

Biocatalytical compositions

The field of the invention

The present invention relates to a composition comprising a solid carrier, a lipase or a
5 fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with
the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase
or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or
a fragment thereof is in the open conformation. The present invention also relates to methods
of producing said composition.

10

Background of the invention

Proteins such as enzymes are frequently needed, e.g. in industrial applications, diagnostics or
for therapeutic use. In order to stabilize the proteins and/or to provide resistance to various
types of stresses it has been suggested in the prior art to immobilize the proteins on the
15 surface of a carrier and to protect them with a layer of protective material. Such an approach
has been described e.g. in WO2015/014888 which discloses a biocatalytical composition
comprising a solid carrier, a functional constituent like an enzyme and a protective layer for
protecting the functional constituent by embedding the functional constituent at least partially
and a process to produce such biocatalytical composition. Nevertheless, there is a need for
20 providing enzymes like lipases which are protected and at the same time have high enzymatic
activity.

Summary of the invention

The present invention provides a composition comprising a solid carrier, a lipase or a
25 fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with
the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase
or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or
a fragment thereof is in the open conformation.

30 The present invention provides also a method of producing a composition, the composition
comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the
solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof,

and a protective layer to protect the lipase or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or a fragment thereof is in the open conformation , the method comprising the following steps:

- (a) providing a solid carrier;
- 5 (b) providing a lipase or a fragment thereof;
- (c) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- (d) allowing the lipase or a fragment thereof of (b) to interact with the agent of (c);
- (e) immobilizing the lipase or a fragment thereof on the solid carrier;
- 10 (f) forming a protective layer on the surface of the solid carrier to protect the lipase or the fragment thereof immobilized on the solid carrier.

It has been surprisingly found that the activity of a lipase immobilized and protected as described in WO2015/014888 can be significantly increased by an agent which interacts with
15 the lid domain of the lipase.

Brief description of the figures

Figure 1) shows a schematic representation of the process for the production of the composition of the invention: a) to a solid carrier, a lipase or a fragment thereof with closed lid and an agent (displayed as round circle) which interacts with the lid domain of the lipase
20 or a fragment thereof is provided and the lipase or a fragment thereof with an open lid is immobilized on the solid carrier; b) and c) a protective layer grows around the immobilized lipase or the fragment with an open lid thereof embedding the immobilized lipase or the fragment thereof.

Figure 2) shows the 3D structure of pancreatic lipase in a) its inactive conformation (with closed lid) and b) active conformation (with opened lid). The active site of pancreatic lipase is covered by a lid that prevents substrates from reaching the enzyme active site (Figure 2a). The opening of the lipase lid is induced by interactions with an agent which interacts with the lid domain of the lipase e.g. bile salts and/or a protein cofactor called colipase, allowing the
25 stabilization of the active conformation of pancreatic lipase (Figure 2b).

Figure 3) shows the kinetics of lipase substrate hydrolysis by recombinant human pancreatic lipase (HRL) with or without colipase (CLPS): a) in its free form; b) immobilized at the

surface of silica nanoparticles (SNPs) and protected in an organosilca layer made of APTES, TEOS and Benzyltriethoxysilane (ATB).

Figure 4) shows the kinetics of lipase substrate hydrolysis by porcine pancreatic lipase (PL) with or without colipase (CLPS) immobilized at the surface of silica nanoparticles (SNPs) and protected in an organosilca layer made of APTES, TEOS and Benzyltriethoxysilane (ATB).

Figure 5) shows the kinetics of lipase substrate hydrolysis by free human recombinant lipase (HRL) using increasing concentrations of sodium taurocholate (NaTc).

Figure 6) shows the kinetics of lipase substrate hydrolysis by porcine pancreatic lipase (PL) immobilized at the surface of silica nanoparticles (SNPs) and protected in an organosilca layer made of APTES, TEOS and Benzyltriethoxysilane (ATB) with or without sodium taurocholate (NaTc).

Figure 7) shows the 3D structure of pancreatic lipase activated by a colipase-mimicking peptide.

Figure 8) shows the added value of the covalent bonding of the enzyme surface to the protective layer. (A) Protein quantification performed on reaction supernatants of NP-1, NP-1(1), NP-1(2). (B) HRL loading per dry weight of SNP. (C) SNP-specific activities expressed in $U_{\mu M/min/g}$ of SNP. (D) HLR-specific activities expressed in $U_{\mu M/min/g}$ of HRL.

Figure 9) shows absorbance of nanoparticles NP-1, NP-1(1), NP-1(2) at 460 nm.

Detailed description of the invention

The present invention relates to a composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or a fragment thereof is in the open conformation.

For the purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and *vice versa*. It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

Features, integers, characteristics, compounds described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any

other aspect, embodiment or example described herein unless incompatible therewith. All of the features disclosed in this specification (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are
5 mutually exclusive. The invention is not restricted to the details of any foregoing embodiments.

The term “comprise” and variations thereof, such as, “comprises” and “comprising” is generally used in the sense of include, that is, as “including, but not limited to”, that is to say
10 permitting the presence of one or more features or components.

The singular forms “a,” “an,” and “the” include plural referents unless the content clearly dictates otherwise.

15 The term "about" refers to a range of values $\pm 10\%$ of a specified value. For example, the phrase "about 200" includes $\pm 10\%$ of 200, or from 180 to 220.

The term “solid carrier” as used herein refers usually to a particle. Preferably the solid carrier is a monodisperse particle or a polydisperse particle, more preferably a monodisperse particle.
20 The solid carrier usually comprises organic particles, inorganic particles, organic-inorganic particles, self-assembling organic particles, silica particles, gold particles, titanium particles and is preferably a silica particle, more preferably a silica nanoparticle (SNP). The particle size of the solid carrier is usually between 1 nm and 1000 μm , preferably between 10 nm and 100 μm , particularly about 50 nm.

25 The term “linker” or “cross-linker” which are used synonymously herein refers to any linking reagents containing groups, which are capable of binding to specific functional groups (e.g. primary amines, sulfhydryls, etc.). A linker in the context of the present invention usually connects the surface of the solid carrier with the lipase. For example, a linker may be
30 immobilized on the surface of the solid carrier e.g. on the silica surface as a carrier material and then the lipase may be bound to an unoccupied binding-site of the linker. Alternatively, the linker may firstly bind to the lipase and then the linker bound to the lipase may bind with its unoccupied binding-site to the solid carrier. Various types of linkers are known in the art,

including but not limited to straight or branched-chain carbon linkers, heterocyclic carbon linkers, peptide linkers, polyether linkers, and linkers that are known in the art as tags.

5 The term “protective layer” as used herein refers to a layer for protecting the functional properties of the lipase or fragment thereof e.g. the lipase or fragment thereof immobilized on the surface of the solid carrier. The protective layer of the present invention is usually built with building blocks at least part of which are monomers capable of interacting with both each other usually by covalent binding and the immobilized lipase usually by non-covalent binding. The protective layer is formed on the surface of the solid carrier to protect the lipase
10 or the fragment thereof immobilized on the solid carrier. The protective layers are usually homogeneous layers where at least 50%, preferably at least 70%, more preferably at least 90% of the lipase or fragment thereof e.g. the lipase or fragment thereof is embedded in the protective layer.

15 The term, "lipase or a fragment thereof" includes naturally occurring lipases or a fragment thereof and also includes artificially engineered lipases or a fragment thereof. Artificially engineered lipases or a fragment thereof are e.g. variants or functionally active fragments of the lipase. The terms “fragment of a lipase”, “fragment thereof” in relation to the lipase and “functionally active fragment of a lipase” are thus used synonymously herein. By “variants or
20 functionally active fragments thereof” in relation to the lipase of the present invention is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the lipase. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the amino acids are contemplated so long
25 as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least about 80% sequence identity more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, most preferably at least about 98% sequence identity to the relevant part of the lipase. A fragment of a lipase as defined herein does usually have the same
30 functional properties as the lipase i.e. the full length enzyme from which it is derived and includes at least the lid domain and the substrate binding region A fragment of a lipase contains usually between 100 and 450 amino acids, preferably between 150 and 400 amino acids, more preferably between 200 and 350 amino acids.

The term “partially embedded lipase” as used herein shall mean that the lipase is not fully covered by the protective layer, thus, the lipase is not fully embedded in the protective layer. In one embodiment less than 50% of the lipase of interest are covered by the protective layer, though typically more at least 70% will be covered, thus improving protection of the lipase. In a preferred embodiment, at least 70%, more preferably at least 80%, even more preferably at least 90%, most preferably at least 95% of the lipase of interest is covered by the protective layer. In another preferred embodiment, around 70% to around 95%, more preferably around 80% to around 95%, even more preferably around 90% to around 95%, most preferably around 90% to around 95, 96, 97, 98 or 99 % of the lipase of interest are covered by the protective layer. In a particularly preferred embodiment, around 70%, particularly around 80%, more particularly around 90%, most particularly around 95% of the lipase of interest is covered by the protective layer. In a more particularly preferred embodiment, around 70%, particularly around 80%, more particularly around 90%, most particularly around 95% of the lipase is covered by the protective layer, wherein the active site is not covered.

The term “fully embedded lipase” as used herein shall mean that the lipase according to the invention is fully, i.e. 100% covered by the protective layer, i.e. that also the active site is covered. Preferably the lipase or a fragment thereof is fully, i.e. 100% covered by the protective layer, i.e. that also the active site is covered.

The term “at least partially embedded lipase” as used herein shall mean that the lipase is at least partially embedded and may be fully embedded by the protective layer. Thus “at least partially embedded lipase” means that the protective layer covers from about 30% and 100% of the lipase or a fragment thereof, preferably from about 50% to about 100%, more preferably from about 80% to about 100%, even more preferably from about 90% to about 100%, most preferably from about 95% to about 100 %, wherein the active site is preferably covered.

The term “agent which interacts with the lid domain of the lipase or a fragment thereof” as used herein refers to an agent which normally binds to the lid domain of the lipase or a fragment thereof and/or to the region of the lipase or a fragment thereof surrounding the lid domain, thereby causing the lid domain to shift the lipase or a fragment thereof to the open conformation and/or to maintain the open conformation of the lipase or the fragment thereof.

The lid domain of lipases is normally an amphipathic structure; in the closed conformation, their hydrophilic side faces the solvent, while the hydrophobic side is directed toward the catalytic pocket (Brocca S., Secundo F., Ossola M., Alberghina L., Carrea G., Lotti M. (2003). Sequence of the lid affects activity and specificity of *Candida rugosa* lipase isoenzymes. Protein Sci. 12, 2312–2319. 10.1110/ps.0304003). As the lipase shifts to the open conformation, the hydrophobic face becomes exposed and contributes to the substrate-binding region. Preferably the agent which interacts with the lid domain of the lipase or a fragment thereof, causes the lipase or a fragment to be locked in its active conformation. When locked in its active conformation the lipase is normally fully activated. The agent which interacts with the lid domain of the lipase or a fragment thereof so that the the lipase or a fragment thereof is in the open conformation include a colipase or a fragment thereof, a colipase-mimicking peptide, and an amphipathic molecule.

The term “amphipathic molecule” as used herein refers to a molecule like a chemical compound containing both polar (water-soluble) and nonpolar (not water-soluble) portions in its structure. It may also relate to a molecule like a chemical compound having both hydrophobic and hydrophilic regions. Amphipathic molecules include bile salts, phospholipids, and nonionic detergents.

The terms “open conformation” or “open conformation of a lipase or a fragment thereof” which are used interchangeably herein refer to the conformation of the lipase or the fragment thereof where substrates can enter the lipases’ active sites and be converted. In the closed conformation entrance of substrates to the active site of the lipase or a fragment thereof and its conversion is limited or not possible. The conformation of the lipase or fragment thereof i.e. whether the lipase is in open or closed confirmation can be determined by X-ray crystallography, enzymatic activity study, site-directed spin labeling (SDSL) methods and electron paramagnetic resonance (EPR).

The term “colipase or a fragment thereof” as used herein includes naturally occurring colipases or a fragment thereof and also includes artificially engineered colipases or a fragment thereof. Artificially engineered colipases or a fragment thereof are e.g. variants or functionally active fragments of the lipase. By “variants or functionally active fragments thereof” in relation to the colipase of the present invention is meant that the fragment or

variant (such as an analogue, derivative or mutant) is capable of exercising the same physiological function as the colipase. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the amino acids are contemplated so long as the

5 modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least about 80% sequence identity more preferably at least about 90% sequence identity, even more preferably at least about 95% sequence identity, most preferably at least about 98% sequence identity to the relevant part of the lipase. A fragment of a colipase as defined herein does have the same functional properties

10 as the colipase from which it is derived. A preferred colipase is the colipase with Uniprot number: P02703.

The term "colipase mimicking peptide" as used herein refers to a peptide consisting of between 10 and 40 amino acids, allowing specific amino acid residues to be geometrically

15 located in the right position to interact with amino acids of the pancreatic lipase structure, inducing stretching of the lipase conformation and opening of the lid, and thereby having the same functional properties as the colipase. A colipase mimicking peptide which can be used in the present invention is preferably the peptide as shown in SEQ ID NO: 1.

20 The term "bile salt" as used herein refers to bile acids conjugated with taurine or glycine and include sodium taurocholate, sodium glycocholate, sodium glycodeoxycholate, sodium taurodeoxycholate, sodium glycochenodeoxycholate, and sodium taurochenodeoxycholate.

The term "nonionic detergent" as used herein refers to a surfactant and include tetra ethylene

25 glycol monoethyl ether, octyl- β -D-glucopyranoside, N,N-dimethyldodecylamine-N-oxide, and β -octylglucoside.

The term "phospholipids" as used herein refers to a class of lipids whose molecule has a hydrophilic "head" containing a phosphate group and two hydrophobic "tails" derived from

30 fatty acids, joined by an alcohol residue (usually a glycerol molecule). Phospholipids include lecithin and lysolecithin.

In a first aspect the present invention provides a composition comprising a solid carrier, a

lipase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or a fragment thereof is in the open conformation.

5

The lipase or a fragment thereof can be immobilized on the surface of the solid carrier by non-covalent binding or covalent binding. Non-covalent binding includes p-p (aromatic) interactions, van der Waals interactions, H-bonding interactions, and electrostatic interactions like e.g. ionic interactions. Preferably, the lipase or fragment thereof is immobilized on the surface of the solid carrier by covalent binding or by covalent binding via a linker.

10

A solution of a lipase or a fragment thereof usually comprises the protein or a fragment thereof in a buffer solution. Buffers which can be used are usually phosphate, chloride, citrate, MES, MOPS, HEPES, PIPES, ACES or mixtures thereof. The solution may additionally contain sugar alcohols or non-ionic surfactants as described herein. A solution of a lipase or a fragment thereof can be prepared by e.g. dissolving the lipase or a fragment thereof in water to reconstitute the stock buffer of lipase or a fragment thereof.

15

In one embodiment the solid carrier is selected from the group of organic particles, inorganic particles, organic-inorganic particles, self-assembling organic particles, silica particles, gold particles, titanium particles and is preferably a silica particle, more preferably a silica nanoparticle (SNP). The particle size is usually measured by measuring the diameter of the particles and is usually between 1 nm and 1000 nm, preferably between 10 nm and 100 nm, particularly about 50 nm. In case the solid carrier is a monodisperse particle, the size is usually between 1 nm and 1000 nm, preferably between 10 nm and 100 nm, particularly about 50 nm. In case the solid carrier is a polydisperse particle, the size is usually between 1 nm and 1000 μm , preferably between 10 nm and 100 μm , particularly between 50 nm and 50 μm . In one embodiment the composition comprises a solid carrier wherein the specific enzyme activity of the lipase on the solid carrier measured as $\text{U}_{\mu\text{M}/\text{min}}/\text{g}$ of solid carrier is at least 20 $\text{U}_{\mu\text{M}/\text{min}}/\text{g}$ of solid carrier, preferably at least 30 $\text{U}_{\mu\text{M}/\text{min}}/\text{g}$ of solid carrier, in particular between 20 and 50 $\text{U}_{\mu\text{M}/\text{min}}/\text{g}$ of solid carrier, more particular between 30 and 40 $\text{U}_{\mu\text{M}/\text{min}}/\text{g}$ of solid carrier.

20

25

30

Usually monodisperse particles or polydisperse particles, preferably monodisperse particles are used as solid carrier in the present invention. In a preferred embodiment the monodisperse particles are spherical monodisperse particles. In a further preferred embodiment, the polydisperse particles are non-spherical polydisperse particles.

5

The solid carrier is usually provided in suspension. Suspension of the solid carrier can be e.g. in water, buffer or non-ionic surfactants or mixtures thereof, preferably in mixtures of water and non-ionic surfactants. Non-ionic surfactants are usually selected from the group consisting of ethoxylated sorbitan esters like PEG-40 sorbitan diisostearate, polysorbate 80 (PS80), polysorbate 20 (PS20), polysorbate 40 (PS40), polysorbate 60 (PS60); block co-polymers like poloxamer 124, poloxamer 188, poloxamer 331, poloxamer 407, fatty acids ethoxylates like PEG-5 oleate, PEG-8 stearate, polyoxyl 40 stearate, polyoxyl 15 hydroxystearate, fatty alcohol ethoxylates like steareth 40; fatty acid esters like ascorbyl palmitate, beeswax, polyglyceryl 3-oleate, propylene glycol monocaprylate, propylene glycol monolaurate; fatty alcohols like cetostearyl alcohol, cetyl alcohol, myristic alcohol, stearyl alcohol; glycerides; pegylated triglycerides; sugar esters and are preferably polysorbates, more preferably polysorbate 80 (PS80). Buffers which can be used in the method of the present invention are phosphate, piperazine-N,N'-bis(2-ethanesulfonic acid), 2-Hydroxy-3-morpholinopropanesulfonic acid, N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid, (3-(N-morpholino)propanesulfonic acid), 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 3-(N,N-Bis[2-hydroxyethyl]amino)-2-hydroxypropanesulfonic acid, N,N-Bis(2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid, N-[Tris(hydroxymethyl)methyl]glycine, Diglycine, 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid, N,N-Bis(2-hydroxyethyl)glycine, N-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid, N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid.

In one embodiment, the agent which interacts with the lid domain of the lipase or a fragment thereof is added to a suspension of the solid carrier, preferably the agent which interacts with the lid domain of the lipase or a fragment thereof is added together with the lipase or a fragment thereof to the suspension of the solid carrier prior to immobilization of the lipase or the fragment thereof on the solid carrier.

30

The immobilization of a lipase on the solid carrier is usually carried out by adding a solution of the lipase to the suspension of the solid carrier. In a preferred embodiment the immobilization of a lipase on the solid carrier is carried out by providing a suspension of the solid carrier and providing a solution of the lipase, wherein the suspension of the solid carrier is incubated with the solution of the lipase to allow the lipase to bind on the surface of the solid carrier. In a more preferred embodiment the immobilization of a lipase on the solid carrier is carried out by providing a suspension of the solid carrier, providing a solution of the lipase and providing a solution of the agent which interacts with the lid domain of the lipase or a fragment thereof, wherein the suspension of the solid carrier is incubated with the solution of the lipase and with the solution of the agent which interacts with the lid domain of the lipase or a fragment thereof to allow the lipase to bind on the surface of the solid carrier.

In a further preferred embodiment the lipase is immobilized on the solid carrier by a linker, preferably a bi-functional cross-linker binding to the lipase and to the surface of the solid carrier.

In one embodiment the surface of the solid carrier is modified to introduce a molecule or functional chemical group as anchoring point i.e. as anchoring point for the lipase or for the linker connecting the lipase to the solid carrier. Preferably, said anchoring point is an amine functional chemical group or moiety. As a non-limiting example, an amino-modified surface of the solid carrier e.g. an amino-modified silica surface may be used as modified solid carrier. Such an amino-modified surface of the solid carrier may be obtained by reacting a solid carrier having a silica surface with an amino silane, e.g. with APTES. Thus, in a preferred embodiment, the solid carrier is a solid carrier having a silica surface with an amino-modified surface, more preferably a solid carrier obtained by reacting the solid carrier having a silica surface with an amino silane, e.g. with APTES. Such a modified carrier may form an amide linkage between the lipase and the amine group at the surface of the carrier material or an amide linkage between the linker and the amine group at the surface of the carrier material. In one embodiment the introduced molecule or functional chemical group as anchoring point is homogeneously distributed on the surface of the solid carrier.

In a further more preferred embodiment the lipase is immobilized on the solid carrier by at

least partly modifying the surface of the solid carrier by introducing a molecule as anchoring point as described supra for the lipase or by using a linker, preferably a bi-functional cross-linker binding to the anchoring point of the solid carrier and the lipase or a fragment thereof.

- 5 In one embodiment the agent which interacts with the lid domain of the lipase or a fragment thereof is selected from the group consisting of a colipase or a fragment thereof, a colipase mimicking peptide, and an amphipathic molecule. Preferably, the agent which interacts with the lid domain of the lipase or a fragment thereof is selected from the group consisting of a colipase or a fragment thereof, a colipase mimicking peptide, and a bile salt, more preferably selected from the group consisting of a colipase or a fragment thereof, a colipase mimicking peptide, and sodium taurocholate, even more preferably selected from the group consisting of a colipase or a fragment thereof, a colipase mimicking peptide as shown in SEQ ID NO:1, and sodium taurocholate.
- 10
- 15 In one embodiment the agent which interacts with the lid domain of the lipase or a fragment thereof interacts specifically with the lid domain of the lipase or a fragment thereof so that the lipase or a fragment thereof shifts to and/or maintains the open conformation.

- In one embodiment from about 50% to 100%, preferably from about 80% to 100%, more preferably from about 90% to 100%, even more preferably about 100% of the lipase or a fragment thereof immobilized on the surface of the solid carrier is in the open conformation.
- 20

- In some embodiments the protective layer has a defined thickness of about 1 to about 200 nm, usually 1 to about 100 nm, preferably about 1 to about 50 nm, more preferably about 1 to about 25 nm, even more preferably about 1 to about 20 nm, in particular about 1 to about 15 nm. The most preferred defined thickness is about 1 to about 10 nm. In some embodiments the layer has a defined thickness of about 5 to about 100 nm, preferably about 5 to about 50 nm, more preferably about 5 to about 25 nm, even more preferably about 5 to about 20 nm, in particular about 5 to about 15 nm. The most preferred defined thickness is about 5 to about 10 nm. The protective layer is usually porous and the pore size is between 1 and 100 nm, preferably between 1 and 20 nm.
- 25
- 30

The protective layer thickness can be measured, by using a microscope such as scanning electron microscope (SEM), transmission electron microscopy (TEM), scanning probe microscopy (SPM), light scattering methods or by ellipsometry.

- 5 In one embodiment, the lipase or a fragment thereof is partially embedded by the protective layer. In a preferred embodiment the lipase or a fragment thereof is at least partially embedded by the protective layer. In a more preferred embodiment the lipase or a fragment thereof is fully embedded by the protective layer. In one embodiment, the protective layer embeds the solid carrier and embeds the lipase or a fragment thereof immobilized on the
- 10 surface of the solid carrier. Preferably, the protective layer fully embeds the solid carrier and fully embeds the lipase or a fragment thereof immobilized on the surface of the solid carrier. If the protective layer fully embeds the solid carrier and fully embeds the lipase or a fragment thereof immobilized on the surface of the solid carrier, the lipase or fragment thereof is fully, i.e. 100% covered by the protective layer, i.e. that also the active site is covered and the solid
- 15 carrier is fully, i.e. 100% covered by the protective layer.

- In a preferred embodiment the lipase or a fragment thereof is a recombinant human pancreatic lipase (HRL) or a fragment thereof or a porcine pancreatic lipase or a fragment thereof, preferably a recombinant human pancreatic lipase (HRL) or a fragment thereof, more
- 20 preferably a full length recombinant human pancreatic lipase (HRL).

- The composition of the present invention is usually produced in a reaction vessel like a reactor. The reaction vessel usually contains a suspension comprising the lipase or a fragment immobilized on the solid carrier and the agent which interacts with the lid domain of the
- 25 lipase or a fragment thereof as described above. The formation of the protective layer is usually carried out by forming the respective protective layer by building blocks, wherein the building blocks build the protective layer in a polycondensation reaction. The polycondensation can be performed in different solvents, preferably in aqueous solution. Polycondensation can be easily controlled and stopped if appropriate, allowing achievement
- 30 of a defined thickness of the protective layer. The choice of the building blocks, which can be used to build the protective layer, may depend on the known structure of the lipase in order to adapt the affinity of the protective layer according to optimal and/or desired parameters. As building blocks for the protective layer usually structural building blocks and protective

building blocks are used to build the protective layer. Structural building blocks which can be used are e.g. tetraethylorthosilicate (designated herein as “TEOS” or “T”). Protective building blocks which can be used are e.g. 3-Aminopropyltriethoxysilane (designated herein as “APTES” or “A”), Propyltriethoxysilane (designated herein as “PTES” or “P”),

5 Isobutyltriethoxysilane (designated as “IBTES”), Hydroxymethyltriethoxysilane (designated herein as “HTMEOS” or H), Benzyltriethoxysilane (designated herein as “BTES”), Ureidopropyltriethoxysilane (designated as “UPTES”), or Carboxyethyltriethoxysilane (designated herein as “CETES”). Structural building blocks are usually precursors of inorganic silica, capable of forming 4 covalent bonds in the layer formed. Protective building

10 blocks are usually organosilanes, bearing an organic moiety endowed with the ability to interact with the lipase. Preferred structural building blocks are tetravalent silanes, in particular tetra-alkoxy-silanes. Preferred protective building blocks are trivalent silanes, in particular tri-alkoxy-silanes. More preferred structural building blocks are mixtures of tetravalent silanes and trivalent silanes, in particular mixtures of tetra-alkoxy-silanes and tri-

15 alkoxy-silanes. Even more preferred structural building blocks are selected from the group consisting of tetraethylorthosilicate, tetra-(2-hydroxyethyl)silane, and tetramethylorthosilicate. Even more preferred protective building blocks are selected from the group consisting of carboxyethylsilanetriol, benzylsilanes, propylsilanes, isobutylsilanes, n-octylsilanes, hydroxysilanes, bis(2-hydroxyethyl)-3-aminopropylsilanes, aminopropylsilanes,

20 ureidopropylsilanes, (N-Acetylglycyl)-3-aminopropylsilanes, hydroxy(polyethyleneoxy)propyl]triethoxysilanes, in particular selected from benzyltriethoxysilane, propyltriethoxysilane, isobutyltriethoxysilane, n-octyltriethoxysilane, hydroxymethyltriethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltriethoxysilane, 3-Aminopropyltriethoxysilane, ureidopropyltriethoxysilane, (N-Acetylglycyl)-3-

25 aminopropyltriethoxysilane, or selected from benzyltrimethoxysilane, propyltrimethoxysilane, isobutyltrimethoxysilane, n-octyltrimethoxysilane, hydroxymethyltrimethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltrimethoxysilane, aminopropyltrimethoxysilane, ureidopropyltrimethoxysilane (N-Acetylglycyl)-3-aminopropyltrimethoxysilane or selected from benzyltrihydroxyethoxysilane, propyltrihydroxyethoxysilane,

30 isobutyltrihydroxyethoxysilane, n-octyltrihydroxyethoxysilane, hydroxymethyltrihydroxyethoxysilane, bis(2-hydroxyethyl)-3-aminopropyltrihydroxyethoxysilane, aminopropyltrihydroxyethoxysilane, Ureidopropyltrihydroxyethoxysilane (N-Acetylglycyl)-3-

aminopropyltrihydroxymethoxysilane.

Particular preferred building blocks are TEOS as structural building block and APTES, benzyltriethoxysilane BTES, and/or HTMEOS, preferably APTES or benzyltriethoxysilane as
5 protective building block. In particular TEOS as structural building block and APTES or benzyltriethoxysilane as protective building block are used to build the protective layer.

The reaction time of the building blocks with the solid carrier carrying the immobilized lipase or fragment thereof can depend on the length of the linker, if a linker is used, and the size of
10 the lipase. The reaction is usually carried out for a time period of between 0.5 to 10 hours, preferably between 1 and 8 hours, more preferably between 2 and 6 hours, even more preferably about 5 hours, preferably in aqueous solution and preferably at room temperature of about 5 to about 25 °C or at about 20 °C. The formation of the protective layer can be
15 stopped by actively stopping the polycondensation reaction e.g. by removing the non-reacted building blocks e.g. by a washing step or by self-stopping of the polycondensation reaction caused by a limited amount of building blocks.

In a further more preferred embodiment the lipase is immobilized on the solid carrier by at least partly modifying the surface of the solid carrier by introducing a molecule as anchoring
20 point as described supra for the lipase and by using a linker, preferably a cross-linker binding to the anchoring point and the lipase.

In one embodiment the introduced molecule as anchoring point and/or the linker are homogeneously distributed on the surface of the solid carrier.

25 In a preferred embodiment the cross-linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimidate, dimethyl pimelimidate, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated
30 sulfhydryls, sulfhydryl-reactive 2-pyridyldithiol, BSOCOES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate)), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate)), DTBP (Dimethyl 3,3'-dithiobispropionimidate·2 HCl), DST (Disuccinimidyl tartarate), Sulfo-LC-SMPT (4-

Sulfosuccinimidyl-6-methyl-a-(2-pyridyldithio)toluamido]hexanoate)), SPDP (N-Succinimidyl 3-(2-pyridyldithio)-propionate), LC-SPDP (Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate), SMPT (4-Succinimidylloxycarbonyl-methyl-a-[2-pyridyldithio]toluene), DPDPB (1,4-Di-[3'-(2'-pyridyldithio)-propionamido]butane), DTME (Dithio-bismaleimidoethane), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane). More preferably said cross-linker is selected from glutaraldehyde, disuccinimidyl tartrate, disuccinimidyl suberate, bis[sulfosuccinimidyl] suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated

10 sulfhydryls (e.g. suflhydryl-reactive 2-pyridyldithio) and a colipase-mimicking peptide, wherein the colipase-mimicking peptide can be functionalized with a chemical group that enable covalent binding to the solid carrier surface. In a more preferred embodiment the cross-linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl

15 adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, BSOE (Bis[2-(succinimidoxycarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate)), DTSSP (3,3 '-Dithiobis[sulfosuccinimidyl]propionate)), DTBP (Dimethyl 3,3 '-dithiobispropionimide-2 HCl), DST (Disuccinimidyl tartarate), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane).

20 More preferably said cross-linker is selected from glutaraldehyde, disuccinimidyl tartrate, disuccinimidyl suberate, bis[sulfosuccinimidyl] suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated sulfhydryls (e.g. suflhydryl-reactive 2-pyridyldithio). Most preferred is glutaraldehyde.

25

After the protective layer has been formed, the suspension obtained can be washed to remove excess agent which interacts with the lid domain of the lipase or a fragment thereof. In one embodiment, after the protective layer has been formed, the solid carrier comprising the lipase and the protective layer is stored. Storing is usually accomplished e.g. by washing the

30 composition formed e.g. with a buffer and storing it suspended or solved in that buffer for a desired time period. In a preferred embodiment the solid carrier comprising the lipase and the protective layer is stored at a constant temperature between 2 to 25 °C. In a further preferred embodiment, the solid carrier comprising the lipase and the protective layer is stored 5 to 48

hours, preferably 10 to 30 hours. More preferably the solid carrier comprising the lipase and the protective layer is stored at a constant temperature between 2 to 25 °C, preferably at room temperature for 10 to 30 hours.

- 5 In a further aspect the present invention provides the composition as described supra for use as a medicament.

- In a further aspect the present invention provides the composition for use in a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme replacement therapy,
10 or for use in a method for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI). In a preferred embodiment the present invention provides the composition for use in a method for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI). In a further preferred embodiment the present invention provides the composition for use in a method of enzyme replacement therapy
15 (ERT), preferably gastrointestinal enzyme replacement therapy.

- Also provided is the use of the composition as described herein for the manufacture of a medicament for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI) in a subject. Also provided is the use of the composition as described
20 herein for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI) in a subject. Also provided is a method for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI) in a subject, comprising administering to said subject a therapeutically effective amount of the composition as described herein. Also provided herein is the use of the composition as described herein for
25 the manufacture of a medicament for a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme replacement therapy. Also provided is the use of the composition as described herein in a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme replacement therapy in a subject. Also provided is a method of enzyme replacement therapy (ERT), preferably gastrointestinal enzyme
30 replacement therapy, in a subject, comprising administering to said subject a therapeutically effective amount of the composition as described herein.

A composition according to the invention is preferably a pharmaceutical composition and comprises a therapeutically effective amount of the composition as described herein and one or more suitable pharmaceutically acceptable carrier. A pharmaceutical composition according to the invention is suitable for oral administration to a subject. If not indicated
5 otherwise, a pharmaceutical composition according to the invention is prepared in a manner known per se.

An exemplary treatment regime entails administration once daily, twice daily, three times daily, every second day, twice per week, once per week. The composition, e.g. the
10 pharmaceutical composition of the invention is usually administered on multiple occasions. Intervals between single dosages can be, for example, less than a day, daily, every second day, twice per week, or weekly. The composition, e.g. the pharmaceutical composition of the invention may be given as a continuous uninterrupted treatment. The composition, e.g. the pharmaceutical composition of the invention may also be given in a regime in which the
15 subject receives cycles of treatment interrupted by a drug holiday or period of non-treatment. Thus, the composition, e.g. the pharmaceutical composition of the invention may be administered according to the selected intervals above for a continuous period of one week or a part thereof, for two weeks, for three weeks for four weeks, for five weeks or for six weeks and then stopped for a period of one week, or a part thereof, for two weeks, for three weeks,
20 for four weeks, for five weeks, or for six weeks. The composition, e.g. the pharmaceutical composition of the present invention may conveniently be administered in unit dosage forms. Units ("U") of enzyme activity can be described in terms of weight or mass of substrate hydrolyzed per unit time.

25 The expression "effective amount" or "therapeutically effective amount" as used herein refers to an amount capable of invoking one or more of the desired effects in a subject receiving the composition of the present invention. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

30

The terms "treatment"/"treating" as used herein includes: (1) delaying the appearance of clinical symptoms of the state, disorder or condition developing in an animal, particularly a mammal and especially a human, that may be afflicted with or predisposed to the state,

disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; (2) inhibiting the state, disorder or condition (e.g. arresting, reducing or delaying the development of the disease, or a relapse thereof in case of maintenance treatment, of at least one clinical or subclinical symptom thereof); and/or (3)
5 relieving the condition (i.e. causing regression of the state, disorder or condition or at least one of its clinical or subclinical symptoms). The benefit to a patient to be treated is either statistically significant or at least perceptible to the patient or to the physician. However, it will be appreciated that when a medicament is administered to a patient to treat a disease, the outcome may not always be effective treatment.

10

As used herein, "delay of progression" means increasing the time to appearance of a symptom. Further, "delay of progression" as used herein includes reversing or inhibition of disease progression. "Inhibition" of disease progression or disease complication in a subject means preventing or reducing the disease progression and/or disease complication in the
15 subject.

Preventive treatments comprise prophylactic treatments. In preventive applications, the pharmaceutical combination of the invention is administered to a subject suspected of having, or at risk for developing the above mentioned diseases or disorders. In therapeutic
20 applications, the pharmaceutical combination is administered to a subject such as a patient already suffering from the above mentioned diseases or disorders in an amount sufficient to cure or at least partially arrest the symptoms of the disease. Amounts effective for this use will depend on the severity and course of the disease, previous therapy, the subject's health status and response to the drugs, and the judgment of the treating physician.

25

In the case wherein the subject's condition does not improve, the pharmaceutical combination of the invention may be administered chronically, which is, for an extended period of time, including throughout the duration of the subject's life in order to ameliorate or otherwise control or limit the symptoms of the subject's disease or condition.

30

In the case wherein the subject's status does improve, the pharmaceutical combination may be administered continuously; alternatively, the dose of drugs being administered may be temporarily reduced or temporarily suspended for a certain length of time (i.e., a "drug holiday"). Once improvement of the patient's condition has occurred, a maintenance dose of the pharmaceutical combination of the invention is administered if necessary. Subsequently,

the dosage or the frequency of administration, or both, is optionally reduced, as a function of the symptoms, to a level at which the improved disease is retained.

In a further aspect the present invention provides a method of producing a composition, the composition comprising a solid carrier, lipase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or a fragment thereof is in the open conformation, the method comprising the following steps:

- 5 (a) providing a solid carrier;
- (b) providing a lipase or a fragment thereof;
- (c) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- (d) allowing the lipase or a fragment thereof of (b) to interact with the agent of (c);
- 15 (e) immobilizing the lipase or a fragment thereof on the solid carrier;
- (f) forming a protective layer on the surface of the solid carrier to protect the lipase or the fragment thereof immobilized on the solid carrier.

Step (a) is usually carried out by providing the solid carrier in suspension in water or a buffer, preferably in water, non-ionic surfactants or a buffer or mixtures thereof, preferably in buffer, more preferably in suspension in water and/or non-ionic surfactants, even more preferably in suspension in water and/or non-ionic surfactants wherein no buffer is present in the suspension, in particular in suspension in mixtures of water and non-ionic surfactants, more particular in suspension in mixtures of water and non-ionic surfactants wherein no buffer is present in the suspension. The suspension can be stirred e.g at 400 rpm, 20°C for 30 min.

Step b) and c) are usually performed separately or can be performed at once e.g. both lipase and agent can be provided in one solution. The immobilization of the lipase on the solid carrier in step e) of the present method is usually carried out by adding a solution of the lipase or a solution containing the lipase and the agent to the suspension of the solid carrier.

30 Preferably a linker to connect the solid carrier with the lipase or a fragment thereof is added to the suspension of the solid carrier prior to adding the solution of the lipase or a fragment thereof to the suspension of the solid carrier. In a preferred embodiment the immobilization of the lipase on the solid carrier is carried out by providing a suspension of the solid carrier and

adding a solution of the lipase or a solution of the lipase or a solution containing the lipase and the agent, wherein the suspension with the added solution of the lipase is incubated to allow the lipase to bind on the surface of the solid carrier. In a more preferred embodiment the immobilization of the lipase or a fragment thereof on the solid carrier in step (e) is carried out

5 by i) adding a linker to the solid carrier provided in step (a), preferably adding a linker to a suspension of the solid carrier provided in step a), and ii) adding the lipase or a fragment thereof provided in step (b), preferably adding a solution of the lipase or a fragment thereof provided in step (b), to the solid carrier and the linker or to the suspension comprising the solid carrier and the linker, wherein the linker connects the solid carrier with the lipase or a

10 fragment thereof in step (e). In one embodiment, a building block of the protective layer, preferably a monomer of a building block of the protective layer, more preferably an organosilane, even more preferably a triethoxysilane, in particular APTES, is added to the solid carrier and the linker or to the suspension comprising the solid carrier and the linker, prior to adding the solution of the lipase or a fragment thereof.

15 In a preferred embodiment the surface of the solid carrier is at least partly modified to improve immobilization of the lipase on the solid carrier. In particular, the surface of the solid carrier is at least partly modified before the lipase is immobilized. The surface of the solid carrier can be at least partly modified by adding a molecule as anchoring point for the lipase to the surface of the solid carrier as described supra.

20 The suspension comprising the solid carrier is usually incubated after each addition step described above to allow a reaction between e.g. the solid carrier and the molecule as anchoring point, the solid carrier and the linker and, the solid carrier comprising the linker and the lipase or a fragment thereof, respectively, so that the lipase or a fragment thereof connects the solid carrier, preferably the surface of the solid carrier, with the lipase or a fragment

25 thereof via the linker, preferably by covalent binding, thereby immobilizing the lipase or a fragment thereof on the solid carrier.

In one embodiment in step (e) the lipase or a fragment thereof is immobilized on the solid carrier by connecting the solid carrier with the lipase or a fragment thereof via a linker,

30 preferably by connecting the solid carrier with the lipase or a fragment thereof via a linker, wherein the solid carrier is connected with the lipase or a fragment thereof by covalent binding between the linker and the solid carrier and between the linker and the lipase or a fragment thereof. Preferably i) a linker is added to solid carrier provided in step (a), and ii) the

lipase or a fragment thereof provided in step (b) is added to the solid carrier and the linker, wherein the linker connects the solid carrier with the lipase or a fragment thereof in step (e). The linker used is as described supra and connects the surface of the solid carrier with the lipase or a fragment thereof by preferably covalent binding. More preferably the linker is

5 added to the solid carrier provided in step (a), in a molar excess to the lipase or a fragment thereof provided in step (b), preferably the linker is added to the solid carrier in step (b), in a 1 fold to 1000 fold molar excess to the lipase or a fragment thereof provided in step (b), more preferably the linker is added to the solid carrier in step (b), in a 2 fold to 300 fold molar excess to the lipase or a fragment thereof provided in step (b), even more preferably the linker

10 is added to the solid carrier in step (b), in a 4 fold to 250 fold molar excess to the lipase or a fragment thereof provided in step (b), in particular the linker is added to the solid carrier in step (b), in a 205 fold molar excess to the lipase or a fragment thereof provided in step (b). In a preferred embodiment the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (e), is present during formation of a protective layer on the

15 surface of the solid carrier in step (f). In a more preferred embodiment the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (e), or a part thereof, covalently binds the protective layer to the lipase or a fragment thereof in step (f). In a furthermore preferred embodiment the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (e) is not removed in step (e) or step (f) or in between

20 step (e) and (f). In a particular embodiment the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (e) is not removed in step (e) or step (f) or in between step (e) and (f) and the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (e), or a part thereof, covalently binds the protective layer to the lipase or a fragment thereof in step (f). The amount of the linker which has not

25 connected the solid carrier with the lipase or a fragment thereof in step (e) after addition of the protein in ii), is usually between 30% and 70%, preferably between 40% and 60 %, more preferably around 50% of the amount of linker added to the solid carrier in step (e). In one embodiment there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the lipase or a fragment thereof to the solid carrier and the linker

30 in ii). In one embodiment there is no washing step between any of steps (a) to (f). In one embodiment there is no washing step between adding the linker to the solid carrier provided in step (e) in (i) and adding the lipase or a fragment thereof to the solid carrier and the linker

in ii) and there is no washing step between any of steps (a) to (f).

In one embodiment the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene
 5 glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, activated
 10 sulfhydryls, sulfhydryl-reactive 2-pyridyldithiol, BSOE (Bis[2-(succinimidocarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3'-
 15 dithiobispropionimide 2 HCl), DST (Disuccinimidyl tartrate), Sulfo-LC-SMPT (4-Sulfosuccinimidyl-6-methyl- α -(2-pyridyldithio)toluamido]hexanoate), SPDP (N-Succinimidyl 3-(2-pyridyldithio)-propionate), LC-SPDP (Succinimidyl 6-(3-[2-pyridyldithio]-propionamido)hexanoate), SMPT (4-Succinimidylcarbonyl-methyl- α -(2-pyridyldithio)toluene), DPDPB (1,4-Di-[3'-(2'-pyridyldithio)-propionamido]butane), DTME
 20 (Dithio-bismaleimidoethane), BMDB (1,4 bismaleimide-2,3-dihydroxybutane) and is preferably glutaraldehyde.

In a preferred embodiment the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene
 20 glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimide, dimethyl pimelimide, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, BSOE (Bis[2-(succinimidocarbonyloxy)ethyl]sulfone), DSP
 (Dithiobis[succinimidyl]propionate), DTSSP (3,3'-Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3'-dithiobispropionimide 2 HCl), DST (Disuccinimidyl tartrate),
 25 BMDB (1,4 bismaleimide-2,3-dihydroxybutane) and is preferably glutaraldehyde.

The formation of the protective layer according to step (f) of the present method is usually carried out by forming the respective protective layer with building blocks, wherein the building blocks build the protective layer in a polycondensation reaction as described supra.
 30

In one embodiment the method comprises the following steps:

- (a) providing a solid carrier;
- (b) providing a lipase or a fragment thereof;

- (c) immobilizing the lipase or a fragment thereof on the solid carrier;
- (d) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- (e) allowing the lipase or a fragment thereof of (c) to interact with the agent of (d);
- 5 (f) forming a protective layer on the surface of the solid carrier to protect the lipase or the fragment thereof immobilized on the solid carrier.

Steps (a) to (f) can be carried out analogously to steps (a) to (f) of the method provided in the further aspect of the present invention described supra, considering that step (c) of the above method corresponds to step (e) of the method provided in the further aspect of the present
10 invention described supra.

In one embodiment the protective layer is formed by building blocks, wherein as building blocks structural building blocks and protective building blocks are used to form the protective layer, wherein the structural building blocks are precursors of inorganic silica,
15 capable of forming 4 covalent bonds in the layer formed and the protective building blocks are organosilanes as described above.

In one embodiment the protective layer embeds from about 30% to about 100% of the lipase.

20 In one embodiment the solid carrier is selected from the group of organic particles, inorganic particles, organic-inorganic particles, self-assembled organic particles, silica particles, gold particles, magnetic particles and titanium particles and is preferably a silica particle, more preferably a silica nanoparticle (SNP).

25 A preferred method of the present invention is a method of producing a composition, the composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or a fragment thereof by embedding the lipase or a fragment thereof, wherein the lipase or a fragment thereof is in the open conformation, the method comprising the following steps:
30

(a) providing a solid carrier, wherein the solid carrier is provided in suspension, preferably wherein the solid carrier is provided in suspension in buffer, water and/or non-ionic

surfactants, more preferably wherein the solid carrier is provided in suspension in mixtures of water and non-ionic surfactants;

(b) providing a lipase or a fragment thereof;

(c) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;

5 (d) allowing the lipase or a fragment thereof of (b) to interact with the agent of (c);

(e) immobilizing the lipase or a fragment thereof on the solid carrier, wherein preferably the surface of the solid carrier is at least partly modified before the lipase or a fragment thereof is immobilized on the solid carrier, wherein i) a linker is added to the suspension of the solid carrier provided in step (a) or i) a linker is added to the suspension of the solid carrier

10 provided in step (a) after the at least partly modification of the surface of the solid carrier and ii) the lipase or a fragment thereof provided in step (b), preferably a solution of the lipase or a fragment thereof provided in step (b) is added to the the suspension of the solid carrier and the linker, wherein the linker connects the solid carrier with the lipase or a fragment thereof in step (e);

15 (f) forming a protective layer on the surface of the solid carrier to protect the lipase or a fragment thereof immobilized on the solid carrier, wherein the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (e), or a part thereof, covalently binds the protective layer to the lipase or a fragment thereof;

Alternatively steps (a) to (f) can be carried out analogously to steps (a) to (f) of the method described supra considering that step (e) of the above method corresponds to step (c) of the method described supra.

Also provided is a composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier wherein the lipase or a fragment thereof is in the open conformation, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or a fragment thereof by embedding the lipase or a fragment thereof, wherein the lipase or a fragment thereof is in the open conformation, wherein the composition is obtainable by the methods, in particular by the preferred methods of the invention as described supra.

30

Examples

Material and Methods:

Reagents:

- Tetraethyl orthosilicate 99%(TEOS), (3-aminopropyl)-triethoxysilane (APTES), ammonium hydroxide (ACS grade, 28–30%), ethanol (ACS grade, anhydrous), glutaraldehyde (grade I, 25% in water), polysorbate 80, recombinant human pancreatic lipase (HRL, certified reference material), porcine pancreatic lipase (4xUSP specifications), 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester), Tris base, colipase, sodium taurocholate hydrate were purchased from Sigma-Aldrich. HRL, porcine pancreatic lipase and colipase were dissolved in water to reconstitute the stock buffer.
- Benzyltriethoxysilane (B, 96%), was purchased from abcr GmbH.
- The trifluoroacetate salt of peptide Glu-Leu-Gly-Gly-Arg-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Arg-Glu-Gly-Gly-Gly-Glu-Arg-Gly-Gly-Gly-Gly-Asn-Gly-Gly-Gly-Gly-Gly (SEQ ID NO: 1) having E-azido-Nle-OH at the carboxy end was purchased from Bachem.

Synthesis of silica nanoparticles:

- Silica nanoparticles (50 nm) have been synthesized following the original Stöber process as described in WO2015/014888 A1. Briefly, ethanol, distilled water (6 M) and ammonium hydroxide (0.13 M) were mixed and stirred at 400 rpm for 1 h. TEOS (0.28 M) was added and the solution was stirred at 400 rpm at 20°C for 22 h. The solution was then centrifuged at 20000 g for 20 min and washed successively with ethanol and water. Particle size measurement was carried out on SEM micrographs acquired at a magnification of 150000x using the image analysis software Olympus stream motion.

Free recombinant human pancreatic lipase activity assay:

- To a solution of recombinant human pancreatic lipase (3.43 μ L, 1 mg/mL) in Tris buffer (0.1 M, pH 8.4, 56.6 μ L) was added 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (60 μ L, 100 μ M). Lipase activity kinetics was monitored by steady-state fluorescence measurement ($\lambda_{ex}/\lambda_{em} = 529/600$ nm) in a dark 96-well plate for 30 min at 37 °C.

Free recombinant human pancreatic lipase activity assay in presence of colipase:

To a solution of recombinant human pancreatic lipase (3.43 μ L, 1 mg/mL) and colipase (0.72 μ L, 3 μ g/mL) in Tris buffer (0.1 M, pH 8.4, 55.8 μ L) was added 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (60 μ L, 100 μ M). Lipase activity kinetics was monitored by steady-state fluorescence measurement ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 529/600$ nm) in a dark 96-well plate for 30 min at 37 °C.

Immobilized and protected recombinant human pancreatic lipase activity assay:

To a solution of immobilized and protected recombinant human pancreatic lipase (20 μ L, 10 mg/mL) in Tris buffer (0.1 M, pH 8.4, 40 μ L) was added 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (60 μ L, 100 μ M). Lipase activity kinetics was monitored by steady-state fluorescence measurement ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 529/600$ nm) in a dark 96-well plate for 30 min at 37 °C.

Immobilized and protected porcine pancreatic lipase activity assay:

To a solution of immobilized and protected porcine pancreatic lipase (15 μ g/mL of enzyme) in Tris buffer (0.1 M, pH 8.4) was added 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (50 μ M). Lipase activity kinetics was monitored by steady-state fluorescence measurement ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 529/600$ nm) in a dark 96-well plate for 30 min at 37 °C.

Example 1

A) Recombinant human pancreatic lipase (HRL) immobilization and shielding

To SNPs (10 mg/mL) in H₂O / PS80 (8 mg/L) was added APTES (3.8 mM). The reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm. Then, glutaraldehyde (3.8 mM) was added and the reaction mixture was stirred for 10 min at 20 °C, 400 rpm. A priming was performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A recombinant human pancreatic lipase solution (525 μ g/mL, 11 μ M) was added and the reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm. An organosilica layer was grown at the surface of the immobilized lipase using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-HRL-ATB were cured overnight in a water bath at 20 °C.

B) Recombinant human pancreatic lipase (HRL) and colipase (CLPS) co-immobilization and shielding:

To SNPs (10 mg/mL) in H₂O / PS80 (8 mg/L) was added APTES (3.8 mM). The reaction
5 mixture was allowed to react for 10 min at 20 °C, 400 rpm. Then, glutaraldehyde (3.8 mM)
was added and the reaction mixture was stirred for 10 min at 20 °C, 400 rpm. A priming was
performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C,
400 rpm. A solution containing recombinant human pancreatic lipase solution (525 µg/mL, 11
10 µM) and colipase (110 µg/mL) was added and the reaction mixture was allowed to react for
10 min at 20 °C, 400 rpm. An organosilica layer was grown at the surface of the immobilized
proteins using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The
resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were
centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-HRL-
CLPS-ATB were cured overnight in a water bath at 20 °C.

15

Activation of recombinant human pancreatic lipase (HRL) with colipase

Activation of recombinant human pancreatic lipase with colipase was studied using a
fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin
ester) (Figure 3a). The faster kinetics of lipase substrate hydrolysis by lipase in the presence
20 of colipase than that of lipase alone shows the proper activation of the lipase enzyme by
colipase.

To generate activated lipase nanoparticles, recombinant human pancreatic lipase was co-
immobilized with colipase, and protected in a hydrophobic organosilica shield on the surface
of silica nanoparticles as described under B) above. Activation of lipase by colipase was
25 assessed using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-
methylresorufin ester) (Figure 3b). The faster kinetics of lipase substrate hydrolysis by lipase
co-immobilized with colipase as described under B) above than that of lipase immobilized
without the colipase as described under A) above shows an activation of the lipase enzyme by
colipase in the shielded nanoparticle being surprisingly more than three times higher than the
30 lipase enzyme without colipase. These results validate the strategy of co-immobilizing lipase
and colipase on the surface of silica nanoparticles to produce nanoparticles bearing
immobilized and protected lipase maintained in its active conformation.

Example 2***A) Porcine pancreatic lipase (PL) immobilization and shielding:***

To SNPs (10 mg/mL) in H₂O / PS80 (8 mg/L) was added APTES (3.8 mM). The reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm. Then, glutaraldehyde (3.8 mM) was added and the reaction mixture was stirred for 10 min at 20 °C, 400 rpm. A priming was performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A porcine pancreatic lipase solution (1.9 mg/mL, 39 µM) was added and the reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm. An organosilica layer was grown at the surface of the immobilized lipase using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-PL-ATB were cured overnight in a water bath at 20 °C.

B) Porcine pancreatic lipase (PL) and colipase (CLPS) co-immobilization and shielding:

To SNPs (10 mg/mL) in H₂O / PS80 (8 mg/L) was added APTES (3.8 mM). The reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm. Then, glutaraldehyde (3.8 mM) was added and the reaction mixture was stirred for 10 min at 20 °C, 400 rpm. A priming was performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A solution containing porcine pancreatic lipase (1.9 mg/mL, 39 µM) and colipase (150 µg/mL) was added and the reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm. An organosilica layer was grown at the surface of the immobilized proteins using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-PL-CLPS-ATB were cured overnight in a water bath at 20 °C.

Activation of porcine pancreatic lipase (PL) with colipase

To generate activated lipase nanoparticles, porcine pancreatic lipase was co-immobilized with colipase, and protected in a hydrophobic organosilica shield on the surface of silica nanoparticles as described under B) above. Activation of lipase by colipase was assessed using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (Figure 4). The faster kinetics of lipase substrate hydrolysis by lipase

co-immobilized with colipase as described under B) above than that of lipase immobilized without colipase as described under A) above shows the activation of the lipase enzyme by colipase in the shielded nanoparticle being surprisingly three times higher than the lipase enzyme without colipase. These results validate the strategy of co-immobilizing lipase and colipase on the surface of silica nanoparticles to produce nanoparticles bearing immobilized and protected lipase maintained in its active conformation. This also demonstrates the versatility of the method with respect to the origin of the enzyme used.

Example 3

10 *Porcine pancreatic lipase (PL) immobilization and shielding in presence of sodium taurocholate (NaTc):*

To SNPs (10 mg/mL) in a mixture of NaTc (2 mM), H₂O and PS80 (8 mg/L) was added APTES (3.8 mM). The reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm. Then, glutaraldehyde (3.8 mM) was added and the reaction mixture was stirred for 10 min at 15 20 °C, 400 rpm. A priming was performed by adding APTES (3.8 mM) and stirring the reaction mixture for 10 min at 20 °C, 400 rpm. A porcine pancreatic lipase (1.9 mg/mL, 39 µM) was added and the reaction mixture was allowed to react for 10 min at 20 °C, 400 rpm. An organosilica layer was grown at the surface of the immobilized lipase using APTES (4.2 mM), TEOS (21.8 mM) and benzyltriethoxysilane (18.9 mM). The resulting suspension was 20 allowed to react for 5 hours at 20 °C, 400 rpm. The particles were centrifuged 5 min at 20000 rcf and washed 3 times in H₂O / PS80 (8 mg/L). SNPs-PL-NaTc-ATB were cured overnight in a water bath at 20 °C.

25 *Activation of recombinant human pancreatic lipase (HRL) with sodium taurocholate (NaTc)*

Activation of recombinant human pancreatic lipase by sodium taurocholate was studied using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (Figure 5). The faster kinetics of lipase substrate hydrolysis by lipase in the presence of increasing concentration of sodium taurocholate than that of lipase alone shows the proper 30 activation of the lipase enzyme by the bile salt.

Activation of porcine pancreatic lipase (PL) with sodium taurocholate (NaTc)

To generate activated lipase nanoparticles, porcine pancreatic lipase was immobilized in presence of sodium taurocholate and protected in a hydrophobic organosilica shield on the surface of silica nanoparticles. Activation of lipase by sodium taurocholate was assessed using a fluorescent lipase substrate, 1,2-Di-O-lauryl-rac-glycero-3-(glutaric acid 6-methylresorufin ester) (Figure 6). The faster kinetics of lipase substrate hydrolysis by lipase immobilized in presence of sodium taurocholate than that of lipase immobilized without the bile salt shows the activation of the lipase enzyme by sodium taurocholate in the shielded nanoparticle being surprisingly almost three times higher than the lipase enzyme without sodium taurocholate. These results validate the strategy of immobilizing lipase in presence of sodium taurocholate on the surface of silica nanoparticles to produce nanoparticles bearing immobilized and protected lipase maintained in its active conformation.

Example 4: Activation of pancreatic lipase (PL) with a colipase-mimicking peptide

The analysis of the lipase-colipase complex has enabled to identify aminoacid residues responsible for lipase-colipase interactions. Based on these findings, we designed a peptide as shown in SEQ ID NO: 1 (Glu-Leu-Gly-Gly-Arg-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Arg-Glu-Gly-Gly-Gly-Glu-Arg-Gly-Gly-Gly-Gly-Asn-Gly-Gly-Gly-Gly-Gly) to mimic colipase interactions with lipase structure, induce conformational changes around the lid and lipase lid opening (Figure 7). The peptide was chemically modified at its carboxy end by adding an azido-Nle-OH group at its carboxy-end to enable cross-linking of the lipase enzyme at the surface of silica nanoparticles by click-chemistry.

Example 5:

Production of NP-1 variants:

The following experiments explored the impact of covalently linking an enzyme to a protective layer on enzyme stability and enzyme activity, respectively.

In a first experiment, nanoparticles (NP-1(1)) were produced in H₂O/PS80 (8 mg/L). Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 69 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.1 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). Then, glutaraldehyde (3.1 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). A priming

- was performed by adding APTES (3.1 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). Sodium taurocholate (2 mM) and human recombinant lipase (0.732 g/L, 15.2 µM) were successively added and the reaction mixture was allowed to react for 10 min at
- 5 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized HRL using APTES (5.7 mM), TEOS (29.9 mM) and benzyltriethoxysilane (25.9 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). NP-1(1) were cured overnight in a water bath at 20°C.
- 10 In a second, comparative experiment, enzyme immobilisation and formation of the protective layer were carried out according to WO2015/014888 A1 to produce nanoparticles (NP-1(2)) in buffer. Nanoparticles were washed after each chemical step resulting in glutaraldehyde removal. To SNPs (10 mg/mL, 69 nm) in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) was added APTES (3.1 mM). The reaction mixture was allowed to react for 10 min at 20°C,
- 15 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). Then, glutaraldehyde (3.1 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). A priming was
- 20 performed by adding APTES (3.1 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). Sodium taurocholate (2 mM) and human recombinant lipase (0.732 g/L, 15.2 µM) were successively added and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An
- 25 organosilica layer was grown at the surface of the immobilized HRL using APTES (5.7 mM), TEOS (29.9 mM) and benzyltriethoxysilane (25.9 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. Particles were washed three times in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L) and resuspended in phosphate buffer (25 mM, pH 7.5), PS80 (8 mg/L). NP-1(2) were cured overnight in a water bath at 20°C.
- 30 In a third experiment, nanoparticles (NP-1) were produced in H₂O/PS80 (8 mg/L). To keep the excess amount of glutaraldehyde which has not linked the solid carrier to the engineered HRL in the reaction mixture, the nanoparticles were not washed between each chemical step. Therefore, glutaraldehyde was still present during layer growth and caused a covalent binding

of the protective layer to the human recombinant lipase. To SNPs (10 mg/mL, 69 nm) in H₂O/PS80 (8 mg/L) was added APTES (3.1 mM). The reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. Then, glutaraldehyde (3.1 mM) was added, and the reaction mixture was stirred for 10 min at 20°C, 400 rpm. A priming was performed by adding APTES (3.1 mM) and stirring the reaction mixture for 10 min at 20°C, 400 rpm. Sodium taurocholate (2 mM) and human recombinant lipase (0.732 g/L, 15.2 µM) were successively added and the reaction mixture was allowed to react for 10 min at 20°C, 400 rpm. An organosilica layer was grown at the surface of the immobilized HRL using APTES (5.7 mM), TEOS (29.9 mM) and benzyltriethoxysilane (25.9 mM). The resulting suspension was allowed to react for 5 hours at 20°C, 400 rpm. Particles were washed three times in H₂O/PS80 (8 mg/L) and resuspended in H₂O/PS80 (8 mg/L). NP-1 were cured overnight in a water bath at 20°C.

The covalent binding of the protective layer to HRL can be observed by the appearance of a yellow/orange color that has an absorbance maximum at 460 nm. This color is due to the formation of an imine bond by reaction between the aldehyde functions of the glutaraldehyde linker and the primary amines of the amino acids of HRL and the organosilica layer. The absorbance of nanoparticles NP-1(1), NP-1(2), and NP-1 at 460 nm was measured after the organosilica layer formation and final particles washing (see Figure 9A). The results showed that NP-1 absorbed much more light than NP-1(2) at this wavelength. Interestingly, NP-1 and NP-1(1) had similar absorbance values, even though NP-1 appeared darker visually. This suggests that the instrument could not distinguish the formation of imine bonds (due to the low enzyme amount used) because of the strong interference from the particles themselves.

To overcome this limitation, we used an inverted microscope to capture images of the NPs (Figure 9B). At the same concentration, NP-1 appeared significantly darker than NP-1(1) under the microscope. This visual confirmation indicates that NP-1 has more imine bonds, likely due to the covalent attachment of the protective layer to the human recombinant lipase.

Enhancing Enzyme Stability and Specific Activity through Covalent Attachment to the protective layer

In a first experiment, nanoparticles NP-1 (1) were produced in non-buffered conditions and included washing after each chemical step (*i.e.* glutaraldehyde removal before layer growth). In a second experiment, nanoparticles NP-1 (2) were produced in buffered conditions and included washing after each chemical step (*i.e.* glutaraldehyde removal before layer growth). In a third experiment, nanoparticles NP-1 were produced in non-buffered conditions without

any intermediate washing steps (*i.e.* unreacted glutaraldehyde still present in the reaction mixture during layer growth).

Protein quantification was performed on the reaction supernatants to determine HRL immobilization yield at the surface of NP-1(1), NP-1(2) and NP-1. The results show that surprisingly enzyme immobilization under conditions where the presence of glutaraldehyde is maintained (NP-1) increases the enzyme immobilization yield by a factor of 4 (Fig. 8A), resulting in a quadrupling of enzyme loading per dry weight of SNP (Fig. 8B) compared to buffered conditions where glutaraldehyde is removed by washing steps (NP-1(2)).

The lipase biocatalytic activity of HRL immobilized and protected on NP-1(1), NP-1(2) and NP-1 was evaluated. Even more surprising than the increase in enzyme immobilization load when glutaraldehyde is maintained, the specific activity of nanoparticles increased thirty-seven-fold compared with buffered conditions where glutaraldehyde is removed by washing steps, and two-fold compared with unbuffered conditions where glutaraldehyde is removed by washing steps (Fig. 8C). These surprising 37- and 2-fold increases in nanoparticle specific activity are accompanied by an extremely surprising 10- and 2-fold increase, respectively, in HRL specific activity ($U_{\mu M/min/g}$ of HRL) of the enzyme protected in the presence of glutaraldehyde compared with buffered and unbuffered conditions where glutaraldehyde is removed by washing steps (Fig. 8D). This result is completely unexpected, as the enzyme is expected to have a much higher activity in the presence of a buffer.

In summary, covalent attachment of the protective layer to the enzyme surface unexpectedly enhances its load, stability and enzyme specific activity compared to enzymes protected with an organosilica layer via electrostatic interactions only.

Claims

1. A composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or a fragment thereof is in the open conformation.
5
2. The composition according to claim 1, wherein the agent which interacts with the lid domain of the lipase or a fragment thereof is selected from the group consisting of a colipase or a fragment thereof, a colipase-mimicking peptide, and an amphipathic molecule.
10
3. The composition according to claim 1 or 2, wherein the agent interacts specifically with the lid domain of the lipase or a fragment thereof so that the lipase or a fragment thereof shifts to and/or maintains the open conformation.
15
4. The composition according to anyone of claims 1-3, wherein about 100% of the lipase or a fragment thereof immobilized on the surface of the solid carrier is in the open conformation.
20
5. The composition of anyone of claims 1-4, wherein the protective layer embeds the solid carrier and embeds the lipase or a fragment thereof immobilized on the surface of the solid carrier.
25
6. The composition of anyone of claims 1-4, wherein the protective layer embeds the solid carrier, embeds the lipase or a fragment thereof immobilized on the surface of the solid carrier and embeds the agent which interacts with the lid domain of the lipase or a fragment thereof.
30
7. The composition of anyone of claims 1-5, for use as a medicament.

8. The composition of anyone of claims 1-5, for use in a method of enzyme replacement therapy (ERT) preferably gastrointestinal enzyme replacement therapy, or for use in a method for the prevention, delay of progression or treatment of exocrine pancreatic insufficiency (EPI).

5

9. A method of producing a composition, the composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or a fragment thereof is in the open conformation, the method comprising the following steps:

10

- (a) providing a solid carrier;
- (b) providing a lipase or a fragment thereof;
- (c) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- (d) allowing the lipase or a fragment thereof of (b) to interact with the agent of (c);
- (e) immobilizing the lipase or a fragment thereof on the solid carrier;
- (f) forming a protective layer on the surface of the solid carrier to protect the lipase or the fragment thereof immobilized on the solid carrier.

15

20

10. A method of producing a composition, the composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or a fragment thereof is in the open conformation, the method comprising the following steps:

25

- (a) providing a solid carrier;
- (b) providing a lipase or a fragment thereof;
- (c) immobilizing the lipase or a fragment thereof on the solid carrier;
- (d) providing an agent which interacts with the lid domain of a lipase or a fragment thereof;
- (e) allowing the lipase or a fragment thereof of (c) to interact with the agent of (d);

30

(f) forming a protective layer on the surface of the solid carrier to protect the lipase or the fragment thereof immobilized on the solid carrier.

- 5 11. The method of claim 9, wherein i) a linker is added to the solid carrier provided in step (a), and ii) the lipase or a fragment thereof provided in step (b) is added to the solid carrier and the linker, wherein the linker connects the solid carrier with the lipase or a fragment thereof in step (e).
- 10 12. The method of claim 11, wherein the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (e), is present during formation of a protective layer on the surface of the solid carrier in step (f).
- 15 13. The method of claim 11, wherein there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the lipase or a fragment thereof to the solid carrier and the linker in ii).
- 20 14. The method of any one of claims 9 and 11-13, wherein there is no washing step between any of steps (a) to (f).
- 25 15. The method of any one of claims 9 and 11-14, wherein the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (e), or a part thereof, covalently binds the protective layer to the lipase or a fragment thereof in step (f).
- 30 16. The method of claim 10, wherein i) a linker is added to the solid carrier provided in step (a), and ii) the lipase or a fragment thereof provided in step (b) is added to the solid carrier and the linker, wherein the linker connects the solid carrier with the lipase or a fragment thereof in step (c).
17. The method of claim 16, wherein the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (c), is present during formation of a protective layer on the surface of the solid carrier in step (f).

18. The method of claim 16, wherein there is no washing step between adding the linker to the solid carrier provided in step (a) in (i) and adding the lipase or a fragment thereof to the solid carrier and the linker in ii).
- 5 19. The method of any one of claims 10 and 16-18, wherein there is no washing step between any of steps (a) to (f).
- 10 20. The method of any one of claims 10 and 16-19, wherein the linker which has not connected the solid carrier with the lipase or a fragment thereof in step (c), or a part thereof, covalently binds the protective layer to the lipase or a fragment thereof in step (f).
- 15 21. The method of any one of claims 9-20, wherein the linker is selected from the group consisting of glutaraldehyde, disuccinimidyl tartrate, bis[sulfosuccinimidyl]suberate, ethylene glycolbis(sulfosuccinimidylsuccinate), dimethyl adipimidate, dimethyl pimelimidate, sulfosuccinimidyl (4-iodoacetyl) aminobenzoate, 1,5-difluoro-2,4-dinitrobenzene, BSO COES (Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone), DSP (Dithiobis[succinimidyl]propionate), DTSSP (3,3 '-Dithiobis[sulfosuccinimidyl]propionate), DTBP (Dimethyl 3,3 '-dithiobispropionimidate-2 HCl), DST (Disuccinimidyl tartarate), BMDB (1,4 bismaleimidyl-2,3-dihydroxybutane).
- 20 22. The method of any one of claims 9-20, wherein the linker is glutaraldehyde.
- 25 23. A composition comprising a solid carrier, a lipase or a fragment thereof immobilized on the surface of the solid carrier, an agent which interacts with the lid domain of the lipase or a fragment thereof, and a protective layer to protect the lipase or the fragment thereof by embedding the lipase or the fragment thereof, wherein the lipase or a fragment thereof is in the open conformation, wherein the composition is obtainable by the method of any one of claims 12-15 or 17-20.
- 30