthesis of LDL-receptor drops and uptake of LDL is reduced. Also, free cholesterol can be used as negative feedback for hydroxymethylglutaryl-CoA (HMG-CoA) reductase and thus inhibiting de-novo cholesterol synthesis. If, because of a genetic defect, hLAL can no longer perform its function, CE and TG will no longer be hydrolysed,resulting in their accumulation and continuous de-novo-cholesterol synthesis, since HMG-CoA reductase is not being suppressed. Deficiency in hLAL causes two rare autosomal recessive lysosomal storage diseases called Wolman disease (WD) and cholesteryl ester storage disease (CESD). Patients suffering from WD die within the first year after birth, contrary to CESD that is less severe because of the presence of residual hLAL activity in the lysosomes. TG and CE accumulate mainly within the blood vessel wall and liver, causing atherosclerotic lesions and hepatosplenomegaly, respectively. Purified hLAL could be used for enzyme replacement therapy to treat CESD, as has been demonstrated by Du *et. al.* (2001) Hum. Mol. Genet. 10, 1639-1648. Apart from the treatment of these genetic diseases, there is also an opportunity to treat patients suffering from atherosclerosis, see for example EP1267914B2. Ideally, recombinant hLAL carries a glycosylation structure which is a mixture of mannose residues and phosphorylated mannose residues. Mannose residues are necessary for efficient binding and uptake by mannose receptors (present on macrophages and foam cells) while phosphorylated mannose residues are necessary for efficient binding and uptake by the mannose-6-phosphate receptors (present on, amongst others, liver cells and vascular endothelial cells). Binding and uptake through mannose receptors and phospho-mannose receptors leads to efficient lysosomal targeting in the target cells. Another requirement for the recombinant production of hLAL is that it is produced at high levels and is biologically active. The latter is not trivial since we showed that the mere production of hLAL in *Pichia*

pastoris leads to a secreted product, which is not biologically active. We have surprisingly shown that the addition of liposomes in the cultivation medium of recombinant yeasts expressing hLAL not only increases its production but importantly leads to the generation of an enzymatically active form of hLAL. Liposomes are known in the art as *in vitro* protein refolding assistants (see Zardeneta G and Horowitz PM (1994) *Analytical Biochemistry* 223, 1-6) but liposomes have never been applied directly in the growth medium of yeasts in the hope of increasing the production of biologically active proteins such as lipases.

Figures

Figure 1:

Fig.1. Optimization of hLAL expression. **A.** Western blot showing the expression levels of 2 clones expressing non-codon optimized hLAL and codon-optimized pLAL. Also 2 clones expressing hLAL or pLAL coupled to their native signal sequence are shown (sshLAL and sspLAL). **B.** Enzyme activity measured in the absence (-) or presence (+) of Hac1p overexpression. **C.** Western blot of hLAL expression without (-) or with (+) Hac1p overexpression. **D.** Western blot of hLAL after PNGaseF digest on secreted proteins. **E.** Enzyme activity present in supernatant three days post-induction at four different pH's. **F.** Western blot of hLAL expression three days-post induction. Lane 1: pH 5,3; Lane 2: pH 5,7; Lane 3: pH 6,0; Lane 4: pH 6,7. **G.** Enzyme activity of hLAL in different induction volumes three days post-induction. Dark grey bars represent no addition of liposomes during induction, while light grey bars represent presence of liposomes. **H.** Western blot shows amount of hLAL present in different volumes without (-) and with (+) liposomes.

Figure 2:

Identification of N-glycans present on secreted glycoproteins and purified hLAL. A and F represent a maltodextrin reference and N-glycans from bovine RNaseB, respectively. B-D. N-glycan profile of the proteins present in growth medium of *P. Pastoris OCH1* deficient strain (B), GS115ΔOCH1GLAHac 1xPNO1 (C), GS115ΔOCH1GLAHac 2xPNO1 (D). E. N-glycan profile of purified hLAL.

Detailed description to the invention

The present invention will be described with respect to particular embodiments and with reference to certain drawings but the invention is not limited thereto but only by the claims. Any reference signs in the claims shall not be construed as limiting the scope. The drawings described are only schematic and are non-limiting. In the drawings, the size of some of the

elements may be exaggerated and not drawn on scale for illustrative purposes. Where the term "comprising" is used in the present description and claims, it does not exclude other elements or steps. Where an indefinite or definite article is used when referring to a singular noun e.g. "a" or "an", "the", this includes a plural of that noun unless something else is specifically stated. Furthermore, the terms first, second, third and the like in the description and in the claims, are used for distinguishing between similar elements and not necessarily for describing a sequential or chronological order. It is to be understood that the terms so used are interchangeable under appropriate circumstances and that the embodiments of the invention described herein are capable of operation in other sequences than described or illustrated herein.

The following terms or definitions are provided solely to aid in the understanding of the invention. Unless specifically defined herein, all terms used herein have the same meaning as they would to one skilled in the art of the present invention. Practitioners are particularly directed to Sambrook et al., Molecular Cloning: A Laboratory Manual, 4th ed., Cold Spring Harbor Press, Plainsview, New York (2012); and Ausubel et al., current Protocols in Molecular Biology (Supplement 100), John Wiley & Sons, New York (2012), for definitions and terms of the art. The definitions provided herein should not be construed to have a scope less than understood by a person of ordinary skill in the art.

Lysosomal Acid Hydrolase (abbreviated herein as LAL) is a member of the lipase family and is a 372 amino acid glycoprotein that is trafficked to the lysosome via the mannose 6-phosphatereceptor system. The cDNA sequence which encodes human LAL has been previously reported (see Anderson RA and Sando GN (1991) *J. Biol. Chem.* 266, 22479-22484). This glycoprotein has six N-glycosylation consensus sequences. All members of the lipase family have conserved GXSXG pentapeptide sequences that contain the active site serine nucleophiles. LAL has two such sequences at residues 97-101 and 151-155 with potential serine nucleophiles at residues 99 and 153, where a key nucleophile resides at the Ser153 residue. LAL cleaves cholesteryl esters and triglycerides *in vitro* using phospholipid/detergent systems.

The present invention provides efficient production of biologically active lysosomal acid lipase produced in the yeast *Pichia pastoris*. Importantly, the invention shows that the biological activity of the recombinant LAL is dependent on the addition of liposomes in the cultivation medium of *Pichia pastoris* during the production. Without limiting the invention towards a particular mechanism or action we believe that one explanation for obtaining biologically active LAL is that the hydrophobic shells of liposomes interact with the hydrophobic loop of the LAL. This interaction could prevent aggregation of the recombinant LAL in the cultivation medium thereby leading to a biologically active LAL. We therefore believe that the conformation of the recombinant LAL is different in the cultivation medium without liposomes compared to

recombinant LAL in the cultivation medium in the presence of liposomes. We believe that the recombinant LAL obtained in the presence of added liposomes in the cultivation medium is a different product with a conformation that cannot be structurally defined. The effect of the addition of liposomes is apparent from the examples since only in the presence of liposomes we could show biological activity of the recombinant LAL.

Liposomes are herein defined as supramolecular structures containing at least one amphipathic component. A particular type of liposomes is small artificial vesicles of spherical shape that can be created from cholesterol and natural non-toxic phospholipids to form lipid bilayer-enveloped compartments with an aqueous interior. Liposome properties differ considerably with lipid composition, surface charge, size, and the method of preparation (see Akbarzadeh A *et al* (2013) *Nanoscale Research Letters* 8:102). The choice of the bilayer components determines the rigidity or fluidity and the charge of the bilayer. Unsaturated phosphatidylcholine species from natural sources (egg or soybean phosphatidylcholine) give more permeable bilayers, whereas the saturated phospholipids with long acyl chains (for example dipalmitoylphosphatidylcholine) form a rigid, rather impermeable bilayer structure. Generally, liposomes are defined as spherical vesicles with particle sizes ranging from 30 nm to several micrometers. They usually consist of one or more lipid bilayers surrounding aqueous units, where the polar head groups are oriented towards the interior and exterior aqueous phases. The skilled person knows that there are several methods to prepare liposomes of different types. For example, for bilayer-enveloped liposomes, basically four stages are common to liposome preparations: i) drying down lipids from an organic solvent, ii) dispersing the lipid in aqueous media, iii) purifying the resulting liposome and iv) analyzing the final product.

In the present invention liposomes are added in the cultivation medium at amounts between 10 µg and 10 mg per ml growth medium.

In one embodiment the present invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast comprising an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

In the present invention when the general embodiments refer to a recombinant yeast then in a specific embodiment a recombinant yeast is selected from the list consisting of *Pichia pastoris*, *Pichia methanolica, Oogataea minuta, Hansenula polymorpha, Saccharomyces cerevisiae, Yarrowia lipolytica, Schizosaccharomyces pombe, Kluyveromyces lactis* and *Arxula adeninivorans*

In another embodiment the present invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast, which is deficient in an alfa-1,6-mannosyltransferase activity, comprising an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

In a particular embodiment said recombinant yeast is selected from the list consisting of *Pichia pastoris*, *Pichia methanolica, Oogataea minuta, Hansenula polymorpha, Saccharomyces cerevisiae, Yarrowia lipolytica, Schizosaccharomyces pombe, Kluyveromyces lactis* and *Arxula adeninivorans.*

In a particular embodiment said lysosomal acid lipase (LAL) is human LAL (hLAL).

In another particular embodiment the alpha-mannosyl-transferase is an OCH1-homologousenzyme. The OCH1 enzyme is described in Nakayama K. *et al* (1992) *EMBO J.* 11(7): 2511-2519 and in US8883445.

In another particular embodiment the recombinant yeast is *Pichia pastoris*.

In another embodiment the invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast comprising an expression construct encoding a HAC1 protein and an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

In a specific embodiment the recombinant yeast comprising an expression construct encoding a HAC1 protein and an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding a LAL protein is selected from the list consisting of *Pichia pastoris*, *Pichia methanolica, Oogataea minuta, Hansenula polymorpha, Schizosaccharomyces pombe, Yarrowia lipolytica, Saccharomyces cerevisiae, Kluyveromyces lactis* and *Arxula adeninivorans*

In yet another embodiment the invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast, deficient in an alfa-1,6-mannosyltransferase, comprising an expression construct encoding a Hac1 protein and an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

The Hac1 protein and its applications are described in Guerfal M. *et al* (2010) *Microbial Cell Factories* 9:49 and in WO2001072783.

In yet another embodiment the invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast, deficient in an OCH1-homologous enzyme, comprising an expression construct encoding a HAC1 protein and an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

In yet another embodiment the invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast, deficient in an alfa-1,6-mannosyltransferase, comprising an expression construct encoding a HAC1 protein, comprising an expression construct encoding a nucleotide sequence encoding a phospho-mannose enzyme such as an MNN4 or PNO1 polypeptide and an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

In yet another embodiment the invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast, deficient in an OCH1 enzyme, comprising an expression construct encoding a HAC1 protein, comprising an expression construct encoding a nucleotide sequence encoding a phospho-mannose enzyme such as an MNN4 or PNO1 polypeptide and an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

In another embodiment the hLAL which is obtained by the previous processes is *in vitro* modified with a mannose decapping enzyme and an alfa-mannosidase enzyme.

A mannose decapping enzyme removes (or hydrolyzes) the terminal mannose from the D-mannose-alpha-1-phospho-6-D-mannose (Man-Pi-6-Man structure) so that a Pi-6-Man glycotype is obtained. The process is described in Tiels P. *et al* (2012) *Nat. Biotechnology* 30, 12, 1225) and examples of mannose decapping enzymes are described in WO2011039634. The Tiels P. *et al* (2012) *Nat. Biotechnology* 30, 12, 1225 reference also describes the *in vitro* use of the alpha--mannosidase enzyme and possible sources of this enzyme.

In yet another embodiment the invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast comprising an expression construct encoding a HAC1 protein, comprising an expression construct encoding a nucleotide sequence encoding a phospho-mannose enzyme such as an MNN4 or PNO1 polypeptide, an expression construct encoding an enzyme hydrolyzing a mannose-1-phospho-6-mannose linkage towards phospho-6-mannose, optionally also an expression construct encoding an alpha-mannosidase enzyme and an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

In yet another embodiment the invention provides a lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast, deficient in an alfa-1,6-mannosyltransferase activity such as OCH1 transferase activity, comprising an expression construct encoding a HAC1 protein, comprising an expression construct encoding a nucleotide sequence encoding a phospho-mannose enzyme such as an MNN4 or PNO1 polypeptide, an expression construct encoding an enzyme hydrolyzing a mannose-1-phospho-6-mannose linkage towards phospho-6-mannose and an expression construct comprising a suitable promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.

In yet another embodiment the LAL protein which is obtained by the previous methods is for use as a medicament.

In yet another embodiment the LAL protein which is obtained by the previous methods is for the treatment of Wolman's disease.

In yet another embodiment the LAL protein which is obtained by the previous methods is for the treatment of cholesteryl ester storage disease.

In yet another embodiment the LAL protein which is obtained by the previous methods is for the treatment of atherosclerosis.

In yet another embodiment the invention provides a method for producing a lipase in a yeast cell, said method comprising i) cultivating a yeast cell, comprising an expression construct comprising a yeast promoter operably linked to a nucleotide sequence encoding said lipase protein, under conditions wherein said yeast promoter is active, ii) adding liposomes to the

cultivation medium and iii) isolating said lipase protein from the growth medium in a purification step.

In a specific embodiment a recombinant yeast is selected from the list consisting of *Pichia pastoris*, *Pichia methanolica, Oogataea minuta, Hansenula polymorpha, Saccharomyces cerevisiae, Yarrowia lipolytica, Schizosaccharomyces pombe, Kluyveromyces lactis* and *Arxula adeninivorans*

In another particular embodiment the invention provides a method for producing a lysosomal acid lipase protein in a yeast cell, said method comprising i) cultivating a yeast cell comprising an expression construct comprising a yeast promoter operably linked to a nucleotide sequence encoding said LAL protein, under conditions wherein said yeast promoter is active, ii) adding liposomes to the cultivation medium and iii) isolating said LAL protein from the growth medium in a purification step.

In a particular embodiment the yeast cell in the method is deficient in an alfa-1,6-mannosyltransferase activity.

In a particular embodiment said alfa-1,6-mannosyltransferase is an OCH1-transferase.

In another embodiment the yeast cell in the methods further comprises an expression construct encoding a HAC1 protein.

In yet another embodiment the yeast cell in the methods further comprises an expression construct encoding an MNN4 or PNO1 polypeptide.

In yet another embodiment the LAL protein produced by the previous methods is *in vitro* modified with a mannose decapping enzyme and an alfa-mannosidase enzyme.

In yet another embodiment the yeast cell in the methods further comprises an expression construct producing an enzyme capable of hydrolyzing a mannose-1-phospho-6-mannose linkage to phospho-6-mannose.

In another embodiment the methods of the invention use a yeast cell which is selected from the list *Pichia pastoris, Pichia methanolica, Oogataea minuta, Hansenula polymorpha, Saccharomyces cerevisiae, Yarrowia lipolytica, Schizosaccharomyces pombe, Kluyveromyces lactis* and *Arxula adeninivorans*.

Genetic engineering of the herein before yeast cells can include, one or more genetic modifications such as: (i) deletion of an endogenous gene encoding an outer chain alfa-1,6-mannosyltransferase activity such as gene encoding for an Outer CHain elongation (OCH1)

protein; (ii) introduction of a recombinant nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation (e.g, a MNN4 polypeptide from *Yarrowia lipolytica, S. cerevisiae, Ogataea minuta, Yarrowia lipolytica, Pichia pastoris*, or *C. albicans*, or PNO1 polypeptide from *P. pastoris*) to increase the phosphorylation of mannose residues; (iii) introduction or expression of an RNA molecule that interferes with the functional expression of an OCH1 protein;(iv) introduction of a recombinant nucleic acid encoding a lipase such as a lysosomal acid lipase, in particular a human lysosomal acid lipase. RNA molecules include, e.g., small-interfering RNA (siRNA), short hairpin RNA (shRNA), anti-sense RNA, or micro RNA (miRNA). Genetic engineering also includes altering an endogenous gene encoding a protein having an N-glycosylation activity to produce a protein having additions (e.g., a heterologous sequence), deletions, or substitutions (e.g., mutations such as point mutations; conservative or non-conservative mutations). Mutations can be introduced specifically (e.g., by site-directed mutagenesis or homologous recombination) or can be introduced randomly (for example, cells can be chemically mutagenized as described in, e.g. Newman and Ferro-Novick (1987) *J. Cell Biol.* 105(4):1587.

Genetic modifications described herein can result in one or more of (i) an increase in one or more activities in the genetically modified cell, (ii) a decrease in one or more activities in the genetically modified cell, or (iii) a change in the localization or intracellular distribution of one or more activities in the genetically modified cell. It is understood that an increase in the amount of a particular activity (e.g. promoting mannosyl phosphorylation) can be due to overexpressing one or more proteins capable of promoting mannosyl phosphorylation, an increase in copy number of an endogenous gene (e.g. gene duplication), or an alteration in the promoter or enhancer of an endogenous gene that stimulates an increase in expression of the protein encoded by the gene. A decrease in one or more particular activities can be due to overexpression of a mutant form (e.g. a dominant negative form), introduction or expression of one or more interfering RNA molecules that reduce the expression of one or more proteins having a particular activity, or deletion of one or more endogenous genes that encode a protein having the particular activity.

To disrupt a gene by homologous recombination, a "gene replacement" vector can be constructed in such a way to include a selectable marker gene. The selectable marker gene can be operably linked, at both 5' and 3' end, to portions of the gene of sufficient length to mediate homologous recombination. The selectable marker can be one of any number of genes which either complement host cell auxotrophy or provide antibiotic resistance, including URA3, LEU2 and HIS3 genes. Other suitable selectable markers include the zeocin resistance gene Zeo^R, which confers zeocin resistance to yeast cells, or the ADE1 gene, which results in reversion of

the pink color observed in ade1 mutants due to the accumulation of an oxidized 5-aminoimidizole ribonucleotide-derived product.

Linearized DNA fragments of the gene replacement vector are then introduced into the cells using methods well known in the art (see below). Integration of the linear fragments into the genome and the disruption of the gene can be determined based on the selection marker and can be verified by, for example, Southern blot analysis. A selectable marker can be removed from the genome of the host cell by, e.g. Cre-loxP systems, TALENs, CRISPR-CAS9 and the like.

Alternatively, a gene replacement vector can be constructed in such a way as to include a portion of the gene to be disrupted, which portion is devoid of any endogenous gene promoter sequence and encodes none or an inactive fragment of the coding sequence of the gene. An "inactive fragment" is a fragment of the gene that encodes a protein having, e.g., less than about 10% (e.g., less than about 9%, less than about 8%, less than about 7%, less than about 6%, less than about 5%, less than about 4%, less than about 3%, less than about 2%, less than about 1%, or 0%) of the activity of the protein produced from the full-length coding sequence of the gene. Such a portion of the gene is inserted in a vector in such a way that no known promoter sequence is operably linked to the gene sequence, but that a stop codon and a transcription termination sequence are operably linked to the portion of the gene sequence. This vector can be subsequently linearized in the portion of the gene sequence and transformed into a cell. By way of single homologous recombination, this linearized vector is then integrated in the endogenous counterpart of the gene.

Expression vectors can be autonomous or integrative. A recombinant nucleic acid (e.g. encoding a mannose decapping enzyme) can be in introduced into the cell in the form of an expression vector such as a plasmid, phage, transposon, cosmid or virus particle. The recombinant nucleic acid can be maintained extrachromosomally or it can be integrated into the yeast cell chromosomal DNA. Expression vectors can contain selection marker genes encoding proteins required for cell viability under selected conditions (e.g., URA3, which encodes an enzyme necessary for uracil biosynthesis or TRP1, which encodes an enzyme required for tryptophan biosynthesis) to permit detection and/or selection of those cells transformed with the desired nucleic acids (see e.g. US4,704,362). Expression vectors can also include an autonomous replication sequence (ARS). For example, US4,837,148 describes autonomous replication sequences which provide a suitable means for maintaining plasmids in *Pichia pastoris*.

Integrative vectors are disclosed, e.g., in US4,882,279. Integrative vectors generally include a serially arranged sequence of at least a first insertable DNA fragment, a selectable marker gene, and a second insertable DNA fragment. The first and second insertable DNA fragments are each

about 200 (e.g. about 250, about 300, about 350, about 400, about 450, about 500, or about 1000 or more) nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of the species to be transformed. A nucleotide sequence containing a gene of interest (e.g. a gene encoding a protein having N-glycosylation activity) for expression is inserted in this vector between the first and second insertable DNA fragments whether before or after the marker gene. Integrative vectors can be linearized prior to yeast transformation to facilitate the integration of the nucleotide sequence of interest into the host cell genome.

Alternatively, a single insertable DNA fragment can be used, in which the vector is cut and thereby linearized. This will lead to integration in the host cell genome with duplication of the insertable DNA fragment.

An expression vector can feature a recombinant nucleic acid under the control of a yeast (e.g. *Yarrowia lipolytica, Arxula adeninivorans, P. pastoris*, or other suitable yeast species) promoter, which enables them to be expressed in fungal cells. Suitable yeast promoters include, e.g., ADC1, TPI1, ADH2, hp4d, PDX, POX2, TEF, AOX1, AOX2, GAP, FLD, MOX and GAL1 (see e.g. Guarente *et al* (1982) *Proc. Natl. Acad. Sci. USA* 79(23):7410) promoters. Additional suitable promoters are described in, e.g., Zhu and Zhang (1999) *Bioinformatics* 15(7-8):608-611 and in US6,265,185.

A promoter can be constitutive or inducible (conditional). A constitutive promoter is understood to be a promoter whose expression is on under the standard culturing conditions. Inducible promoters are promoters that are responsive to one or more induction cues. For example, an inducible promoter can be chemically regulated (e.g., a promoter whose transcriptional activity is regulated by the presence or absence of a chemical inducing agent such as an alcohol, tetracycline, a steroid, a metal, or other small molecule) or physically regulated (e.g., a promoter whose transcriptional activity is regulated by the presence or absence of a physical inducer such as light or high or low temperatures). An inducible promoter can also be indirectly regulated by one or more transcription factors that are themselves directly regulated by chemical or physical cues.

It is understood that other genetically engineered modifications can also be conditional. For example, a gene can be conditionally deleted using, e.g., a site-specific DNA recombinase such as the Cre-loxP system (see, e.g., Gossen et al. (2002) Ann. Rev. Genetics 36:153-173 and US20060014264).

A recombinant nucleic acid can be introduced into a cell described herein using a variety of methods such as the spheroplast technique or the whole-cell lithium chloride yeast transformation method. Other methods useful for transformation of plasmids or linear nucleic

acid vectors into cells are described in, for example, US4,929,555; Hinnen *et al.* (1978) *Proc. Nat. Acad. Sci. USA* 75:1929; Ito *et al.* (1983) *J. Bacteriol.* 153:163; US4,879,231; and Sreekrishna *et al.* (1987) *Gene* 59:115, the disclosures of each of which are incorporated herein by reference in their entirety. Electroporation and PEG1000 whole cell transformation procedures may also be used, as described by Cregg and Russel, *Methods in Molecular Biology*: *Pichia* Protocols, Chapter 3, Humana Press, Totowa, N.J., pp. 27-39 (1998).

Transformed yeast cells can be selected for by using appropriate techniques including, but not limited to, culturing auxotrophic cells after transformation in the absence of the biochemical product required (due to the cell's auxotrophy), selection for and detection of a new phenotype, or culturing in the presence of an antibiotic which is toxic to the yeast in the absence of a resistance gene contained in the transformants. Transformants can also be selected and/or verified by integration of the expression cassette into the genome, which can be assessed by e.g. Southern blot or PCR analysis.

Prior to introducing the vectors into a target cell of interest, the vectors can be grown (e.g., amplified) in bacterial cells such as Escherichia coli (E. coli) as described above. The vector DNA can be isolated from bacterial cells by any of the methods known in the art which result in the purification of vector DNA from the bacterial milieu. The purified vector DNA can be extracted extensively with phenol, chloroform, and ether, to ensure that no E. coli proteins are present in the plasmid DNA preparation, since these proteins can be toxic to mammalian cells.

In some embodiments, the genetically engineered fungal cell lacks the OCH1 gene or gene products (e.g., mRNA or protein) thereof, and is deficient in OCH1 activity. In some embodiments, the genetically engineered cell expresses a polypeptide capable of promoting mannosyl phosphorylation (e.g., a MNN4 polypeptide from *Yarrowia lipolytica, S. cerevisiae, Ogataea minuta, Pichia pastoris*, or *C. albicans*, or a PNO1 polypeptide from *P. pastoris*). For example, the fungal cell can express a MNN4 polypeptide from *Y. lipolytica* (Genbank. Acccession Nos: XM.sub.--503217, Genolevures Ref: YALI0D24101g). In some embodiments, the genetically engineered cell is deficient in OCH1 activity and expresses a polypeptide capable of promoting mannosyl phosphorylation.

Methods for detecting glycosylation of a target molecule include DNA sequencer-assisted (DSA), fluorophore-assisted carbohydrate electrophoresis (FACE) or matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). For example, an analysis can utilize DSA-FACE in which, for example, glycoproteins are denatured followed by immobilization on, e.g., a membrane. The glycoproteins can then be reduced with a suitable reducing agent such as dithiothreitol (DTT) or beta-mercaptoethanol. The sulfhydryl groups of the proteins can be carboxylated using an acid such as iodoacetic acid. Next, the N-glycans can

be released from the protein using an enzyme such as N-glycosidase F. N-glycans, optionally, can be reconstituted and derivatized by reductive amination. The derivatized N-glycans can then be concentrated. Instrumentation suitable for N-glycan analysis includes, e.g., the ABI PRISM. 377 or 3130 DNA sequencer (Applied Biosystems). Data analysis can be performed using, e.g. GENESCAN. 3.1 software (Applied Biosystems). Optionally, isolated mannoproteins or glycans derived thereof can be further treated with one or more enzymes to confirm their N-glycan status. Additional methods of N-glycan analysis include, e.g. mass spectrometry (e.g. MALDI-TOF-MS), high-pressure liquid chromatography (HPLC) on normal phase, reversed phase and ion exchange chromatography (e.g., with pulsed amperometric detection when glycans are not labeled and with UV absorbance or fluorescence if glycans are appropriately labeled). See also Callewaert *et al* (2001) *Glycobiology* 11(4):275-281 and Freire *et al* (2006) *Bioconjug. Chem.* 17(2):559-564.

Pharmaceutical Compositions and Methods of Treatment

A recombinant LAL enzyme obtained according to the methods of the invention, preferentially after in vitro treatment with a mannose decapping and an alfa-mannosidase enzyme, can be incorporated into a pharmaceutical composition containing a therapeutically effective amount of the LAL enzyme and one or more adjuvants, excipients, carriers, and/or diluents. Acceptable diluents, carriers and excipients typically do not adversely affect a recipient's homeostasis (e.g., electrolyte balance). Acceptable carriers include biocompatible, inert or bioabsorbable salts, buffering agents, oligo- or polysaccharides, polymers, viscosity-improving agents, preservatives and the like. One exemplary carrier is physiologic saline (0.15 M NaCl, pH 7.0 to 7.4). Another exemplary carrier is 50 mM sodium phosphate, 100 mM sodium chloride. Further details on techniques for formulation and administration of pharmaceutical compositions can be found in, e.g., Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.). Supplementary active compounds can also be incorporated into the compositions.

Administration of a pharmaceutical composition containing a recombinant LAL enzyme of the invention can be systemic or local. Pharmaceutical compositions can be formulated such that they are suitable for parenteral and/or non-parenteral administration. Specific administration modalities include subcutaneous, intravenous, intramuscular, intraperitoneal, transdermal, intrathecal, oral, rectal, buccal, topical, nasal, ophthalmic, intra-articular, intra-arterial, sub-arachnoid, bronchial, lymphatic, vaginal, and intra-uterine administration.

Administration can be by periodic injections of a bolus of the pharmaceutical composition or can be uninterrupted or continuous by intravenous or intraperitoneal administration from a reservoir which is external (e.g., an IV bag) or internal (e.g., a bioerodable implant, a bioartificial organ, or a colony of implanted altered N-glycosylation molecule production cells). See e.g. US4,407,957,

5,798,113, and 5,800,828. Administration of a pharmaceutical composition can be achieved using suitable delivery means such as: a pump (see, e.g. *Annals of Pharmacotherapy*, 27:912 (1993); *Cancer*, 41:1270 (1993); *Cancer Research*, 44:1698 (1984); microencapsulation (see e.g. US4,352,883; 4,353,888; and 5,084,350); continuous release polymer implants (see, e.g. USNo4,883,666); macroencapsulation (see e.g. US5,284,761, 5,158,881, 4,976,859 and 4,968,733 and WO92/19195, WO 95/05452); injection, either subcutaneously, intravenously, intra-arterially, intramuscularly, or to other suitable site; or oral administration, in capsule, liquid, tablet, pill, or prolonged release formulation.

Examples of parenteral delivery systems include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, pump delivery, encapsulated cell delivery, liposomal delivery, needle-delivered injection, needle-less injection, nebulizer, aerosolizer, electroporation, and transdermal patch.

Formulations suitable for parenteral administration conveniently contain a sterile aqueous preparation of the altered N-glycosylation molecule, which preferably is isotonic with the blood of the recipient (e.g. physiological saline solution). Formulations can be presented in unit-dose or multi-dose form.

Formulations suitable for oral administration can be presented as discrete units such as capsules, cachets, tablets, or lozenges, each containing a predetermined amount of the altered N-glycosylation molecule; or a suspension in an aqueous liquor or a non-aqueous liquid, such as a syrup, an elixir, an emulsion, or a draught.

A LAL enzyme obtained according to the methods of the invention suitable for topical administration can be administered to a mammal (e.g., a human patient) as, e.g., a cream, a spray, a foam, a gel, an ointment, a salve, or a dry rub. A dry rub can be rehydrated at the site of administration. Such molecules can also be infused directly into (e.g., soaked into and dried) a bandage, gauze, or patch, which can then be applied topically. Such molecules can also be maintained in a semi-liquid, gelled, or fully-liquid state in a bandage, gauze, or patch for topical administration (see e.g., US307,717).

Therapeutically effective amounts of a pharmaceutical composition can be administered to a subject in need thereof in a dosage regimen ascertainable by one of skill in the art. For example, a composition can be administered to the subject, e.g., systemically at a dosage from 0.01 µg/kg to 10,000 µg/kg body weight of the subject, per dose. In another example, the dosage is from 1 µg/kg to 100 µg/kg body weight of the subject, per dose. In another example, the dosage is from 1 µg/kg to 30 µg/kg body weight of the subject, per dose, e.g. from 3 µg/kg to 10 µg/kg body weight of the subject, per dose.

In order to optimize therapeutic efficacy, a LAL molecule of the invention can be first administered at different dosing regimens. The unit dose and regimen depend on factors that include, e.g. the species of mammal, its immune status, the body weight of the mammal. Typically, levels of such a molecule in a tissue can be monitored using appropriate screening assays as part of a clinical testing procedure, e.g. to determine the efficacy of a given treatment regimen.

The frequency of dosing for a LAL protein is within the skills and clinical judgment of medical practitioners (e.g., doctors or nurses). Typically, the administration regime is established by clinical trials which may establish optimal administration parameters. However, the practitioner may vary such administration regimes according to the subject's age, health, weight, sex and medical status. The frequency of dosing can be varied depending on whether the treatment is prophylactic or therapeutic.

Toxicity and therapeutic efficacy of such molecules or pharmaceutical compositions thereof can be determined by known pharmaceutical procedures in, for example, cell cultures or experimental animals. These procedures can be used, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Pharmaceutical compositions that exhibit high therapeutic indices are preferred. While pharmaceutical compositions that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to normal cells (e.g., non-target cells) and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in appropriate subjects (e.g., human patients). The dosage of such pharmaceutical compositions lies generally within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For a pharmaceutical composition used as described herein (e.g., for treating a metabolic disorder in a subject), the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the pharmaceutical composition which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

As defined herein, a "therapeutically effective amount" of a LAL molecule of the invention is an amount of the molecule that is capable of producing a medically desirable result (e.g., amelioration of one or more symptoms of atherosclerosis) in a treated subject. A therapeutically effective amount (i.e., an effective dosage) can includes milligram or microgram amounts of the compound per kilogram of subject or sample weight (e.g., about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram).

The subject can be any mammal, e.g., a human (e.g., a human patient) or a non-human primate (e.g., chimpanzee, baboon, or monkey), a mouse, a rat, a rabbit, a guinea pig, a gerbil, a hamster, a horse, a type of livestock (e.g., cow, pig, sheep, or goat), a dog or a cat.

A molecule or pharmaceutical composition thereof described herein can be administered to a subject as a combination therapy with another treatment, e.g. in a treatment for atherosclerosis. For example, the combination therapy can include administering to the subject (e.g. a human patient) one or more additional agents that provide a therapeutic benefit to the subject who has, or is at risk of developing atherosclerosis. Thus, the pharmaceutical composition comprising LAL and the one or more additional agents can be administered at the same time. Alternatively, the pharmaceutical composition comprising LAL can be administered first and the one or more additional agents administered second, or vice versa.

Any of the pharmaceutical compositions described herein can be included in a container, pack, or dispenser together with instructions for administration.

It is to be understood that although particular embodiments, specific configurations as well as materials and/or molecules, have been discussed herein for cells and methods according to the present invention, various changes or modifications in form and detail may be made without departing from the scope and spirit of this invention. The following examples are provided to better illustrate particular embodiments, and they should not be considered limiting the application. The application is limited only by the claims.

Examples

1. Recombinant expression of lysosomal acid lipase in *Pichia pastoris*

In this example we expressed the human LAL in the yeast *P. pastoris*. Thereto the *och1p* deficient GS115 strain was transformed with a linearized expression vectors containing variants of the hLAL coding sequence (see materials and methods section). We first evaluated whether codon optimization and presence or absence of its own signal sequence, fused in frame after the pre-pro sequence of the α-mating factor, was beneficial for the yield. After clone selection

and induction experiments, the best results were obtained when the own signal sequence was absent (Fig 1A). Furthermore, codon optimisation did not improve the yield, as the codon optimized pLAL gave a similar signal as non-codon optimized hLAL on western blot (Fig. 1A)). The strain expressing non-codon optimized hLAL was chosen to continue with.

Different strategies were undertaken to optimize the expression levels, such as artificial induction of the Unfolded Protein Response (UPR) by overexpression of the transcription factor Hac1. This resulted in higher enzyme activity of hLAL present in the induction medium as well as in a higher yield (Fig. 1B-C). To measure activity, liposomes containing MUO were used. For different experiments, fresh liposomes were generated and each time the size distribution was measured using DLS. After detection on western blot three distinctive bands were observed. As hLAL has six glycosylation sites, possibly these bands represented different glycoforms. Therefore, deglycosylation of yeast-secreted proteins was perfomed using PNGaseF, which cleaves between the innermost N-acetyl-glucosamine and asparagine residues of N-linked high mannose glycans from glycoproteins. After treatment, all three bands shifted downwards and only one band remained (Fig. 1D). This showed that not all N-glycosylation sites were occupied for part of the produced hLAL. Keeping these last results in mind and looking at the western blot of hLAL after Hac1 overexpression, UPR induction did not only increase the yield of hLAL but more fully glycosylated enzyme has been produced since the upper band was more intense and the lowest band almost disappeared.

Next, it was investigated whether the pH played a role in determining the yield of hLAL. Therefore, four different pH's of culture medium during induction were evaluated (Fig. 1E-F). Although no substantial differences in enzyme activity were measured, a faint band of around 85 kDa appeared on western blot at high pH. This may indicate the possibility of aggregate formation. Therefore, pH 6,2 was chosen as optimal expression pH and was used for all other expression experiments.

Before purification experiments can be done, expression behaviour needed to be evaluated when the volumes were scaled up from 2 ml in 24 deep-well blocks to maximal 250 ml in a 2 l shake flask. Therefore, cultures were induced in either 12.5 ml, 50 ml, 100 ml or 250 ml BMMY and 12,5 ml was shaken in a 125 ml baffled shake flask, 50 ml in a 500 ml baffled shake flask, 100 ml and 250 ml were shaken in a 2 l baffled shake flask. For each condition activity was measured (Fig. 1G). We speculated that the lipase may be stabilized at the medium:air interface, which has a much larger area relative to volume at small scale than at larger scale. Consequently, we speculated that adding another source of hydrophobic surface would enable production at larger scale. Therefore, the effect of the addition of liposomes during induction was tested. Much to our surprise, this worked very well and under such conditions the enzyme was produced at similar yields and activity as the small scale (Fig. 1G-H).

2. Glycan engineering of the recombinant lysosomal acid lipase

In order to target enzymes to the lysosomal compartments it is known that enzymes have to bind to the mannose-phosphate receptor (MPR) and/or to the mannose receptor present on cells. High avidity for the MPR is obtained when high Man-6-Pi residues are present on an enzyme. As known in the art *P. pastoris* is able to produce highly phosphorylated glycans if the phosphomannosylation encoding enzyme *PNO1* is overexpressed (see Tiels *et al* (2012) *Nat. biotech*, 30,1225–1231).

Therefore, a recombinant *Pichia pastoris* strain has been created which not only expressed hLAL but also contained extra copies of *PNO1*. The effect of overexpression was evaluated by preparing N-glycans from total secreted proteins. As indicated in Fig. 2B, the wild type *OCH1* deficient strain expressing hLAL mainly produced neutral $Man_8GlcNAc_2$ glycans. When *PNO1* was overexpressed, mono- and double phosphomannosylated N-glycans appeared (Fig. 2C). We showed that after a second transformation with *PNO1* the total amount of phospomannosylated glycans increased compared to the neutral glycans. Also, a slight increase in percentage of double-phosphomannosylated glycans was observed (Fig. 2D). A very similar N-glycan composition was observed for purified hLAL expressed by GS115ΔOCH1hLALHac2xPNO1. Figure 2, panel C shows that a mixture of Man-6-Pi- and mannose-glycans is present on the recombinant hLAL, the first glycan structures bind with high affinity with the mannose-6-P receptors while the second glycan structures bind with high affinity with the mannose receptors. Please note that in order to be clinically applicable for the treatment of atherosclerosis, Wolman's disease or cholesterol ester storage disease that the recombinant hLAL of Figure 2 still needs to be treated with a "mannose-uncapping enzyme" (i.e. the hydrolysis of the mannose-1-phospho-6-mannose linkage towards phospo-6-mannose) and an alpha-mannosidase enzyme. These latter steps can be conveniently carried out *in vitro* and this procedure is for example described in Tiels P. *et al* (2012) *Nat. Biotechnology* 30, 12, 1225-1231.

Materials and methods

Strains

strain	**Reference**
GS115ΔOCH1	Vervecken *et al* (2004) *Appl. Environ. Microbiol.* 70, 2639-2646)
GS115ΔOCH1sshLAL	This document
GS115ΔOCH1hLAL	This document
GS115ΔOCH1hLALHac	This document
GS115ΔOCH1sspLAL	This document
GS115ΔOCH1pLAL	This document

strain	Reference
GS115ΔOCH1pLALHac	This document
GS115ΔOCH1hLALHac 1xPNO1	This document
GS115ΔOCH1hLALHac 2xPNO1	This document

Plasmids

Plasmid	Reference
pPIC9	Invitrogen
pPIC9-sshLAL	This document
pPIC9-hLAL	This document
pPIC9-sspLAL	This document
pPIC9-pLAL	This document
pPICHygpPHAC1spliced	M. Guerfal *et a*l (2010) *Microb. Cell Factories* 9, 49
pGAPNorPNO1	This document
pGAPKanPNO1	This document

hLAL introduction in *P. pastoris*

The nucleotide sequence encoding human hLAL followed by a 6xHis-tag and flanked by AvrII and NotI restriction sites has been chemically synthesized, both codon optimized or not, for *P. pastoris* expression by GenScript. The synthetic ORF of hLAL was fused in frame after the prepro sequence of the α mating factor in the pPIC9 vector (Invitrogen), resulting in the pPIC9-sspLAL and pPIC9-sshLAL for codon and non-codon optimized ORF, respectively. To remove the LAL signal sequence, the ORF was digested with SpeI and NotI and fused in AvrII and NotI digested pPIC9 vector. The resulting vectors were called pPIC9-hLAL and pPIC9-pLAL. GS115ΔOCH1 was transformed with all four created vectors, linearized with PmeI for integration at the *AOX1* locus. Transformation was performed according to the protocol from the *Pichia* Expression kit (Invitrogen Cat. No. K1710-01). Clones were selected on MM-His (6.7 g/L YNB w/o amino acids, 20 g/L glucose, 0.77 g/L CSM-His), and screened for expression. The best performing clone (GS115ΔOCH1hLAL) was selected for further engineering.

Overexpression hac1

GS115ΔOCH1hLAL was transformed with pPICHygPpHac1spliced after linearization with PmeI for targeting to the *AOX1* locus. 24 clones selected with hygromycin were screened for increased expression compared to the wild type GS115ΔOCH1hLAL. The best performing clone (GS115ΔOCH1hLALHac) was selected to work further with.

Expression experiments

To optimize the expression, the strains were grown over night in YPD (20 g/L dextrose, 20 g/L bacto-peptone and 10 g/L yeast extract) followed by dilution in BMGY (Buffered Glycerol-complex Medium: 100 mM potassium phosphate pH 6.2 containing 13.4 g/L YNB without amino acids, 10 g/L yeast extract, 20 g/L peptone and 10 g/L glycerol) to obtain a culture with OD 0,1. After 48h of growth, stationary phase was reached and BMGY was changed to BMMY (Buffered Methanol-complex Medium: 100 mM potassium phosphate pH 6.2 containing 13.4 g/L YNB without amino acids, 10 g/L yeast extract, 20 g/L peptone and 10 g/L methanol). Every 12 h 1 % MeOH was added. Depending on the experiment, strains were grown in 2 ml deep well blocks or baffled shake flasks and liposomes were added to BMMY were needed. To create liposomes, 3.3 mmol (2.55 g) of L-alpha-phosphatidylcholine was dissolved in 5 ml chloroform and after evaporation, 840 ml BMMY containing 2.4 mM taurodeoxycholic acid (1 g) was added. The solution was shaken until the L-alpha-phosphatidylcholine was resuspended and was cooled on ice. Before use, BMMY with liposomes was filtered through a 0.22 µm bottle top filter. For hLAL detection on western blot, Anti-Lysosomal acid lipase antibody (ab89771, Abcam) was used.

PNGaseF digest

A PNGaseF digest was performed on precipitated yeast-secreted proteins. The proteins were precipitated using 10 % deoxycholic acid of a 5 mg/ml stock solution. This solution was cooled on ice during 10 min, followed by the addition of 10 % tri-chloroacetic acid (200 g in 90.4 ml H_2O stock solution). Again the sample was cooled on ice during 20 min. After 30 min of centrifugation at 13,000 rpm, the precipitate was washed twice with ice cold aceton and once with 70 % ethanol. The pellet was dried en resuspended in PBS. Tris pH 8 was added to 25 µg precipitated proteins to a final concentration of 100 mM, as well as 10x denaturing buffer (0.5% SDS, 40 mM DTT; P0704S; NEB). This mixture was boiled for 5 min and cooled down on ice. 10x NP40 (1% NP-40 in MilliQ-H_2O), 10x G7 (50 mM sodium phosphate) (P0704S; NEB) and 1000 U of PNGaseF were added and incubated overnight at 37 °C. The next day 500 U of PNGaseF was spiked and incubated for 2 hours extra.

Enzyme assay of hLAL

The activity of hLAL was measured as described previously by Sheriff *et. al.*[4]. In short, to create an artificial substrate for hLAL, 4-methyl-umbelliferyloleate (MUO) was incorporated in liposomes. First, 25 µmol MUO was dissolved in 1 ml hexane and 40 µmol L-alpha-phosphatidylcholine was dissolved in 1 ml chloroform. Both solutions were mixed and evaporated under N_2. The residue was resuspended in 10 ml double distilled water containing 2.4 mM taurodeoxycholic acid. After 24 h at 4 °C the suspension was sonicated for 3 min at 50

Watt to obtain a homogenous solution. The size distribution of the obtained MUO-containing liposomes was measured with DLS and had a Z-average of about 110-120 d.nm.

The reaction mixture (50 µl) containing hLAL and 1 mM of MUO in 0.2 M NaAc pH 5.5 was incubated at 37 °C for 10 min. Every 2 min, a sample from the reaction was stopped by the addition of 0.1 M Tris HCl pH 7.6. Release of 4-methyl-umbelliferone (MU) was measured using a fluorometer at an excitation wavelength of 360 nm and emission wavelength of 460 nm. One unit of enzyme is defined as the amount of enzyme needed to convert 1 nmol of MUO to MU in 1 h at 37 °C.

Overexpression PNO1 in GLA expressing strain

The vector pGAPNorPNO1 integrated in the endogenous *PNO1* locus of the GS115ΔOCH1hLALHac strain after linearization with XhoI. To increase the copy number even more, the resulting strain GS115ΔOCH1hLALHac1 1xPNO1 was transformed with pGAPKanPNO1. The pGAPKanPNO1 plasmid was linearized in the GAP promoter using AvrII. This final strain was called GS115ΔOCH1hLALHac1 2xPNO1. After each transformation the best performing clone out of 12 was selected to continue with.

Carbohydrate analysis and qPCR

N-glycans present on yeast-secreted proteins were analyzed by capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) using an ABI 3130 capillary DNA sequencer as described previously[8]. In short, the proteins were blotted on a PVDF membrane and disulfide bridges were disrupted trough reduction and carboxymethylation. PNGaseF solution was added to release the N-glycans from the blotted proteins. These glycans were labelled with the fluorescent dye APTS for detection.

Purification of hLAL

To purify hLAL, GS115ΔOCH1hLALHac 2xPNO1 was first grown in 250 ml BMGY pH 6.2 in a 2 l shake flask during 48 h at 28 °C. BMGY was changed to 250 ml BMMY containing liposomes for induction during 48 h in a 2 l shake flask. To create liposomes in BMMY, 1 mmol (0.76 g) of L-alpha-phosphatidylcholine was dissolved in 5 ml chloroform and after evaporation, 250 ml BMMY containing 2.4 mM taurodeoxycholic acid (0.31 g) was added. The culture was induced by the addition of 1 % methanol every 12 h. After centrifugation (5000g, 5 min) of the induced culture, the supernatant was filtered using a 0.22 µm bottle top filter. The filtered medium was loaded on a 1 ml HisTrap HP column (17-5247-01, GE healtcare) using an equilibration buffer pH 7 (20 mM NaH_2PO_4; 0,5 M NaCl; 10 mM imidazole; 0,1% CHAPS; pH 7). Imidazol was used

to elute the proteins (20 mMNaH_2PO_4; 20 mM NaCl; 200 mM imidazole pH 6.2). The fractions containing the enzyme were pooled and used for glycan analysis.

Claims

1. A lysosomal acid lipase (LAL) protein obtained by i) cultivating a recombinant yeast comprising an expression construct comprising a promoter operably linked to a nucleotide sequence encoding said LAL protein under conditions wherein said promoter is active in said recombinant yeast, ii) adding liposomes to the growth medium and iii) isolating said LAL protein from the growth medium in a purification step.
2. A LAL protein according to claim 1 wherein said recombinant yeast is deficient in an alfa-1,6-mannosyltransferase activity.
3. A LAL protein according to claims 1 or 2 wherein said recombinant yeast is deficient in OCH1 transferase activity.
4. A LAL protein according to claims 1, 2 or 3 wherein said recombinant yeast further comprises an expression construct encoding a functional HAC1 protein.
5. A LAL protein according to claims 1, 2, 3 or 4 wherein said recombinant yeast further comprises an expression construct encoding an MNN4 or PNO1 polypeptide.
6. A LAL protein obtained and isolated according to claims 1, 2, 3, 4 or 5 which is further treated with a mannose decapping enzyme and an alfa-mannosidase.
7. A LAL protein according to any one of claims 1, 2, 3, 4, 5 or 6 for use as a medicament.
8. A pharmaceutical composition comprising a LAL protein according to claims 1, 2, 3, 4, 5 or 6.
9. A LAL protein according to any one of claims 1, 2, 3, 4, 5 or 6 for the treatment of Wolman's disease.
10. A LAL protein according to any one of claims 1, 2, 3, 4, 5 or 6 for the treatment of cholesteryl ester storage disease.
11. A LAL protein according to any one of claims 1, 2, 3, 4, 5 or 6 for the treatment of atherosclerosis.
12. A method to produce a lipase in a recombinant yeast cell, said method comprising i) cultivating a recombinant yeast cell, comprising an expression construct comprising a yeast promoter operably linked to a nucleotide sequence encoding said lipase protein, under conditions wherein said yeast promoter is active, ii) adding liposomes to the cultivation medium and iii) isolating said lipase protein from the growth medium in a purification step.
13. A method to produce a LAL protein in a yeast cell, said method comprising i) cultivating a yeast cell comprising an expression construct comprising a yeast promoter operably linked to a nucleotide sequence encoding said LAL protein, under conditions wherein said yeast promoter is active, ii) adding liposomes to the cultivation medium and iii) isolating said LAL protein from the growth medium in a purification step.

14. A method according to claim 13 wherein said yeast cell is deficient in an alfa-1,6-mannosyltransferase activity.
15. A method according to claim 14 wherein said alfa-1,6-mannosyltransferase is an OCH1 transferase.
16. A method according to claims 12, 13, 14 or 15 wherein said yeast cell further comprises an expression construct encoding a HAC1 protein.
17. A method according to claims 12, 13, 14, 15 or 16 further comprising an expression construct encoding an MNN4 or PNO1 polypeptide.
18. A method according to claims 12, 13, 14, 15, 16 or 17 further comprising an expression construct encoding a hydrolyse capable of hydrolyzing a mannose-1-phospho-6-mannose linkage to phospho-6-mannose.
19. A method according to claim 18 further comprising an expression construct encoding an alpha-mannosidase.
20. A method according to any one of claims 12-19 wherein said yeast cell is selected from the list *Pichia pastoris, Pichia methanolica, Oogataea minuta, Hansenula polymorpha, Saccharomyces cerevisiae, Kluyveromyces lactis, Schizosaccharomyces pombe. Yarrowia lipolytica* and *Arxula adeninivorans*.

Figure 1

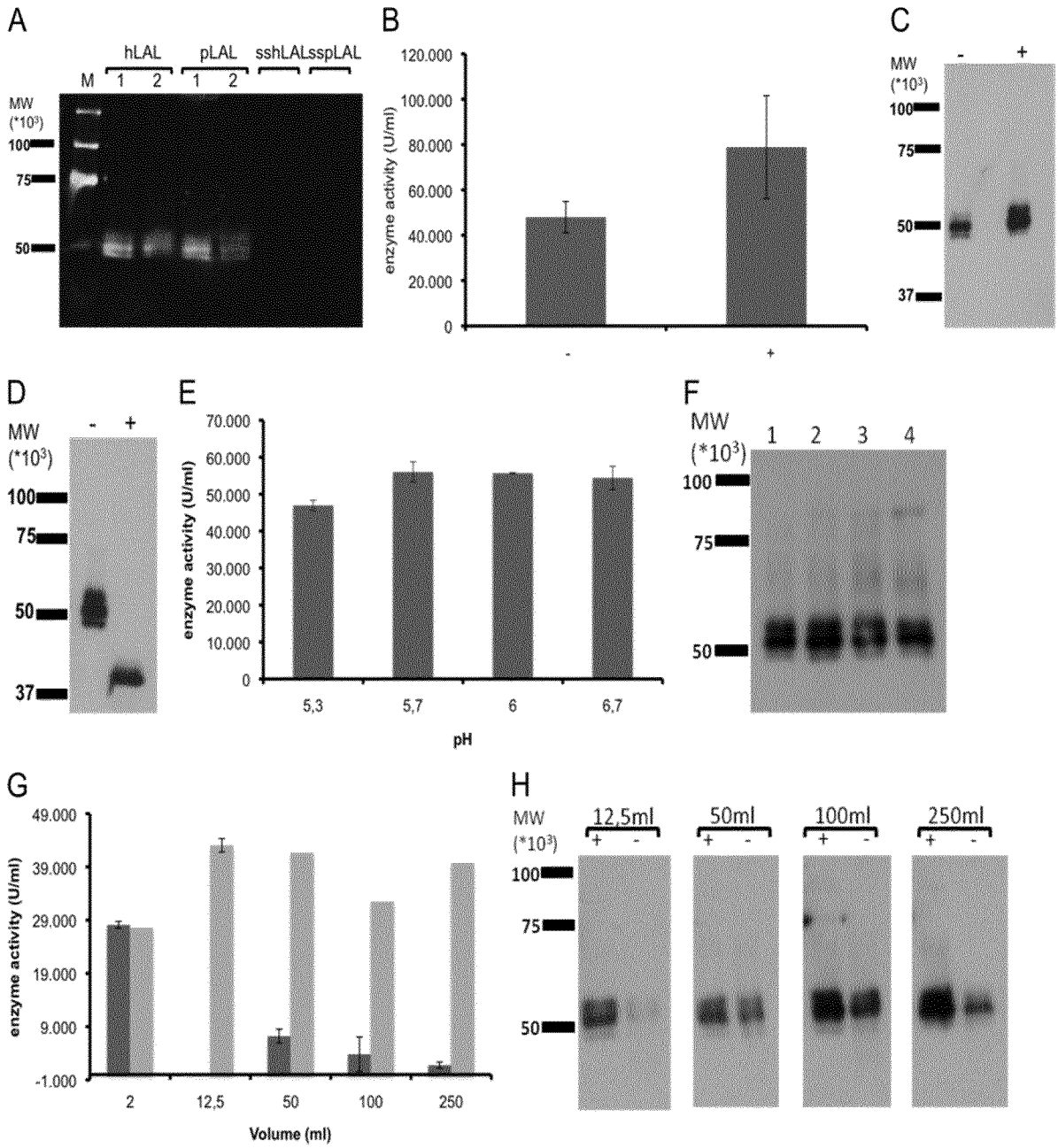
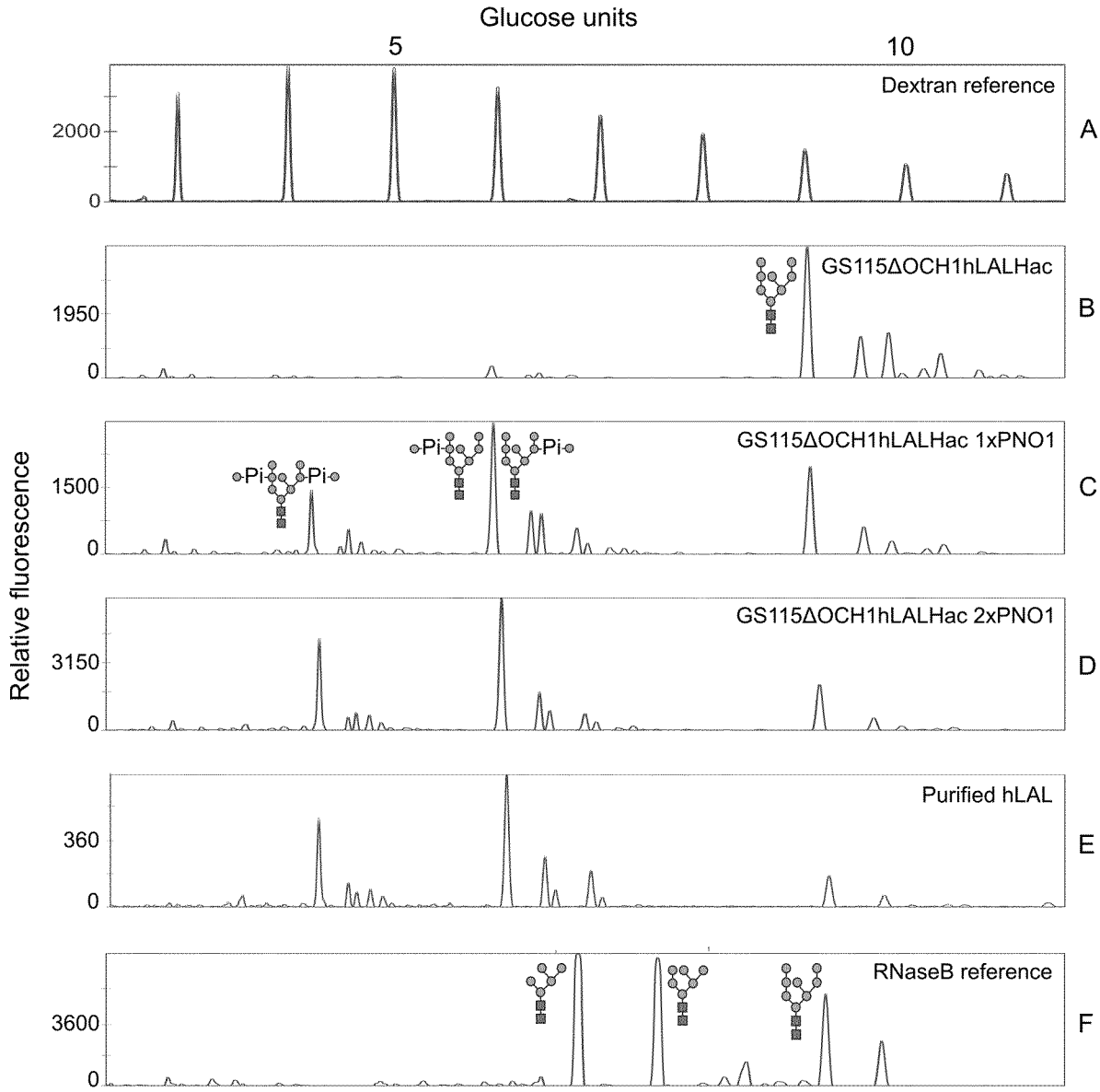

A
hLAL
pLAL
sshLAL
sspLAL
M
1
2
1
2
MW
(*10³)
100
75
50
B
enzyme activity (U/ml)
120.000
100.000
80.000
60.000
40.000
20.000
0
-
+
C
MW
(*10³)
-
+
100
75
50
37
D
MW
(*10³)
-
+
100
75
50
37
E
enzyme activity (U/ml)
70.000
60.000
50.000
40.000
30.000
20.000
10.000
0
5,3
5,7
6
6,7
pH
F
MW
(*10³)
1
2
3
4
100
75
50
G
enzyme activity (U/ml)
49.000
39.000
29.000
19.000
9.000
-1.000
2
12,5
50
100
250
Volume (ml)
H
MW
(*10³)
12,5ml
50ml
100ml
250ml
+
-
100
75
50

Figure 2


Glucose units
5
10
Dextran reference
2000
0
A
GS115ΔOCH1hLALHac
1950
0
B
GS115ΔOCH1hLALHac 1xPNO1
Pi
Pi
Pi
Pi
1500
0
C
GS115ΔOCH1hLALHac 2xPNO1
3150
0
D
Purified hLAL
360
0
E
RNaseB reference
3600
0
F
Relative fluorescence

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/078317

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/20 A61K38/46 C12R1/84
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 A61K C12R C12P

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 practicable, search terms used)

EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 March 2016	10/03/2016

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	Pilat, Daniel

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 2007/037248 A1 (BOBROWICZ PIOTR [US] ET AL) 15 February 2007 (2007-02-15) abstract paragraphs [0007], [0021], [0161], [0169], [0314]; table 1	2,3,5

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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A	US 2011/092374 A1 (CALLEWAERT NICO [BE] ET AL) 21 April 2011 (2011-04-21) abstract GalGnM3IL-10,Man-II; paragraphs [0009], [0010], [0041], [0068]; claims 3,7,; table 3	2,3
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/078317

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