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(54) **Title**: MANNOSIDASES CAPABLE OF UNCAPPING MANNOSE-1-PHOSPHO-6-MANNOSE LINKAGES AND DE-MANNOSYLATING PHOSPHORYLATED N-GLYCANS AND METHODS OF FACILITATING MAMMALIAN CELLULAR UPTAKE OF GLYCOPROTEINS

(57) **Abstract**: The invention provides mannosidases capable of uncapping mannose-l-phospho- 6-mannose moieties and demannosylating phosphorylated N-glycans, methods of using such mannosidases, glycoproteins produced using the methods, as well as methods of facilitating mammalian cellular uptake of glycoproteins.

Mannosidases Capable of Uncapping Mannose-1-Phospho-6-Mannose Linkages and Demannosylating Phosphorylated N-glycans and Methods of Facilitating Mammalian Cellular Uptake of Glycoproteins

TECHNICAL FIELD

This invention relates to mannosidases that can (i) hydrolyze a mannose-1-phospho-6-mannose linkage or moiety to phospho-6-mannose and (ii) hydrolyze a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety of such phosphate containing glycans. The invention also relates to methods of facilitating mammalian cellular uptake of glycoproteins.

BACKGROUND

High performance expression systems are required to produce most biopharmaceuticals (e.g., recombinant proteins) currently under development. The biological activity of many of these biopharmaceuticals is dependent on their post-translational modification (e.g., phosphorylation or glycosylation). A yeast-based expression system combines the ease of genetic manipulation and fermentation of a microbial organism with the capability to secrete and to modify proteins. However, recombinant glycoproteins produced in yeast cells exhibit mainly heterogeneous high-mannose and hyper-mannose glycan structures, which can be detrimental to protein function, downstream processing, and subsequent therapeutic use, particularly where glycosylation plays a biologically significant role.

SUMMARY

This document is based on, *inter alia*, the discovery (i) of a mannosidase that can hydrolyze a mannose-1-phospho-6-mannose linkage or moiety to phospho-6-mannose (also referred to as "mannose-6-phosphate" herein) ("uncap") and hydrolyze a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety of such phosphate containing glycans ("demannosylate"); and (ii) that both uncapping and

demannosylation (either by separate enzymes or a single enzyme) are required to achieve mammalian cellular uptake of glycoproteins.

In one aspect, this document features a method for uncapping a mannose-1-phospho-6-mannose linkage or moiety and demannosylating a phosphorylated N-glycan on a glycoprotein. The method includes providing the glycoprotein having a phosphorylated N-glycan containing the mannose-1-phospho-6-mannose linkage or moiety; and contacting the glycoprotein with a mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety. The mannosidase can be a family 38 glycosyl hydrolase. The mannosidase can be from *Canavalia ensiformis* or *Yarrowia lipolytica*.

This document also features a method of demannosylating phosphorylated N-glycans. The method includes providing a glyoprotein comprising a phosphorylated N-glycan; and contacting the glycoprotein with a mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety. The mannosidase can be a family 38 glycosyl hydrolase. The mannosidase can be from *Canavalia ensiformis* or *Yarrowia lipolytica*.

The methods described herein can further include after the providing and contacting steps, contacting a mammalian cell with the glycoprotein that includes the demannosylated phosphorylated N-glycan, wherein, after the contacting, the glycoprotein is transported to the interior of the mammalian cell (e.g., a human cell).

The methods described herein further can include isolating the glycoprotein produced in the methods. The protein can be a human protein expressed in a fungal organism. For example, the fungal organism can be *Yarrowia lipolytica* or *Arxula adeninivorans*. The fungal organism also can be a methylotrophic yeast (e.g., *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, or *Hansenula polymorpha*) or a filamentous fungus (e.g., *Aspergillus caesiellus, Aspergillus candidus, Aspergillus carneus, Aspergillus clavatus, Aspergillus deflectus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus niger, Aspergillus*

ochraceus, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus penicilloides, Aspergillus restrictus, Aspergillus sojae, Aspergillus sydowi, Aspergillus tamari, Aspergillus terreus, Aspergillus ustus, or *Aspergillus versicolor*). The protein can be a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein. For example, the lysosomal protein can be a lysosomal enzyme such as a lysosomal enzyme associated with a lysosomal storage disorder (LSD). A LSD can be Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

This document also features a method of producing a target protein having an uncapped mannose-6-phosphate linkage or moiety and demannosylated phosphorylated N-glycans in a fungal organism. The method includes providing a fungal cell genetically engineered to include a nucleic acid encoding a mannosidase that can hydrolyze a mannose-1-phospho-6-mannose linkage or moiety to a phospho-6-mannose moiety and hydrolyze a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety of such a phosphate containing glycan; and introducing into the cell a nucleic acid encoding a target protein.

This document also features an isolated fungal cell genetically engineered to produce glycoproteins that include an uncapped mannose-6-phosphate and a demannosylated phosphorylated N-glycan. The fungal cell can be *Yarrowia lipolytica* or *Arxula adeninivorans*. The fungal cell also can be a methylotrophic yeast (e.g., *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, or *Hansenula polymorpha*) or a

filamentous fungus (e.g., *Aspergillus caesiellus, Aspergillus candidus, Aspergillus carneus, Aspergillus clavatus, Aspergillus deflectus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus niger, Aspergillus ochraceus, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus penicilloides, Aspergillus restrictus, Aspergillus sojae, Aspergillus sydowi, Aspergillus tamari, Aspergillus terreus, Aspergillus ustus,* or *Aspergillus versicolor*). The fungal cell can include a nucleic acid encoding a mannosidase, the mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety. The fungal cell further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation. The fungal cell can be genetically engineered to be deficient in OCH1 activity. The fungal cell further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation, and wherein the fungal cell is genetically engineered to be deficient in OCH1 activity. The mannosidase can include a secretion signal and/or a targeting signal to target the mannosidase to an intracellular compartment.

A fungal cell further can include a nucleic acid encoding a target protein, wherein the target protein is a glycoprotein. The target protein can be a human protein. The target protein can be a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein. The lysosomal protein can be a lysosomal enzyme. The target protein can be a protein associated with a LSD such as Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder,

chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

A polypeptide capable of promoting mannosyl phosphorylation can be a MNN4 polypeptide (e.g., a *Yarrowia liplytica*, *S. cerevisiae*, *Ogataea minuta*, *Pichia pastoris*, or *C. albicans* polypeptide). The polypeptide capable of promoting mannosyl phosphorylation can be a *P. pastoris* PNO1 polypeptide.

In yet another aspect, this document features a substantially pure culture of *Yarrowia lipolytica*, *Pichia pastoris*, *Hansenula polymorpha*, *Ogataea minuta*, *Pichia methanolica*, *Arxula adeninivorans*, or *Aspergillus niger* cells, a substantial number of which are genetically engineered to produce glycoproteins that contain uncapped mannose-6-phosphate linkages or moieties and demannosylated phosphorylated N-glycans. Substantial number indicates that more than about 40% of the total number of viable cells in the culture are genetically engineered. The cells can include a nucleic acid encoding a mannosidase, the mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety. The cells further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation. The cells can be genetically engineered to be deficient in OCH1 activity. The cells further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation, and can be genetically engineered to be deficient in OCH1 activity. The mannosidase can include a secretion signal and/or a targeting signal to target the mannosidase to an intracellular compartment.

This document also features a method of directing a glycoprotein to the interior of a mammalian cell. The method includes providing a glycoprotein wherein its mannose-6-phosphate linkages have been demannosylated, and contacting the cell with the demannosylated glycoprotein. The glycoprotein can be demannosylated with a family 47 or family 92 glycosyl hydrolase. The glycoprotein can be demannosylated with a mannosidase from *Aspergillus satoi* or *Cellulosimicrobium cellulans*. The glycoprotein can be demannosylated with a family 38 glycosyl hydrolase such as a mannosidase from *Canavalia ensiformis* or *Yarrowia lipolytica*.

In another aspect, this document features a method of directing a glycoprotein to the interior of a mammalian cell. The method includes providing a glycoprotein having a phosphorylated N-glycan, wherein the glycoprotein does not substantially bind to a mannose-6-phosphate receptor on the cell; contacting the glycoprotein with a mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated to produce a demannosylated glycoprotein, wherein the glycoprotein after the demannosylation, substantially binds to the mannose-6-phosphate receptor on the cell; and contacting the cell with the demannosylated glycoprotein. The glycoprotein can be demannosylated with a family 47 or family 92 glycosyl hydrolase. The mannosidase can be from *Aspergillus satoi* or *Cellulosimicrobium cellulans*. The glycoprotein can be demannosylated with a family 38 glycosyl hydrolase such as a mannosidase from *Canavalia ensiformis* or *Yarrowia lipolytica*.

In yet another aspect, this document features a method of converting a glycoprotein from a first form that does not substantially bind to a mannose-6-phosphate receptor on a mammalian cell to a second form that does substantially bind to a mannose-6-phosphate receptor on a mammalian cell, wherein in the first form, the glycoprotein includes one or more N-glycans containing one or more terminal mannose residues that are linked at the 1 position to a mannose residue that contains a phosphate residue at the 6 position. The method includes contacting the first form of the glycoprotein with a mannosidase that demannosylates terminal mannose residues. The mannosidase can have uncapping and demannosylating activities. For example, the mannosidase can be from *Canavalia ensiformis* or *Yarrowia lipolytica*. In some embodiments, the mannosidase does not have uncapping activity (e.g., a mannosidase from *Aspergillus satoi* or *Cellulosimicrobium cellulans*).

This document also features a method of directing a glycoprotein to the interior of a mammalian cell, the glycoprotein includes one or more mannose-1-phospho-6-mannose linkages or moieties. The method includes contacting the cell with the glycoprotein after (a) uncapping the one or more mannose-1-phospho-6-mannose linkages or moieties to mannose-6-phosphate on the glycoprotein, wherein, after uncapping, the glycoprotein

does not substantially bind to a mannose-6-phosphate receptor on the cell and, after step (a), (b) demannosylating phosphorylated N-glycans on the glycoprotein, wherein after both the uncapping and the demannosylation, the glycoprotein does substantially bind to a mannose-6-phosphate receptor on the cell. Steps (a) and (b) can be catalyzed by two different enzymes (e.g., a *Cellulosimicrobium cellulans* mannosidase such as CcMan5 and a *Canavalia ensiformis* mannosidase) or by a single enzyme.

In another aspect, this document features a method of directing a glycoprotein to the interior of a mammalian cell. The method includes providing a glycoprotein having uncapped and demannosylated phosphorylated N-glycans, and contacting the mammalian cell with the glycoprotein.

In the methods described herein, the glycoprotein can be a human protein.

In the methods described herein, the glycoprotein can be a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein. The lysosomal protein can be a lysosomal enzyme (e.g., acid alpha glucosidase or alpha galactosidase). The glycoprotein can be associated with a LSD (e.g., Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia).

The document also features a glycoprotein capable of being transported to the interior of a mammalian cell, wherein the glycoprotein has been treated with any of the methods described herein, as well as a mammalian cell (e.g., human cell) that includes such a glycoprotein. In another aspect, this document features a method of treatment that includes administering such a glycoprotein to a subject in need thereof.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the exemplary methods and materials are described below. All publications, patent applications, patents, Genbank® Accession Nos, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present application, including definitions, will control. The materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1A is a depiction of the codon optimized nucleotide sequence of human alpha glucosidase (GAA) with lip2 pre sequence in bold (SEQ ID NO:1). FIG. 1B is a depiction of the amino acid sequence of human GAA with the lip2 pre sequence in bold, where the * represents the stop codon (SEQ ID NO:2).

FIG. 2 is a schematic of a *Y. lipolytica* expression vector used for cloning of huGAA.

FIG. 3A is a depiction of the nucleotide sequence of the open reading frame (ORF) of *Yarrowia lipolytica* AMS1 with a C-terminal His-tag (SEQ ID NO:3). FIG. 3B is a depiction of the nucleotide sequence of the ORF of *Yarrowia lipolytica* AMS1 with N-terminal His-tag (SEQ ID NO:4). FIG. 3C is a depiction of the amino acid sequence of the *Yarrowia lipolytica* AMS1 polypeptide (SEQ ID NO:5).

FIG. 4 is a schematic of the potential final hydrolysis products from 8-amino-1,3,6,-pyrenetrisulfonic acid (APTS)-labeled sugars derived from an MNN4 overexpressing *Yarrowia lipolytica* strain, which contains $Man_8GlcNAc_2$ (M8), the monophosphorylated ManP-$Man_8GlcNAc_2$ (MP-M8) and/or the diphosphorylated $(ManP)_2$-$Man_8GlcNAc_2$ ($(MP)_2$-M8) sugars (referred to as MNN4 sugars or MNN4 N-

glycans) assuming that the alpha-mannosidases can also fully remove mannose residues from the MNN4 N-glycans.

FIG. 5 is a series of electropherograms depicting the N-glycan analysis of MNN4 N-glycans treated with Jack bean (Jb) alpha-mannosidase. Analysis was performed using DNA sequencer-assisted, fluorophore-assisted carbohydrate electrophoresis (DSA-FACE). "M1," "M2," "M3," "M4," "M5," "M6," "M8," and "M9" refer to the number of mannose residues conjugated to the base N-acetylglucosamine structure. The Y-axis represents the relative fluorescence units as an indication of the amount of each N-glycan structure. The X-axis represents the relative mobility of each N-glycan structure through a capillary.

FIG. 6 is a series of electropherograms showing de-mannosylation and phosphate uncapping activity using AMS1 from *Yarrowia lipolytica* (YlAms1).

FIG. 7 is a series of electropherograms depicting the N-glycan profiles of huGAA before and after the Jack bean alpha-1,2-mannosidase treatment.

FIG. 8A is a depiction of the nucleotide sequence of the open reading frame (ORF) of DsbA-*Cellulosimicrobium cellulans* mannosidase 5 (CcMan5) (SEQ ID NO:6). FIG. 8B is a depiction of the amino acid sequence of the CcMan5 polypeptide with signal sequence in bold (SEQ ID NO: 7). FIG. 8C is a depiction of the amino acid sequence of the CcMan5 polyeptpide without signal sequence (SEQ ID NO:8). The predicted molecular weight of the CcMan5 polypeptide without the signal sequence is 173 kDa.

FIG. 9A is a depiction of the nucleotide sequence of the ORF of DsbA-*C. cellulans* mannosidase 4 (CcMan4) (SEQ ID NO: 9). FIG. 9B is a depiction of the amino acid sequence of the CcMan4 polypeptide with signal sequence in bold (SEQ ID NO: 10). The predicted molecular weight of the CcMan4 polypeptide without the signal sequence is 184 kDa.

FIG. 10 is a schematic of the plasmids pLSAHCcMan5 and pLSAHCcMan4.

FIG. 11 is a series of electropherograms depicting the N-glycan analysis of human alpha glucosidase (GAA) treated with CcMan4 and/or CcMan5.

FIG. 12 is a schematic representation of the capped N-glycans, where P refers to phosphate, a filled square refers to a GlcNac moiety, an open circle refers to a beta-linked mannose, and a filled circle refers to an alpha-linked mannose.

FIG. 13 is a series of electropherograms depicting the N-glycan analysis of Myozyme® treated with CcMan4.

FIG. 14 is a series of electropherograms depicting the N-glycan analysis of human alpha glucosidase (GAA) treated with CcMan4 and/or CcMan5.

FIG. 15 is a series of electropherograms depicting the N-glycan analysis of human GAA treated with JbMan.

FIG. 16 is a series of electropherograms depicting the N-glycan analysis of human GAA treated with JbMan.

FIG. 17 is a line graph of the intracellular GAA activity (U/mg) of Myozyme® (diamonds) or human GAA treated with CcMan5 (squares), CcMan4 (triangles), CcMan4 and CcMan5 (×), or JbMan (✱) at the indicated concentration of enzyme (U/mL). Each data point represents the average of duplicates per dose ± the standard deviation. Data points marked with an asterisk are results from a single stimulation condition per dose.

FIG. 18 is a line graph of the intracellular GAA activity (U/mg) of Myozyme® (diamonds), Myozyme® plus M6P (squares), Myozyme® treated with CcMan4 (triangles), Myozyme® treated with CcMan4, plus M6P (×), human GAA treated with CcMan4 and CcMan5 (✱), human GAA treated with CcMan4 and CcMan5, plus M6P (circles), human GAA treated with JbMan (l), or human GAA treated with JbMan, plus M6P ( ) at the indicated concentration of enzyme (U/mL). Each data point represents the average of duplicates per dose ± the standard deviation. Data points marked with an asterisk are results from a single stimulation condition per dose.

FIG. 19 is a bar graph of the intracellular GAA activity (U/mg) in Pompe fibroblasts incubated with Myozyme, JbMan, or the combination of CcMan4 and CcMan5 for either 14 hours or 46 hours. The average of duplicates ± the standard deviation is presented.

FIG. 20 is a series of electropherograms depicting the N-glycan analysis of human GAA treated with CcMan5 and JbMan.

FIG. 21 is a line graph of the intracellular activity of purified, uncapped and demannosylated huGAA versus the intracellular activity of Myozyme® after extracellular stimulation of the cells with the huGAA and Myozyme respectively.. The amount of enzyme (expressed as enzyme activity units) added to the cells was converted to enzyme concentration (expressed as nM) and plotted versus the specific activity (expressed in U/mg) for the calculations of the K_{uptake}. K_{uptake} and the standard deviation were calculated in GraphPrism using non-linear regression through 14 data points (2 data points per concentration) for huGAA and through 12 data points for Myozyme®.

FIG. 22 is a depiction of the amino acid sequence of a mannosidase from *Aspergillus saitoi* (SEQ ID NO: 11).

DETAILED DESCRIPTION

In general, this document provides methods and materials for hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to phospho-6-mannose (also referred to as "mannose-6-phosphate" herein) ("uncapping") and hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety of such a phosphate containing glycan ("demannosylating"). Also provided are methods of facilitating uptake of a glycoprotein by a mammalian cell as both uncapping and demannosylation (either by separate enzymes or a single enzyme) are required to achieve mammalian cellular uptake of glycoproteins. The methods and materials described herein are particularly useful for producing agents for treating patients with lysosomal storage disorders (LSDs), a diverse group of hereditary metabolic disorders characterized by the accumulation of storage products in the lysosomes due to impaired activity of catabolic enzymes involved in their degradation. The build-up of storage products leads to cell dysfunction and progressive clinical manifestations. Deficiencies in catabolic enzymes can be corrected by enzyme replacement therapy (ERT), provided that the administered enzyme can be targeted to the lysosomes of the diseased cells. Lysosomal enzymes typically are glycoproteins that are synthesized in the endoplasmic reticulum (ER), transported via the secretory pathway to the Golgi, and then recruited to the lysosomes. Using the methods and materials described herein, a microbial based production process

can be used to obtain therapeutic proteins with demannosylated phosphorylated N-glycans. Thus, the methods and materials described herein are useful for preparing glycoproteins for the treatment of metabolic disorders such as LSDs.

Mannosidases

This document provides isolated nucleic acids encoding mannosidases that can (i) hydrolyze a mannose-1-phospho-6-mannose linkage or moiety to phospho-6-mannose and/or (ii) hydrolyze a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety of such a phosphate containing glycan. The terms "nucleic acid" and "polynucleotide" are used interchangeably herein, and refer to both RNA and DNA, including cDNA, genomic DNA, synthetic DNA, and DNA (or RNA) containing nucleic acid analogs. Polynucleotides can have any three-dimensional structure. A nucleic acid can be double-stranded or single-stranded (i.e., a sense strand or an antisense strand). Non-limiting examples of polynucleotides include genes, gene fragments, exons, introns, messenger RNA (mRNA), transfer RNA, ribosomal RNA, siRNA, micro-RNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers, as well as nucleic acid analogs.

"Polypeptide" and "protein" are used interchangeably herein and mean any peptide-linked chain of amino acids, regardless of length or post-translational modification. Typically, a polypeptide described herein (e.g., a mannosidase or an uncapped and demannosylated target protein) is isolated when it constitutes at least 60%, by weight, of the total protein in a preparation, e.g., 60% of the total protein in a sample. In some embodiments, a polypeptide described herein consists of at least 75%, at least 90%, or at least 99%, by weight, of the total protein in a preparation.

An "isolated nucleic acid" refers to a nucleic acid that is separated from other nucleic acid molecules that are present in a naturally-occurring genome, including nucleic acids that normally flank one or both sides of the nucleic acid in a naturally-occurring genome (e.g., a yeast genome). The term "isolated" as used herein with respect to nucleic acids also includes any non-naturally-occurring nucleic acid sequence, since such

non-naturally-occurring sequences are not found in nature and do not have immediately contiguous sequences in a naturally-occurring genome.

An isolated nucleic acid can be, for example, a DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a DNA molecule that exists as a separate molecule (e.g., a chemically synthesized nucleic acid, or a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., any paramyxovirus, retrovirus, lentivirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include an engineered nucleic acid such as a DNA molecule that is part of a hybrid or fusion nucleic acid. A nucleic acid existing among hundreds to millions of other nucleic acids within, for example, cDNA libraries or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not considered an isolated nucleic acid.

The term "exogenous" as used herein with reference to nucleic acid and a particular host cell refers to any nucleic acid that does not occur in (and cannot be obtained from) that particular cell as found in nature. Thus, a non-naturally-occurring nucleic acid is considered to be exogenous to a host cell once introduced into the host cell. It is important to note that non-naturally-occurring nucleic acids can contain nucleic acid subsequences or fragments of nucleic acid sequences that are found in nature provided that the nucleic acid as a whole does not exist in nature. For example, a nucleic acid molecule containing a genomic DNA sequence within an expression vector is non-naturally-occurring nucleic acid, and thus is exogenous to a host cell once introduced into the host cell, since that nucleic acid molecule as a whole (genomic DNA plus vector DNA) does not exist in nature. Thus, any vector, autonomously replicating plasmid, or virus (e.g., retrovirus, adenovirus, or herpes virus) that as a whole does not exist in nature is considered to be non-naturally-occurring nucleic acid. It follows that genomic DNA fragments produced by PCR or restriction endonuclease treatment as well as cDNAs are considered to be non-naturally-occurring nucleic acid since they exist as separate

molecules not found in nature. It also follows that any nucleic acid containing a promoter sequence and polypeptide-encoding sequence (e.g., cDNA or genomic DNA) in an arrangement not found in nature is non-naturally-occurring nucleic acid. A nucleic acid that is naturally-occurring can be exogenous to a particular cell. For example, an entire chromosome isolated from a cell of yeast x is an exogenous nucleic acid with respect to a cell of yeast y once that chromosome is introduced into a cell of yeast y.

A nucleic acid encoding a mannosidase can have at least 70% sequence identity (e.g., at least 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100% sequence identity) to a nucleotide sequence set forth in SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:6, or SEQ ID NO:9. In some embodiments, nucleic acids described herein can encode mannosidase polypeptides that have at least 70% (e.g., at least 75, 80, 85, 90, 95, 99, or 100 percent) identity to an amino acid sequence set forth in SEQ ID NOs: 5, 7, 8, 10, or 11. For example, a nucleic acid can encode a mannosidase having at least 90% (e.g., at least 95 or 98%) identity to the amino acid sequence set forth in SEQ ID NOs: 5, 7, 8, 10, 11 or a portion thereof. For example, a nucleic acid can encode a mannosidase having at least 90% identity to amino acid residues 1 to 774 of SEQ ID NO:8. The percent identity between a particular amino acid sequence and the amino acid sequence set forth in SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:11 can be determined as follows. First, the amino acid sequences are aligned using the BLAST 2 Sequences (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTP version 2.0.14. This stand-alone version of BLASTZ can be obtained from Fish & Richardson's web site (e.g., www.fr.com/blast/) or the U.S. government's National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ. Bl2seq performs a comparison between two amino acid sequences using the BLASTP algorithm. To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt); and all other options are left at their default setting.

For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq –i c:\seq1.txt –j c:\seq2.txt –p blastp –o c:\output.txt. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not share homology, then the designated output file will not present aligned sequences. Similar procedures can be following for nucleic acid sequences except that blastn is used.

Once aligned, the number of matches is determined by counting the number of positions where an identical amino acid residue is presented in both sequences. The percent identity is determined by dividing the number of matches by the length of the full-length mannosidase polypeptide amino acid sequence followed by multiplying the resulting value by 100.

It is noted that the percent identity value is rounded to the nearest tenth. For example, 78.11, 78.12, 78.13, and 78.14 is rounded down to 78.1, while 78.15, 78.16, 78.17, 78.18, and 78.19 is rounded up to 78.2. It also is noted that the length value will always be an integer.

It will be appreciated that a number of nucleic acids can encode a polypeptide having a particular amino acid sequence. The degeneracy of the genetic code is well known to the art; i.e., for many amino acids, there is more than one nucleotide triplet that serves as the codon for the amino acid. For example, codons in the coding sequence for a given mannosidase polypeptide can be modified such that optimal expression in a particular species (e.g., bacteria or fungus) is obtained, using appropriate codon bias tables for that species.

Hybridization also can be used to assess homology between two nucleic acid sequences. A nucleic acid sequence described herein, or a fragment or variant thereof, can be used as a hybridization probe according to standard hybridization techniques. The hybridization of a probe of interest (e.g., a probe containing a portion of a *Yarrowia lipolytica* AMS1 nucleotide sequence) to DNA or RNA from a test source is an indication of the presence of DNA or RNA (e.g., an AMS1 nucleotide sequence) corresponding to the probe in the test source. Hybridization conditions are known to those skilled in the

art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 6.3.1-6.3.6, 1991. Moderate hybridization conditions are defined as equivalent to hybridization in 2X sodium chloride/sodium citrate (SSC) at 30°C, followed by a wash in 1 X SSC, 0.1% SDS at 50°C. Highly stringent conditions are defined as equivalent to hybridization in 6X sodium chloride/sodium citrate (SSC) at 45°C, followed by a wash in 0.2 X SSC, 0.1% SDS at 65°C.

Other mannosidase polypeptide candidates suitable for use herein can be identified by analysis of nucleotide and polypeptide sequence alignments. For example, performing a query on a database of nucleotide or polypeptide sequences can identify homologs and/or orthologs of mannosidase polypeptides. Sequence analysis can involve BLAST, Reciprocal BLAST, or PSI-BLAST analysis of nonredundant databases using known mannosidase amino acid sequences. Those polypeptides in the database that have greater than 40% sequence identity can be identified as candidates for further evaluation for suitability as a mannosidase polypeptide. Amino acid sequence similarity allows for conservative amino acid substitutions, such as substitution of one hydrophobic residue for another or substitution of one polar residue for another. If desired, manual inspection of such candidates can be carried out in order to narrow the number of candidates to be further evaluated.

This document also provides (i) biologically active variants and (ii) biologically active fragments or biologically active variants thereof, of the mannosidases described herein. Biologically active variants of mannosidases can contain additions, deletions, or substitutions relative to the sequences set forth in SEQ ID NOs: 5, 7, 8, 10, and 11. Proteins with substitutions will generally have not more than 50 (e.g., not more than one, two, three, four, five, six, seven, eight, nine, ten, 12, 15, 20, 25, 30, 35, 40, or 50) conservative amino acid substitutions. A conservative substitution is the substitution of one amino acid for another with similar characteristics. Conservative substitutions include substitutions within the following groups: valine, alanine and glycine; leucine, valine, and isoleucine; aspartic acid and glutamic acid; asparagine and glutamine; serine, cysteine, and threonine; lysine and arginine; and phenylalanine and tyrosine. The non-polar hydrophobic amino acids include alanine, leucine, isoleucine, valine, proline,

phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Any substitution of one member of the above-mentioned polar, basic or acidic groups by another member of the same group can be deemed a conservative substitution. By contrast, a non-conservative substitution is a substitution of one amino acid for another with dissimilar characteristics.

Deletion variants can lack one, two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid segments (of two or more amino acids) or non-contiguous single amino acids.

Additions (addition variants) include fusion proteins containing: (a) a mannosidase set forth in SEQ ID NOs: 5, 7, 8, 10, 11 or a fragment thereof; and (b) internal or terminal (C or N) irrelevant or heterologous amino acid sequences. In the context of such fusion proteins, the term "heterologous amino acid sequences" refers to an amino acid sequence other than (a). A heterologous sequence can be, for example a sequence used for purification of the recombinant protein (e.g., FLAG, polyhistidine (e.g., hexahistidine), hemagluttanin (HA), glutathione-S-transferase (GST), or maltose-binding protein (MBP)). Heterologous sequences also can be proteins useful as diagnostic or detectable markers, for example, luciferase, green fluorescent protein (GFP), or chloramphenicol acetyl transferase (CAT). In some embodiments, the fusion protein contains a signal sequence from another protein. In certain host cells (e.g., yeast host cells), expression and/or secretion of the target protein can be increased through use of a heterologous signal sequence. In some embodiments, the fusion protein can contain a carrier (e.g., KLH) useful, e.g., in eliciting an immune response for antibody generation) or endoplasmic reticulum or Golgi apparatus retention signals. Heterologous sequences can be of varying length and in some cases can be a longer sequences than the full-length target proteins to which the heterologous sequences are attached.

Biologically active fragments or biologically active variants of the mannosidases have at least 40% (e.g., at least: 50%; 60%; 70%; 75%; 80%; 85%; 90%; 95%; 97%;

98%; 99%; 99.5%, or 100% or even greater) of the mannosidase activity (e.g., uncapping and/or demannosylating) of the wild-type, full-length, mature protein. For example, a biologically active fragment of a mannosidase that can hydrolyze a mannose-1-phospho-6-mannose linkage or moiety to phospho-6-mannose can contain residues 1 to 774 of SEQ ID NO:8.

The mannosidases described herein can be used to produce uncapped and demannosylated target molecules. The methods can be performed *in vitro* or *in vivo*.

Methods of Demannosylating, or Uncapping and Demannosylating Glycoproteins

As described herein, glycoproteins containing a phosphorylated N-glycan can be demannosylated, and glycoproteins containing a phosphorylated N-glycan containing a mannose-1-phospho-6-mannose linkage or moiety can be uncapped and demannosylated by contacting the glycoprotein with a mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety. Non-limiting examples of such mannosidases include a *Canavalia ensiformis* (Jack bean) mannosidase and a *Yarrowia lipolytica* mannosidase (e.g., AMS1). Both the Jack bean and AMS1 mannosidase are family 38 glycoside hydrolases.

The Jack bean mannosidase is commercially available, for example, from Sigma-Aldrich (St. Louis, MO) as an ammonium sulfate suspension (Catalog No. M7257) and a proteomics grade preparation (Catalog No. M5573). As described in Example 8, such commercial preparations can be further purified, for example, by gel filtration chromatography to remove contaminants such as phosphatases. The Jack bean mannosidase contains a segment with the following amino acid sequence NKIPRAGWQIDPFGHSAVQG (SEQ ID NO:12). See Howard *et al.*, *J. Biol. Chem.*, 273(4):2067–2072, 1998.

The *Yarrowia lipolytica* AMS1 mannosidase can be recombinantly produced. The nucleic acid sequences encoding AMS1 with a C- or N-terminal polyhistidine tag are set forth in SEQ ID NOs. 3 and 4, respectively (see also FIGs. 3A and 3B). The amino acid sequence of the AMS1 polypeptide is set forth in SEQ ID NO:5 (see also FIG. 3C).

Isolated nucleic acid molecules encoding mannosidase polypeptides can be produced by standard techniques. For example, polymerase chain reaction (PCR) techniques can be used to obtain an isolated nucleic acid containing a nucleotide sequence described herein. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers that are identical or similar in sequence to opposite strands of the template to be amplified. Various PCR strategies also are available by which site-specific nucleotide sequence modifications can be introduced into a template nucleic acid. Isolated nucleic acids also can be chemically synthesized, either as a single nucleic acid molecule (e.g., using automated DNA synthesis in the 3' to 5' direction using phosphoramidite technology) or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a single, double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector. Isolated nucleic acids also can be obtained by mutagenesis of, e.g., a naturally occurring DNA.

To recombinantly produce a mannosidase polypeptide, a vector is used that contains a promoter operably linked to nucleic acid encoding the mannosidase polypeptide. As used herein, a "promoter" refers to a DNA sequence that enables a gene to be transcribed. The promoter is recognized by RNA polymerase, which then initiates transcription. Thus, a promoter contains a DNA sequence that is either bound directly by, or is involved in the recruitment, of RNA polymerase. A promoter sequence can also include "enhancer regions," which are one or more regions of DNA that can be bound with proteins (namely, the trans-acting factors, much like a set of transcription factors) to enhance transcription levels of genes (hence the name) in a gene-cluster. The enhancer, while typically at the 5' end of a coding region, can also be separate from a promoter

sequence and can be, e.g., within an intronic region of a gene or 3' to the coding region of the gene.

As used herein, "operably linked" means incorporated into a genetic construct (e.g., vector) so that expression control sequences effectively control expression of a coding sequence of interest.

Expression vectors can be introduced into host cells (e.g., by transformation or transfection) for expression of the encoded polypeptide, which then can be purified. Expression systems that can be used for small or large scale production of mannosidase polypeptides include, without limitation, microorganisms such as bacteria (e.g., *E. coli*) transformed with recombinant bacteriophage DNA, plasmid DNA, or cosmid DNA expression vectors containing the nucleic acid molecules, and fungal (e.g., *S. cerevisiae*, *Yarrowia lipolytica*, *Arxula adeninivorans*, *Pichia pastoris*, *Hansenula polymorpha*, or *Aspergillus*) transformed with recombinant fungal expression vectors containing the nucleic acid molecules. Useful expression systems also include insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the nucleic acid molecules, and plant cell systems infected with recombinant virus expression vectors (e.g., tobacco mosaic virus) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing the nucleic acid molecules. Mannosidase polypeptides also can be produced using mammalian expression systems, which include cells (e.g., immortalized cell lines such as COS cells, Chinese hamster ovary cells, HeLa cells, human embryonic kidney 293 cells, and 3T3 L1 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., the metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter and the cytomegalovirus promoter).

Typically, recombinant mannosidase polypeptides are tagged with a heterologous amino acid sequence such FLAG, polyhistidine (e.g., hexahistidine), hemagluttanin (HA), glutathione-S-transferase (GST), or maltose-binding protein (MBP) to aid in purifying the protein. Other methods for purifying proteins include chromatographic techniques such as ion exchange, hydrophobic and reverse phase, size exclusion, affinity, hydrophobic charge-induction chromatography, and the like (see, e.g., Scopes, Protein

Purification: Principles and Practice, third edition, Springer-Verlag, New York (1993); Burton and Harding, *J. Chromatogr. A* 814:71-81 (1998)).

In some embodiments, the uncapping and demannosylating steps are catalyzed by two different enzymes. For example, uncapping of a mannose-1-phospho-6 mannose linkage or moiety can be performed using a mannosidase from *Cellulosimicrobium cellulans* (e.g., CcMan5). The amino acid sequence of the CcMan5 polypeptide containing signal sequence is set forth in SEQ ID NO: 7. The amino acid sequence of the CcMan5 polypeptide without signal sequence is set forth in SEQ ID NO:8. A nucleic acid sequence encoding a CcMan5 polypeptide is set forth in SEQ ID NO:6. In some embodiments, a biologically active fragment of the CcMan5 polypeptide is used. For example, a biologically active fragment can includes residues 1-774 of the amino acid sequence set forth in SEQ ID NO:8. See also WO 2011/039634. The CcMan5 mannosidase is a family 92 glycoside hydrolase.

Demannosylation of an uncapped glycoprotein can be catalyzed using a mannosidase from *Aspergillus satoi* (As) (also known as *Aspergillus phoenicis*) or a mannosidase from *Cellulosimicrobium cellulans* (e.g., CcMan4). The *Aspergillus satoi* mannosidase is a family 47 glycoside hydrolase and the CcMan4 mannosidase is a family 92 glycoside hydrolase. The amino acid sequence of the *Aspergillus satoi* mannosidase is set forth in SEQ ID NO:11 (see FIG. 22) and in GenBank Accession No. BAA08634. The amino acid sequence of the CcMan4 polypeptide is set forth in SEQ ID NO: 10. The nucleotide sequence set forth in SEQ ID NO:9 encodes the polypeptide of SEQ ID NO:10.

Demannosylation of an uncapped glycoprotein also can be catalyzed using a mannosidase from the family 38 glycoside hydrolases such as a *Canavalia ensiformis* (Jack bean) mannosidase or a *Yarrowia lipolytica* mannosidase (e.g., AMS1). For example, CcMan5 can be used to uncap a mannose-1-phospho-6 mannose moiety on a glycoprotein and the Jack bean mannosidase can be used to demannosylate the uncapped glycoprotein.

To produce demannosylated glycoproteins, or uncapped and demannosylated glycoproteins, a target molecule containing a mannose-1-phospho-6 mannose linkage or

moiety is contacted under suitable conditions with a suitable mannosidase(s) and/or a cell lysate containing a suitable recombinantly produced mannosidase(s). Suitable mannosidases are described above. The cell lysate can be from any genetically engineered cell, including a fungal cell, a plant cell, or animal cell. Non-limiting examples of animal cells include nematode, insect, plant, bird, reptile, and mammals such as a mouse, rat, rabbit, hamster, gerbil, dog, cat, goat, pig, cow, horse, whale, monkey, or human.

Upon contacting the target molecule (e.g., a glycoprotein) with the purified mannosidases and/or cell lysate, the mannose-1-phospho-6-mannose linkage or moiety can be hydrolyzed to phospho-6-mannose and the terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety of such a phosphate containing glycan can be hydrolyzed to produces an uncapped and demannosylated target molecule. In some embodiments, one mannosidase is used that catalyzes both the uncapping and demannosylating steps. In some embodiments, one mannosidase is used to catalyze the uncapping step and a different mannosidase is used to catalyze the demannosylating step. The methods described in Example 5 can be used to determine if the target molecule has been uncapped and demannosylated. Following processing by the mannosidase, the target molecule can be isolated.

Suitable methods for obtaining cell lysates that preserve the activity or integrity of the mannosidase activity in the lysate can include the use of appropriate buffers and/or inhibitors, including nuclease, protease and phosphatase inhibitors that preserve or minimize changes in N-glycosylation activities in the cell lysate. Such inhibitors include, for example, chelators such as ethylenediamine tetraacetic acid (EDTA), ethylene glycol bis(P-aminoethyl ether) N,N,N1,Nl-tetraacetic acid (EGTA), protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, antipain and the like, and phosphatase inhibitors such as phosphate, sodium fluoride, vanadate and the like. Appropriate buffers and conditions for obtaining lysates containing enzymatic activities are described in, e.g., Ausubel et al. Current Protocols in Molecular Biology (Supplement 47), John Wiley & Sons, New York (1999); Harlow and Lane, Antibodies: A Laboratory Manual Cold Spring Harbor Laboratory Press (1988); Harlow and Lane, Using

Antibodies: A Laboratory Manual, Cold Spring Harbor Press (1999); Tietz Textbook of Clinical Chemistry, 3rd ed. Burtis and Ashwood, eds. W.B. Saunders, Philadelphia, (1999).

A cell lysate can be further processed to eliminate or minimize the presence of interfering substances, as appropriate. If desired, a cell lysate can be fractionated by a variety of methods well known to those skilled in the art, including subcellular fractionation, and chromatographic techniques such as ion exchange, hydrophobic and reverse phase, size exclusion, affinity, hydrophobic charge-induction chromatography, and the like.

In some embodiments, a cell lysate can be prepared in which whole cellular organelles remain intact and/or functional. For example, a lysate can contain one or more of intact rough endoplasmic reticulum, intact smooth endoplasmic reticulum, or intact Golgi apparatus. Suitable methods for preparing lysates containing intact cellular organelles and testing for the functionality of the organelles are described in, e.g., Moreau *et al.* (1991) *J. Biol. Chem.* 266(7):4329-4333; Moreau *et al.* (1991) *J. Biol. Chem.* 266(7):4322-4328; Rexach *et al.* (1991) *J. Cell Biol.* 114(2):219-229; and Paulik *et al.* (1999) *Arch. Biochem. Biophys.* 367(2):265-273.

Target molecules, as used herein, refer to (i) any molecule containing terminal mannose-1-phospho-6 mannose linkage or moiety; (ii) any molecule, when expressed in a cell of fungal origin, that contains a mannose-1-phospho-6 mannose linkage or moiety; (iii) any molecule containing a terminal alpha-1,2 mannose, alpha-1,3 mannose, and/or alpha-1,6 mannose linkage or moiety of a phosphate containing glycan; or (iv) any molecule, when expressed in a cell of fungal origin, that contains a terminal alpha-1,2 mannose, alpha-1,3 mannose, and/or alpha-1,6 mannose linkage or moiety of a phosphate containing glycan. In some embodiments, the target protein is a human glycoprotein. Suitable target proteins can include pathogen proteins such as tetanus toxoid or diptheria toxoid; viral surface proteins such as cytomegalovirus (CMV) glycoproteins B, H and gCIII, human immunodeficiency virus 1 (HIV-1) envelope glycoproteins, Rous sarcoma virus (RSV) envelope glycoproteins, herpes simplex virus (HSV) envelope glycoproteins, Epstein Barr virus (EBV) envelope glycoproteins, varicella-zoster virus (VZV) envelope

glycoproteins, human papilloma virus (HPV) envelope glycoproteins, Influenza virus glycoproteins, and Hepatitis family surface antigen; lysosomal proteins (e.g., acid alpha glucosidase, alpha galatosidase, glucocerebrosidase, cerebrosidase, or galactocerebrosidase); insulin; glucagons; growth factors; cytokines; chemokines; and antibodies or fragments thereof. Growth factors include, e.g., vascular endothelial growth factor (VEGF), Insulin-like growth factor (IGF), bone morphogenic protein (BMP), Granulocyte-colony stimulating factor (G-CSF), Granulocyte-macrophage colony stimulating factor (GM-CSF), Nerve growth factor (NGF); a Neurotrophin, Platelet-derived growth factor (PDGF), Erythropoietin (EPO), Thrombopoietin (TPO), Myostatin (GDF-8), Growth Differentiation factor-9 (GDF9), basic fibroblast growth factor (bFGF or FGF2), Epidermal growth factor (EGF), Hepatocyte growth factor (HGF). Cytokines include, for example, interleukins such as IL-1 to IL-33 (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, or IL-15). Chemokines include, e.g., I-309, TCA-3, MCP-1, MIP-1α, MIP-1β, RANTES, C10, MRP-2, MARC, MCP-3, MCP-2, MRP-2, CCF18, MIP-1γ, Eotaxin, MCP-5, MCP-4, NCC-1, Ckβ10, HCC-1, Leukotactin-1, LEC, NCC-4, TARC, PARC, or Eotaxin-2. Also included are tumor glycoproteins (e.g., tumor-associated antigens), for example, carcinoembryonic antigen (CEA), human mucins, HER-2/neu, and prostate-specific antigen (PSA) [Henderson and Finn, *Advances in Immunology*, 62, pp. 217-56 (1996)].

In some embodiments, the target protein can be one associated with a lysosomal storage disorder, which target proteins include, e.g., acid alpha glucosidase, alpha galactosidase, alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, arylsulfatase B, arylsulfatase A, alpha-N-acetylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminide-N-acetyltransferase, beta-D-glucoronidase, hyaluronidase, alpha-L-mannosidase, alpha-neuraminidase, phosphotransferase, acid lipase, acid ceramidase, sphingomyelinase, thioesterase, cathepsin K, and lipoprotein lipase.

In some embodiments, the target proteins are fusion proteins in which the target protein is fused to another polypeptide sequence, or to a polymer, a carrier, an adjuvant, an immunotoxin, or a detectable (e.g., fluorescent, luminescent, or radioactive) moiety.

For example, a target protein can be joined to a polymer such as polyethyleneglycol to increase the molecular weight of small proteins and/or increase circulation residence time.

Upon contact of a mammalian cell with a target molecule containing uncapped and demannosylated phosphorylated N-glycans, the target molecule can be transported to the interior of the mammalian cell (e.g., a human cell). A glycoprotein having an uncapped, but not demannosylated, phosphorylated N-glycan does not substantially bind mannose-6-phosphate receptors on mammalian cells, and as such, is not efficiently transported to the interior of the cell. As used herein, "does not substantially bind" means that less than 15% (e.g., less than 14%, 12%, 10%, 8%, 6%, 4%, 2%, 1%, 0.5%, or less, or 0%) of the glycoprotein molecules bind to mannose-6-phosphate receptors on mammalian cells. However, if such a glycoprotein is contacted with a mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated, a demannosylated glycoprotein is produced that substantially binds to the mannose-6-phosphate receptor on the mammalian cells and is efficiently transported to the interior of the cell. As used herein "substantially binds" means that 15% or more (e.g., greater than 16%, 18%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%) of the glycoprotein molecules bind to mannose-6-phosphate receptors on mammalian cells. It is understood that a preparation (e.g., a recombinant host cell or a cell-free preparation) containing an enzyme that uncaps but does not demannosylate phosphorylated N-glycans could be contaminated with an enzyme that demannosylates phosphorylated N-glycans. A target protein sample after contact with such a preparation can contain protein molecules with some phosphorylated N-glycans that are uncapped only and others that are uncapped and demannosylated. Naturally those protein molecules containing uncapped and demannosylated phosphorylated N-glycans can substantantially bind to mannose-6-phosphate receptors. The above definition of "does not substantially bind" does not apply to such a target protein sample since the phosphorylated N-glycans on the protein molecules cannot be characterized as uncapped but not demannosylated.

As set forth in Examples 9 and 12, target molecules that are uncapped and demannosylated are more efficiently taken up by mammalian cells than target molecules containing uncapped phosphorylated N-glycans. For example, an uncapped and demannosylated target molecule can be taken up at least 10 times (e.g., at least 15, 20, 25, or 30 times) more efficient than an uncapped glycoprotein.

Thus, this document provides methods of converting a glycoprotein from a first form that does not bind to a mannose-6-phosphate receptor on a mammalian cell to a second form that does bind to a mannose-6-phosphate receptor on a mammalian cell. In the first form, the glycoprotein comprises one or more N-glycans containing one or more mannose residues that are linked at the 1 position to a mannose residue that contains a phosphate residue at the 6 position. In such methods, the first form of the glycoprotein is contacted with a mannosidase that demannosylates the terminal mannose residues to result in the mannose containing the phosphate at the 6 position to become the terminal mannose. In some embodiments, the mannosidase has both uncapping and demannosylating activity (e.g., *Canavalia ensiformis* (Jack bean) or *Yarrowia lipolytica* AMS1 mannosidase). In some embodiments, the mannosidase does not have uncapping activity (e.g., a mannosidase from *Aspergillus satoi* or a mannosidase from *Cellulosimicrobium cellulans* (e.g., CcMan4)).

Transport of a glycoprotein to the interior of the cell can be assessed using a cell uptake assay such as the one set forth in Example 9. For example, mammalian cells and a target molecule containing uncapped and demannosylated phosphorylated N-glycans can be incubated, then the cells washed and lysed. Cell lysates can be assessed for the presence of the target molecule (e.g., by Western blotting) or by activity of the target molecule in the cell lysate. For example, when the target molecule is a glucosidase such as human alpha glucosidase, uptake can be assessed in fibroblasts deficient in acid alpha glucosidase activity. Intracellular activity of alpha glucosidase can be assessed using the 4-methylumbelliferyl-alpha-D-glucopyranoside (4-MUG) assay. See, Example 3. Cleavage of the substrate 4-MUG by a glucosidase leads to the generation of the fluorigenic product 4-MU, which can be visualized or detected by irradiation with UV light.

In Vivo Methods of Uncapping and Demannosylating Glycoproteins

Genetically engineered cells described herein can be used to produce uncapped and demannosylated target molecules. For example, a cell based method can include the steps of introducing into a fungal cell genetically engineered to include a nucleic acid encoding a mannosidase that is capable of hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to phospho-6-mannose, a nucleic acid encoding a target molecule, wherein the cell produces the target molecule containing uncapped phosphorylated N-glycans. Such phosphorylated N-glycans can be demannosylated as described above. Another cell based method can include the steps of introducing into a fungal cell genetically engineered to include a nucleic acid encoding a mannosidase that is capable of (i) hydrolyzing a mannose-1-phospho-6-mannose linkage or moiety to phospho-6-mannose and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage or moiety of a phosphate containing glycan, a nucleic acid encoding a target molecule, wherein the cell produces uncapped and demannosylated target molecules. In some embodiments, the nucleic acids encoding the mannosidase and target molecule contain a secretion sequence such that the mannosidase and target molecule are co-secreted.

Genetically engineered cells described herein contain a nucleic acid encoding a mannosidase. Cells suitable for *in vivo* production of target molecules can be of fungal origin, including *Yarrowia lipolytica*, *Arxula adeninivorans*, methylotrophic yeast (such as a methylotrophic yeast of the genus *Candida*, *Hansenula*, *Oogataea*, *Pichia* or *Torulopsis*) or filamentous fungi of the genus *Aspergillus*, *Trichoderma*, *Neurospora*, *Fusarium*, or *Chrysosporium*. Exemplary fungal species include, without limitation, *Pichia anomala, Pichia bovis, Pichia canadensis, Pichia carsonii, Pichia farinose, Pichia fermentans, Pichia fluxuum, Pichia membranaefaciens, Pichia membranaefaciens, Candida valida, Candida albicans, Candida ascalaphidarum, Candida amphixiae, Candida Antarctica, Candida atlantica, Candida atmosphaerica, Candida blattae, Candida carpophila, Candida cerambycidarum, Candida chauliodes, Candida corydalis, Candida dosseyi, Candida dubliniensis, Candida ergatensis, Candida fructus, Candida*

glabrata, Candida fermentati, Candida guilliermondii, Candida haemulonii, Candida insectamens, Candida insectorum, Candida intermedia, Candida jeffresii, Candida kefyr, Candida krusei, Candida lusitaniae, Candida lyxosophila, Candida maltosa, Candida membranifaciens, Candida milleri, Candida oleophila, Candida oregonensis, Candida parapsilosis, Candida quercitrusa, Candida shehatea, Candida temnochilae, Candida tenuis, Candida tropicalis, Candida tsuchiyae, Candida sinolaborantium, Candida sojae, Candida viswanathii, Candida utilis, Oogataea minuta, Pichia membranaefaciens, Pichia silvestris, Pichia membranaefaciens, Pichia chodati, Pichia membranaefaciens, Pichia menbranaefaciens, Pichia minuscule, Pichia pastoris, Pichia pseudopolymorpha, Pichia quercuum, Pichia robertsii, Pichia saitoi, Pichia silvestrisi, Pichia strasburgensis, Pichia terricola, Pichia vanriji, Pseudozyma Antarctica, Rhodosporidium toruloides, Rhodotorula glutinis, Saccharomyces bayanus, Saccharomyces bayanus, Saccharomyces momdshuricus, Saccharomyces uvarum, Saccharomyces bayanus, Saccharomyces cerevisiae, Saccharomyces bisporus, Saccharomyces chevalieri, Saccharomyces delbrueckii, Saccharomyces exiguous, Saccharomyces fermentati, Saccharomyces fragilis, Saccharomyces marxianus, Saccharomyces mellis, Saccharomyces rosei, Saccharomyces rouxii, Saccharomyces uvarum, Saccharomyces willianus, Saccharomycodes ludwigii, Saccharomycopsis capsularis, Saccharomycopsis fibuligera, Saccharomycopsis fibuligera, Endomyces hordei, Endomycopsis fobuligera. Saturnispora saitoi, Schizosaccharomyces octosporus, Schizosaccharomyces pombe, Schwanniomyces occidentalis, Torulaspora delbrueckii, Torulaspora delbrueckii, Saccharomyces dairensis, Torulaspora delbrueckii, Torulaspora fermentati, Saccharomyces fermentati, Torulaspora delbrueckii, Torulaspora rosei, Saccharomyces rosei,Torulaspora delbrueckii, Saccharomyces rosei, Torulaspora delbrueckii, Saccharomyces delbrueckii, Torulaspora delbrueckii, Saccharomyces delbrueckii, Zygosaccharomyces mongolicus, Dorulaspora globosa, Debaryomyces globosus, Torulopsis globosa, Trichosporon cutaneum, Trigonopsis variabilis, Williopsis californica, Williopsis saturnus, Zygosaccharomyces bisporus, Zygosaccharomyces bisporus, Debaryomyces disporua. Saccharomyces bisporas, Zygosaccharomyces bisporus, Saccharomyces bisporus, Zygosaccharomyces mellis, Zygosaccharomyces priorianus, Zygosaccharomyces rouxiim, Zygosaccharomyces

rouxii, Zygosaccharomyces barkeri, Saccharomyces rouxii, Zygosaccharomyces rouxii, Zygosaccharomyces major, Saccharomyces rousii, Pichia anomala, Pichia bovis, Pichia Canadensis, Pichia carsonii, Pichia farinose, Pichia fermentans, Pichia fluxuum, Pichia membranaefaciens, Pichia pseudopolymorpha, Pichia quercuum, Pichia robertsii, Pseudozyma Antarctica, Rhodosporidium toruloides, Rhodosporidium toruloides, Rhodotorula glutinis, Saccharomyces bayanus, Saccharomyces bayanus, Saccharomyces bisporus, Saccharomyces cerevisiae, Saccharomyces chevalieri, Saccharomyces delbrueckii, Saccharomyces fermentati, Saccharomyces fragilis, Saccharomycodes ludwigii, Schizosaccharomyces pombe, Schwanniomyces occidentalis, Torulaspora delbrueckii, Torulaspora globosa, Trigonopsis variabilis, Williopsis californica, Williopsis saturnus, Zygosaccharomyces bisporus, Zygosaccharomyces mellis, or *Zygosaccharomyces rouxii*. Exemplary filamentous fungi include various species of *Aspergillus* including, but not limited to, *Aspergillus caesiellus, Aspergillus candidus, Aspergillus carneus, Aspergillus clavatus, Aspergillus deflectus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus niger, Aspergillus ochraceus, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus penicilloides, Aspergillus restrictus, Aspergillus sojae, Aspergillus sydowi, Aspergillus tamari, Aspergillus terreus, Aspergillus ustus,* or *Aspergillus versicolor*. Such cells, prior to the genetic engineering as specified herein, can be obtained from a variety of commercial sources and research resource facilities, such as, for example, the American Type Culture Collection (Rockville, MD). Target molecules include proteins such as any of the target proteins described herein (see above).

Genetic engineering of a cell can include, in addition to an exogenous nucleic acid encoding a mannosidase, one or more genetic modifications such as: (i) deletion of an endogenous gene encoding an Outer CHain elongation (OCH1) protein; (ii) introduction of a recombinant nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation (e.g, a MNN4 polypeptide from *Yarrowia lipolytica*, *S. cerevisiae*, *Ogataea minuta*, *Pichia pastoris*, or *C. albicans*, or PNO1 polypeptide from *P. pastoris*) to increasing phosphorylation of mannose residues; (iii) introduction or expression of an RNA molecule that interferes with the functional expression of an OCH1 protein; (iv)

introduction of a recombinant nucleic acid encoding a wild-type (e.g., endogenous or exogenous) protein having a N-glycosylation activity (i.e., expressing a protein having an N-glycosylation activity); (v) introduction of a recombinant nucleic acid encoding a target molecule described above; or (v) altering the promoter or enhancer elements of one or more endogenous genes encoding proteins having N-glycosylation activity to thus alter the expression of their encoded proteins. RNA molecules include, e.g., small-interfering RNA (siRNA), short hairpin RNA (shRNA), anti-sense RNA, or micro RNA (miRNA). Genetic engineering also includes altering an endogenous gene encoding a protein having an N-glycosylation activity to produce a protein having additions (e.g., a heterologous sequence), deletions, or substitutions (e.g., mutations such as point mutations; conservative or non-conservative mutations). Mutations can be introduced specifically (e.g., by site-directed mutagenesis or homologous recombination) or can be introduced randomly (for example, cells can be chemically mutagenized as described in, e.g., Newman and Ferro-Novick (1987) *J. Cell Biol.* 105(4):1587.

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

To disrupt a gene by homologous recombination, a "gene replacement" vector can be constructed in such a way to include a selectable marker gene. The selectable marker

gene can be operably linked, at both 5' and 3' end, to portions of the gene of sufficient length to mediate homologous recombination. The selectable marker can be one of any number of genes which either complement host cell auxotrophy or provide antibiotic resistance, including URA3, LEU2 and HIS3 genes. Other suitable selectable markers include the CAT gene, which confers chloramphenicol resistance to yeast cells, or the lacZ gene, which results in blue colonies due to the expression of β-galactosidase. Linearized DNA fragments of the gene replacement vector are then introduced into the cells using methods well known in the art (see below). Integration of the linear fragments into the genome and the disruption of the gene can be determined based on the selection marker and can be verified by, for example, Southern blot analysis. A selectable marker can be removed from the genome of the host cell by, e.g., Cre-loxP systems (see below).

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

Expression vectors can be autonomous or integrative. A recombinant nucleic acid (e.g., one encoding a mannosidase) can be in introduced into the cell in the form of an expression vector such as a plasmid, phage, transposon, cosmid or virus particle. The recombinant nucleic acid can be maintained extrachromosomally or it can be integrated into the yeast cell chromosomal DNA. Expression vectors can contain selection marker

genes encoding proteins required for cell viability under selected conditions (e.g., URA3, which encodes an enzyme necessary for uracil biosynthesis or TRP1, which encodes an enzyme required for tryptophan biosynthesis) to permit detection and/or selection of those cells transformed with the desired nucleic acids (see, e.g., U.S. Pat. No. 4,704,362). Expression vectors can also include an autonomous replication sequence (ARS). For example, U.S. Pat. No. 4,837,148 describes autonomous replication sequences which provide a suitable means for maintaining plasmids in *Pichia pastoris*.

Integrative vectors are disclosed, e.g., in U.S. Pat. No. 4,882,279. Integrative vectors generally include a serially arranged sequence of at least a first insertable DNA fragment, a selectable marker gene, and a second insertable DNA fragment. The first and second insertable DNA fragments are each about 200 (e.g., about 250, about 300, about 350, about 400, about 450, about 500, or about 1000 or more) nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of the species to be transformed. A nucleotide sequence containing a gene of interest (e.g., a gene encoding a protein having N-glycosylation activity) for expression is inserted in this vector between the first and second insertable DNA fragments whether before or after the marker gene. Integrative vectors can be linearized prior to yeast transformation to facilitate the integration of the nucleotide sequence of interest into the host cell genome.

An expression vector can feature a recombinant nucleic acid under the control of a yeast (e.g., *Yarrowia lipolytica, Arxula adeninivorans, P. pastoris,* or other suitable fungal species) promoter, which enables them to be expressed in fungal cells. Suitable yeast promoters include, e.g., ADC1, TPI1, ADH2, hp4d, POX, and Gal10 (see, e.g., Guarente *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79(23):7410) promoters. Additional suitable promoters are described in, e.g., Zhu and Zhang (1999) *Bioinformatics* 15(7-8):608-611 and U.S. Patent No. 6,265,185.

A promoter can be constitutive or inducible (conditional). A constitutive promoter is understood to be a promoter whose expression is constant under the standard culturing conditions. Inducible promoters are promoters that are responsive to one or more induction cues. For example, an inducible promoter can be chemically regulated (e.g., a promoter whose transcriptional activity is regulated by the presence or absence of a

chemical inducing agent such as an alcohol, tetracycline, a steroid, a metal, or other small molecule) or physically regulated (e.g., a promoter whose transcriptional activity is regulated by the presence or absence of a physical inducer such as light or high or low temperatures). An inducible promoter can also be indirectly regulated by one or more transcription factors that are themselves directly regulated by chemical or physical cues.

It is understood that other genetically engineered modifications can also be conditional. For example, a gene can be conditionally deleted using, e.g., a site-specific DNA recombinase such as the Cre-loxP system (see, e.g., Gossen *et al.* (2002) *Ann. Rev. Genetics* 36:153-173 and U.S. Application Publication No. 20060014264).

A recombinant nucleic acid can be introduced into a cell described herein using a variety of methods such as the spheroplast technique or the whole-cell lithium chloride yeast transformation method. Other methods useful for transformation of plasmids or linear nucleic acid vectors into cells are described in, for example, U.S. Patent No. 4,929,555; Hinnen *et al.* (1978) *Proc. Nat. Acad. Sci. USA* 75:1929; Ito *et al.* (1983) *J. Bacteriol.* 153:163; U.S. Patent No. 4,879,231; and Sreekrishna *et al.* (1987) *Gene* 59:115, the disclosures of each of which are incorporated herein by reference in their entirety. Electroporation and PEG1000 whole cell transformation procedures may also be used, as described by Cregg and Russel, Methods in Molecular Biology: Pichia Protocols, Chapter 3, Humana Press, Totowa, N.J., pp. 27-39 (1998).

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

Prior to introducing the vectors into a target cell of interest, the vectors can be grown (e.g., amplified) in bacterial cells such as *Escherichia coli* (*E. coli*) as described above. The vector DNA can be isolated from bacterial cells by any of the methods known in the art which result in the purification of vector DNA from the bacterial milieu.

The purified vector DNA can be extracted extensively with phenol, chloroform, and ether, to ensure that no *E. coli* proteins are present in the plasmid DNA preparation, since these proteins can be toxic to mammalian cells.

In some embodiments, the genetically engineered fungal cell lacks the OCH1 gene or gene products (e.g., mRNA or protein) thereof, and is deficient in OCH1 activity. In some embodiments, the genetically engineered cell expresses a polypeptide capable of promoting mannosyl phosphorylation (e.g., a MNN4 polypeptide from *Yarrowia lipolytica*, *S. cerevisiae*, *Ogataea minuta*, *Pichia pastoris*, or *C. albicans*, or a PNO1 polypeptide from *P. pastoris*). For example, the fungal cell can express a MNN4 polypeptide from *Y. lipolytica* (Genbank® Acccession Nos: XM_503217, Genolevures Ref: YALI0D24101g). In some embodiments, the genetically engineered cell is deficient in OCH1 activity and expresses a polypeptide capable of promoting mannosyl phosphorylation.

Following uncapping and demannosylation, the target molecule can be isolated. In some embodiments, the target molecule is maintained within the yeast cell and released upon cell lysis. In some embodiments, the target molecule is secreted into the culture medium via a mechanism provided by a coding sequence (either native to the exogenous nucleic acid or engineered into the expression vector), which directs secretion of the molecule from the cell. The presence of the uncapped and demannosylated target molecule in the cell lysate or culture medium can be verified by a variety of standard protocols for detecting the presence of the molecule. For example, where the altered target molecule is a protein, such protocols can include, but are not limited to, immunoblotting or radioimmunoprecipitation with an antibody specific for the altered target protein (or the target protein itself), binding of a ligand specific for the altered target protein (or the target protein itself), or testing for a specific enzyme activity of the altered target protein (or the target protein itself).

In some embodiments, following isolation, the uncapped and demannosylated target molecule can be attached to a heterologous moiety, e.g., using enzymatic or chemical means. A "heterologous moiety" refers to any constituent that is joined (e.g., covalently or non-covalently) to the altered target molecule, which constituent is different

from a constituent originally present on the altered target molecule. Heterologous moieties include, e.g., polymers, carriers, adjuvants, immunotoxins, or detectable (e.g., fluorescent, luminescent, or radioactive) moieties. In some embodiments, an additional N-glycan can be added to the altered target molecule.

Methods for detecting glycosylation of a target molecule include DNA sequencer-assisted (DSA), fluorophore-assisted carbohydrate electrophoresis (FACE) or surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). For example, an analysis can utilize DSA-FACE in which, for example, glycoproteins are denatured followed by immobilization on, e.g., a membrane. The glycoproteins can then be reduced with a suitable reducing agent such as dithiothreitol (DTT) or β-mercaptoethanol. The sulfhydryl groups of the proteins can be carboxylated using an acid such as iodoacetic acid. Next, the N-glycans can be released from the protein using an enzyme such as N-glycosidase F. N-glycans, optionally, can be reconstituted and derivatized by reductive amination. The derivatized N-glycans can then be concentrated. Instrumentation suitable for N-glycan analysis includes, e.g., the ABI PRISM® 377 DNA sequencer (Applied Biosystems). Data analysis can be performed using, e.g., GENESCAN® 3.1 software (Applied Biosystems). Isolated mannoproteins can be further treated with one or more enzymes such as calf intestine phosphatase to confirm their N-glycan status. Additional methods of N-glycan analysis include, e.g., mass spectrometry (e.g., MALDI-TOF-MS), high-pressure liquid chromatography (HPLC) on normal phase, reversed phase and ion exchange chromatography (e.g., with pulsed amperometric detection when glycans are not labeled and with UV absorbance or fluorescence if glycans are appropriately labeled). See also Callewaert *et al.* (2001) *Glycobiology* 11(4):275-281 and Freire *et al.* (2006) *Bioconjug. Chem.* 17(2):559-564.

Cultures of Engineered Cells

This document also provides a substantially pure culture of any of the genetically engineered cells described herein. As used herein, a "substantially pure culture" of a genetically engineered cell is a culture of that cell in which less than about 40% (i.e., less than about : 35%; 30%; 25%; 20%; 15%; 10%; 5%; 2%; 1%; 0.5%; 0.25%; 0.1%; 0.01%;

0.001%; 0.0001%; or even less) of the total number of viable cells in the culture are viable cells other than the genetically engineered cell, e.g., bacterial, fungal (including yeast), mycoplasmal, or protozoan cells. The term "about" in this context means that the relevant percentage can be 15% percent of the specified percentage above or below the specified percentage. Thus, for example, about 20% can be 17% to 23%. Such a culture of genetically engineered cells includes the cells and a growth, storage, or transport medium. Media can be liquid, semi-solid (e.g., gelatinous media), or frozen. The culture includes the cells growing in the liquid or in/on the semi-solid medium or being stored or transported in a storage or transport medium, including a frozen storage or transport medium. The cultures are in a culture vessel or storage vessel or substrate (e.g., a culture dish, flask, or tube or a storage vial or tube).

The genetically engineered cells described herein can be stored, for example, as frozen cell suspensions, e.g., in buffer containing a cryoprotectant such as glycerol or sucrose, as lyophilized cells. Alternatively, they can be stored, for example, as dried cell preparations obtained, e.g., by fluidized bed drying or spray drying, or any other suitable drying method.

Metabolic Disorders

Uncapped and demannosylated molecules can be used to treat a variety of metabolic disorders. A metabolic disorder is one that affects the production of energy within individual human (or animal) cells. Most metabolic disorders are genetic, though some can be "acquired" as a result of diet, toxins, infections, etc. Genetic metabolic disorders are also known as inborn errors of metabolism. In general, the genetic metabolic disorders are caused by genetic defects that result in missing or improperly constructed enzymes necessary for some step in the metabolic process of the cell. The largest classes of metabolic disorders are disorders of carbohydrate metabolism, disorders of amino acid metabolism, disorders of organic acid metabolism (organic acidurias), disorders of fatty acid oxidation and mitochondrial metabolism, disorders of porphyrin metabolism, disorders of purine or pyrimidine metabolism, disorders of steroid

metabolism disorders of mitochondrial function, disorders of peroxisomal function, and lysosomal storage disorders (LSDs).

Examples of metabolic disorders that can be treated through the administration of one or more uncapped and demannosylated molecules (or pharmaceutical compositions of the same) can include hereditary hemochromatosis, oculocutaneous albinism, protein C deficiency, type I hereditary angioedema, congenital sucrase-isomaltase deficiency, Crigler-Najjar type II, Laron syndrome, hereditary Myeloperoxidase, primary hypothyroidism, congenital long QT syndrome, tyroxine binding globulin deficiency, familial hypercholesterolemia, familial chylomicronemia, abeta-lipoproteinema, low plasma lipoprotein A levels, hereditary emphysema with liver injury, congenital hypothyroidism, osteogenesis imperfecta, hereditary hypofibrinogenemia, alpha-1antichymotrypsin deficiency, nephrogenic diabetes insipidus, neurohypophyseal diabetes insipidus, adenosine deaminase deficiency, Pelizaeus Merzbacher disease, von Willebrand disease type IIA, combined factors V and VIII deficiency, spondylo-epiphyseal dysplasia tarda, choroideremia, I cell disease, Batten disease, ataxia telangiectasias, ADPKD-autosomal dominant polycystic kidney disease, microvillus inclusion disease, tuberous sclerosis, oculocerebro-renal syndrome of Lowe, amyotrophic lateral sclerosis, myelodysplastic syndrome, Bare lymphocyte syndrome, Tangier disease, familial intrahepatic cholestasis, X-linked adreno-leukodystrophy, Scott syndrome, Hermansky-Pudlak syndrome types 1 and 2, Zellweger syndrome, rhizomelic chondrodysplasia puncta, autosomal recessive primary hyperoxaluria, Mohr Tranebjaerg syndrome, spinal and bullar muscular atrophy, primary ciliary diskenesia (Kartagener's syndrome), giantism and acromegaly, galactorrhea, Addison's disease, adrenal virilism, Cushing's syndrome, ketoacidosis, primary or secondary aldosteronism, Miller Dieker syndrome, lissencephaly, motor neuron disease, Usher's syndrome, Wiskott-Aldrich syndrome, Optiz syndrome, Huntington's disease, hereditary pancreatitis, anti-phospholipid syndrome, overlap connective tissue disease, Sjögren's syndrome, stiff-man syndrome, Brugada syndrome, congenital nephritic syndrome of the Finnish type, Dubin-Johnson syndrome, X-linked hypophosphosphatemia, Pendred syndrome, persistent hyperinsulinemic hypoglycemia of infancy, hereditary spherocytosis,

aceruloplasminemia, infantile neuronal ceroid lipofuscinosis, pseudoachondroplasia and multiple epiphyseal, Stargardt-like macular dystrophy, X-linked Charcot-Marie-Tooth disease, autosomal dominant retinitis pigmentosa, Wolcott-Rallison syndrome, Cushing's disease, limb-girdle muscular dystrophy, mucoploy-saccharidosis type IV, hereditary familial amyloidosis of Finish, Anderson disease, sarcoma, chronic myelomonocytic leukemia, cardiomyopathy, faciogenital dysplasia, Torsion disease, Huntington and spinocerebellar ataxias, hereditary hyperhomosyteinemia, polyneuropathy, lower motor neuron disease, pigmented retinitis, seronegative polyarthritis, interstitial pulmonary fibrosis, Raynaud's phenomenon, Wegner's granulomatosis, preoteinuria, CDG-Ia, CDG-Ib, CDG-Ic, CDG-Id, CDG-Ie, CDG-If, CDG-IIa, CDG-IIb, CDG-IIc, CDG-IId, Ehlers-Danlos syndrome, multiple exostoses, Griscelli syndrome (type 1 or type 2), or X-linked non-specific mental retardation. In addition, metabolic disorders can also include lysosomal storage disorders such as, but not limited to, Fabry disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM_1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM_2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease (types A, B, and C), Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis (types II ,III, and IV), cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

Symptoms of a metabolic disorder are numerous and diverse and can include one or more of, e.g., anemia, fatigue, bruising easily, low blood platelets, liver enlargement, spleen enlargement, skeletal weakening, lung impairment, infections (e.g., chest infections or pneumonias), kidney impairment, progressive brain damage, seizures, extra thick meconium, coughing, wheezing, excess saliva or mucous production, shortness of breath, abdominal pain, occluded bowel or gut, fertility problems, polyps in the nose, clubbing of the finger/toe nails and skin, pain in the hands or feet, angiokeratoma,

decreased perspiration, corneal and lenticular opacities, cataracts, mitral valve prolapse and/or regurgitation, cardiomegaly, temperature intolerance, difficulty walking, difficulty swallowing, progressive vision loss, progressive hearing loss, hypotonia, macroglossia, areflexia, lower back pain, sleep apnea, orthopnea, somnolence, lordosis, or scoliosis. It is understood that due to the diverse nature of the defective or absent proteins and the resulting disease phenotypes (e.g., symptomatic presentation of a metabolic disorder), a given disorder will generally present only symptoms characteristic to that particular disorder. For example, a patient with Fabry disease can present a particular subset of the above-mentioned symptoms such as, but not limited to, temperature intolerance, corneal whirling, pain, skin rashes, nausea, or dirarrhea. A patient with Gaucher syndrome can present with splenomegaly, cirrhosis, convulsions, hypertonia, apnea, osteoporosis, or skin discoloration.

In addition to the administration of one or more uncapped and demannosylated molecules described herein, a metabolic disorder can also be treated by proper nutrition and vitamins (e.g., cofactor therapy), physical therapy, and pain medications.

Depending on the specific nature of a given metabolic disorder, a patient can present these symptoms at any age. In many cases, symptoms can present in childhood or in early adulthood. For example, symptoms of Fabry disease can present at an early age, e.g., at 10 or 11 years of age.

As used herein, a subject "at risk of developing a metabolic disorder" is a subject that has a predisposition to develop a disorder, i.e., a genetic predisposition to develop metabolic disorder as a result of a mutation in a enzyme such as acid alpha glucosidase, alpha galactosidase, alpha-L-iduronidase, beta-D-galactosidase, beta-glucosidase, beta-hexosaminidase, beta-D-mannosidase, alpha-L-fucosidase, arylsulfatase B, arylsulfatase A, alpha-N-acteylgalactosaminidase, aspartylglucosaminidase, iduronate-2-sulfatase, alpha-glucosaminide-N-acetyltransferase, beta-D-glucoronidase, hyaluronidase, alpha-L-mannosidase, alpha-neurominidase, phosphotransferase, acid lipase, acid ceramidase, sphinogmyelinase, thioesterase, cathepsin K, or lipoprotein lipase. Clearly, subjects "at risk of developing a metabolic disorder" are not all the subjects within a species of interest.

A subject "suspected of having a disorder" is one having one or more symptoms of a metabolic disorder such as any of those described herein.

Pharmaceutical Compositions and Methods of Treatment

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

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

Administration can be by periodic injections of a bolus of the pharmaceutical composition or can be uninterrupted or continuous by intravenous or intraperitoneal administration from a reservoir which is external (e.g., an IV bag) or internal (e.g., a bioerodable implant, a bioartificial organ, or a colony of implanted altered N-glycosylation molecule production cells). See, e.g., U.S. Pat. Nos. 4,407,957, 5,798,113, and 5,800,828. Administration of a pharmaceutical composition can be achieved using suitable delivery means such as: a pump (see, e.g., Annals of Pharmacotherapy, 27:912

(1993); Cancer, 41:1270 (1993); Cancer Research, 44:1698 (1984); microencapsulation (see, e.g., U.S. Pat. Nos. 4,352,883; 4,353,888; and 5,084,350); continuous release polymer implants (see, e.g., Sabel, U.S. Pat. No. 4,883,666); macroencapsulation (see, e.g., U.S. Pat. Nos. 5,284,761, 5,158,881, 4,976,859 and 4,968,733 and published PCT patent applications WO92/19195, WO 95/05452); injection, either subcutaneously, intravenously, intra-arterially, intramuscularly, or to other suitable site; or oral administration, in capsule, liquid, tablet, pill, or prolonged release formulation.

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

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

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

An uncapped and demannosylated molecule suitable for topical administration can be administered to a mammal (e.g., a human patient) as, e.g., a cream, a spray, a foam, a gel, an ointment, a salve, or a dry rub. A dry rub can be rehydrated at the site of administration. Such molecules can also be infused directly into (e.g., soaked into and dried) a bandage, gauze, or patch, which can then be applied topically. Such molecules can also be maintained in a semi-liquid, gelled, or fully-liquid state in a bandage, gauze, or patch for topical administration (see, e.g., U.S. Patent No. 4,307,717).

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

dose. In another example, the dosage is from 1 μg/kg to 100 μg/kg body weight of the subject, per dose. In another example, the dosage is from 1 μg/kg to 30 μg/kg body weight of the subject, per dose, e.g., from 3 μg/kg to 10 μg/kg body weight of the subject, per dose.

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

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

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

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in appropriate subjects (e.g., human patients). The

dosage of such pharmaceutical compositions lies generally within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For a pharmaceutical composition used as described herein (e.g., for treating a metabolic disorder in a subject), the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (i.e., the concentration of the pharmaceutical composition which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

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

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

A molecule or pharmaceutical composition thereof described herein can be administered to a subject as a combination therapy with another treatment, e.g., a treatment for a metabolic disorder (e.g., a lysosomal storage disorder). For example, the combination therapy can include administering to the subject (e.g., a human patient) one or more additional agents that provide a therapeutic benefit to the subject who has, or is at risk of developing, (or suspected of having) a metabolic disorder (e.g., a lysosomal storage disorder). Thus, the compound or pharmaceutical composition and the one or

more additional agents can be administered at the same time. Alternatively, the molecule can be administered first and the one or more additional agents administered second, or vice versa.

It will be appreciated that in instances where a previous therapy is particularly toxic (e.g., a treatment for a metabolic disorder with significant side-effect profiles), administration of a molecule described herein can be used to offset and/or lessen the amount of the previously therapy to a level sufficient to give the same or improved therapeutic benefit, but without the toxicity.

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

The following are examples of the practice of the invention. They are not to be construed as limiting the scope of the invention in any way.

EXAMPLE 1

Generation of a human alpha glucosidase expression strain

Y. lipolytica strain OXYY1589 was constructed as follows and contains three copies of the human alpha glucosidase gene (huGAA, also known as acid alpha glucosidase or acid maltase EC3.2.1.3) and two copies of the *Y. lipolytica* MNN4 gene. The genotype of strain OXY1589 is as follows:

MatA, leu2-958, ura3-302, xpr2-322,
gut2-744, ade2-844

POX2-Lip2pre-huGAA:URA3Ex::zeta
POX2-Lip2pre-huGAA:LEU2Ex::zeta
POX2-Lip2pre-hGM-CSF:GUTEx::zeta
YlMNN4-POX2-hp4d-YLMNN4 :ADE2*::PT targeted*

All transformations were carried out according to well established protocols with modifications for the different selective markers. Unless otherwise specified, the huGAA integration fragment was obtained by NotI restriction digestion of the expression plasmid in order to remove the kanamycin resistance gene. The fragments resulting from the restriction digest were separated by agarose gel electrophoresis followed by Qiagen

column purification of the huGAA fragment. Three stable integrative transformations were performed in order to obtain the final huGAA production strain OXYY1589.

Y. lipolytica codon optimized huGAA expression vector: The nucleotide sequence encoding the 110 kDA huGAA precursor was chemically synthesized and codon optimized for *Y. lipolytica* expression. Table 1 shows the codon usage for *Y. lipolytica*. Data was derived from 2,945,919 codons present in 5,967 coding sequences. The contents of Table 1 were obtained from a Codon Usage Database, which can be found at world wide web at kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=284591.

TABLE 1

Yarrowia lipolytica Codon Usage Table

UUU 15.9(46804)	CU 21.8(64161)	AU 6.8(20043)	GU 6.1(17849)
UUC 23.0(67672)	CC 20.6(60695)	AC 23.1(68146)	GC 6.1(17903)
UUA 1.8(5280)	CA 7.8(22845)	AA 0.8(2494)	GA 0.4(1148)
UUG 10.4(30576)	CG 15.4(45255)	AG 0.8(2325)	GG 12.1(35555)
CUU 13.2(38890)	CU 17.4(51329)	AU 9.6(28191)	GU 6.0(17622)
CUC 22.6(66461)	CC 23.3(68633)	AC 14.4(42490)	GC 4.4(12915)
CUA 5.3(15548)	CA 6.9(20234)	AA 9.8(28769)	GA 21.7(63881)
CUG 33.5(98823)	CG 6.8(20042)	AG 32.1(94609)	GG 7.7(22606)
AUU 22.4(66134)	CU 16.2(47842)	AU 8.9(26184)	GU 6.7(19861)
AUC 24.4(71810)	CC 25.6(75551)	AC 31.3(92161)	GC 9.8(28855)
AUA 2.2(6342)	CA 10.5(30844)	AA 12.4(36672)	GA 8.4(24674)
AUG 22.6(66620)	CG 8.5(25021)	AG 46.5(136914)	GG 2.4(7208)
GUU 15.8(46530)	CU 25.5(75193)	AU 21.5(63259)	GU 16.6(48902)
GUC 21.5(63401)	CC 32.7(96219)	AC 38.3(112759)	GC 21.8(64272)
GUA 4.0(11840)	CA 11.2(32999)	AA 18.8(55382)	GA 20.9(61597)
GUG 25.7(75765)	CG 8.9(26190)	AG 46.2(136241)	GG 4.4(12883)

Tablefields are shown as [triplet] [frequency: per thousand] ([number]).

In the synthetic construct, the pre- and the pro-huGAA signal peptides were eliminated such that the protein starts at amino acid 57. The synthetic open reading frame (ORF) of huGAA (FIG. 1A) was fused in frame at the 5' end to the 3'end of the *Y. lipolytica* LIP2 signal sequence (pre), followed by the coding sequence of two Xxx-Ala cleavage sites, and flanked by BamHI and AvrII restriction sites for cloning into the

expression vector. In the construct, the fused polypeptide encoding sequence was under the control of the inducible POX2 promoter. The complete amino acid sequence of the fusion construct is shown on FIG. 1B.

A general schematic of the *Y. lipolytica* expression vector is presented in FIG. 2. The bacterial moiety is derived from the plasmid pHSS6, and contains a bacterial origin of replication (ori) and the kanamycin-resistance gene that confers resistance to kanamycin (KanR). The integration cassette comprises a) the selection marker for transformation to *Yarrowia lipolytica* (URA3; LEU2; GUT2), b) the expression cassette composed of a promoter, c) a multiple cloning site (MCS) to insert huGAA in frame with signal sequence and d) the terminator of the LIP2 gene. The integration cassette is flanked by zeta sequences for stable non-homologous integration into the *Y. lipolytica* genome. Two NotI restriction sites enable the isolation of the expression cassette before transformation. Plasmids pRAN034, pRAN036 and OXYP183 were used to generate huGAA expression vectors pRAN058, pRAN059 and pRAN060, respectively, containing URA3, LEU2 and GUT2 transformation markers, respectively.

Tandem YlMNN4 expression vector OXYP1470B: The *Y. lipolytica* MNN4 (YlMNN4) gene was cloned under control of the inducible pPOX2 promoter and under control of the (semi)constitutive hp4d promoter. These two expression cassettes of YlMNN4 were subcloned in one vector as a tandem construct carrying flanking regions (PT) of the ADE2 gene for targeted integration into the *ADE2* locus of the genome and the ADE2 gene as a selection marker.

Intermediate Strain OXYY1569: The first transformation was a co-transformation of strain G014 of *Y. lipolytica* with the expression cassettes purified from the pRAN058 and pRAN059 vectors, using the URA3 and LEU2 markers to produce intermediate recombinant strain OXYY1569. Thus, OXYY1569 carries two expression constructs of huGAA under control of the pPOX2 promoter randomly integrated in the genome of strain G014.

OXYY1569 was selected as follows. Integration of the huGAA DNA into the genome of *Y. lipolytica* was confirmed by PCR screening of genomic DNA. Primers for the PCR reactions were designed to amplify a 2552bp fragment of the huGAA nucleotide

sequence. Southern blot analysis of the genomic DNA also was performed in order to confirm the integration of at least 2 copies of huGAA DNA. In particular, genomic DNAs from OXYY1569 clones were digested with Hind III and probed with an huGAA DIG labeled specific probe.

In order to select a clone secreting high levels of huGAA, several randomly selected clones with confirmed integration of at least two copies of the huGAA DNA were grown in shake flasks under POX2 inducing conditions using a medium containing 1% yeast extract, 2% peptone and 5% emulsified oleic acid. In all cases, the culture supernatant was collected 72h post-induction and screened in a standard Western blot and enzyme activity assay analysis using the 4-MUG assay described in Example 3. N-Glycan analysis of OXYY1569 indicated the predominant structure in OXYY1569 was $Man_8GlcNAc_2$.

Intermediate Strain OXYY1584: Recombinant strain OXYY1569 was transformed with the expression cassette excised from plasmid OXYP1479B in order to integrate two copies of the *Y. lipolytica* MNN4 gene into its genome to produce OXYY1584. The expression cassette was excised from plasmid OXYP1479B with a SacII/XmaI restriction digest. The expression cassette was designed for targeted integration into the ADE2 locus of the *Y. lipolytica* genome. The recombinant strain was selected after Southern blotting and glycan analysis to evaluate the strain behavior with respect to the increased phosphorylation. Genomic DNA of several arbitrarily chosen transformants was digested with SpeI and probed with a MNN4 specific DIG labeled probe. Correct targeted integration of the MNN4 expression cassette into the ADE2 locus of *Y. lipolytica* genome produced 4207bp and 5683bp bands after SpeI digestion. Positive clones were grown in a standard shake flask procedure. N-glycan analysis of secreted proteins was performed in order to select the intermediate clone OXYY1584. Compared to the parent stain OXXY1569, the predominant structures after MNN4 over-expression were $Man_8GlcNAc_2(PMan)_1$ and $Man_8GlcNAc_2$ $(PMan)_2$.

Production strain OXYY1589: To generate the final prototrophic production strain OXYY1589, a third copy of huGAA was integrated into the genome of recombinant OXYY1584 strain. The transformation was performed with a Not I excised

expression cassette from pRAN069. The genomic DNA of transformants was first screened by PCR for the presence of the additional copy of huGAA. To evaluate huGAA production, arbitrarily selected PCR positive clones were further analyzed for expression after a standard shake flask cultivation. The clone expressing the highest level of huGAA (OXYY1589) was chosen after Western blot analysis and enzymatic activity assay (4-MUG assay described in Example 3). It also was reconfirmed that the conversion levels of M8 to MP2-M8 and MP-M8 N-glycans was not influenced by the presence of the additional huGAA expression cassette.

EXAMPLE 2

Fed Batch Cultivation of Strain OXYY1589

To produce huGAA from strain OXYY1589 (Example 1), a fed batch process was established using a 10 L stirred tank, with a working volume of 6-8 liters. The process was divided in two phases:

1) Batch growth on glucose for biomass formation

2) Product formation by induction with help of a limited oleic acid feed.

Typically the batch phase was about 20 hours (h) and the production phase approximately 72 hours. At the end of the process, the culture broth was centrifuged and the supernatant was collected. The supernatant was used as starting material for the purification of huGAA (see Example 3).

The following parameters were controlled during the fermentation. Aeration was maintained at a constant value of 1.5 vvm air (volume per volume per minute). Dissolved oxygen (DO) was initially kept at 30%. The stirring was increased from 600 to 1200 rpm depending on the DO levels. Once it reached the maximum of 1200 rpm, the speed was kept constant and the DO-setpoint was set to 10%. To maintain 10% DO, oxygen was spiked into the reactor with a maximal percentage of 50%. Foam evolution was controlled by a foam probe. If foam was detected, antifoam was added to the bioreactor. The pH was controlled by adding 14% (v/v) ammonia (base) or 10% phosphoric acid to maintain a constant value of pH 6.8. The temperature was kept constant at 28°C throughout the whole process.

Biomass was monitored by measurement of optical density at 600 nm (OD600). The samples were diluted 2 – 1000 times in distilled water to obtain values in the linear range of the spectrophotometer. Product formation was detected by Western blot analysis and specific enzymatic activity tests.

EXAMPLE 3

Purification of recombinant huGAA (rhGAA)

The supernatant after cultivation (see Example 2) was clarified via depth filtration. The resulting material then was concentrated 20 times via tangential flow filtration (TFF) and diafiltered against 20 mM sodium phosphate pH 6 and 100 mM NaCl using a 10kDa MWCO membrane (Millipore).

Purification of rhGAA was started by adding ammonium sulphate up to a concentration of 1 M. After centrifugation, the supernatant was loaded on a Toyopearl-Phenyl 650M (Tosoh Biosciences) packed XK16/40 column. A linear gradient from 1 to 0 M ammonium sulphate was applied for elution. Those fractions that contained rhGAA were then pooled and subjected to a buffer exchange into 10 mM BIS-TRIS pH 6. Further purification was achieved via anion exchange chromatography on a source 30Q packed Tricorn 10/50 or XK25/20 column (GE Healthcare) using a linear salt gradient from 0 to 1 M NaCl. The resulting GAA-containing fractions were then concentrated before loading onto a final Hiload 16/60 superdex 200 gel filtration column (GE Healthcare) that was pre-equilibrated with 50 mM sodium phosphate pH 6 and 200 mM NaCl. Fractions were selected on the basis of specific activity and purity on Coomassie-stained SDS-PAGE gels and then combined and concentrated to a final concentration of 5-10 mg/ml. Proteins were concentrated using 15 ml Amicon Ultra centrifugal devices (Millipore) with a MWCO of 10 kDa.

The 4-methylumbelliferyl-alpha-D-glucopyranoside (4-MUG) assay was used to screen rhGAA. Cleavage of the substrate 4-MUG by a glucosidase leads to the generation of the fluorigenic product 4-MU, which can be visualized or detected by irradiation with UV light. The reactions for the qualitative screening for rhGAA were started by adding the reaction buffer consisting of 0.35 mM 4-MUG, 0.1% BSA and 100

mM sodium acetate pH 4 in a 10:1 or 20:1 volume proportion to 10 or 5 µl of the elution fraction. All reactions were done in 96-well flat-bottom microtiter plates. After an incubation period of 30 minutes to 1 hour at 37°C, an equal volume of 100 mM glycine pH 11 was added to stop the reaction and the release of the fluorogenic reaction product 4-methylumbelliferone (4MU) was observed under UV-light. Specific activities (units/mg protein) were determined using a colorimetric assay with the synthetic substrate p-nitrophenyl-α-D-glucopyranoside (PNPG) that measures the enzymatic release of the yellow coloured p-nitrophenolate reaction product. The reactions were started by mixing 10 µl of enzyme solution and 90 µl of substrate reaction buffer (2 mM PNPG in 150 mM citrate-phosphate buffer pH 4, 1% BSA) in reaction wells of a microtiter plate and were subsequently incubated at 37°C. After incubating for 1 to 2 hours, an equal volume of stop buffer, 10% sodium carbonate pH 12, was added to quench the reaction and bring the released p-nitrophenol (PNP) in its ionized state. Background-corrected absorbances and p-nitrophenolate standards were measured at a wavelength of 405 nm and specific activities were calculated. Protein concentrations were determined by the bicinchoninic acid (BCA) method. One unit was defined as the amount of enzyme that catalyzes the conversion of 1 nmol of PNPG to 1 nmol PNP and D-glucose per min at 37°C at a final substrate concentration of 2 mM in a citrate-phosphate buffer, pH 4.0.

EXAMPLE 4

Cloning and expression of YlAMS1

The Ams1 gene from *Yarrowia lipolytica* (YlAms1) was PCR amplified from *Yarrowia* genomic DNA using gene specific primers. A HIS6-tag coding sequence was fused to the 3' end of the YlAms1 ORF such that YlAMS1 protein with a C-terminal His-tag could be produced, and was also fused to the 5' end of the YlAms1 ORF such that YLAMS1 protein with an N-terminal His tag could be produced. Both ORFs were cloned under control of the semi constitutive hp4d promoter (FIG. 3A and FIG. 3B) and the expression cassettes were transformed into *Yarrowia lipolytica*. Cells were grown in complex medium (YPD) and harvested after 72 h growth. After disrupting the cells by sonication, the AMS1 protein was purified using a NTA column. Purified material was

analyzed for activity using PNP-mannose as a substrate. Active fractions were pooled and kept for glycan analysis.

EXAMPLE 5

De-mannosylation and phosphate uncapping of APTS-labeled phosphorylated N-glycans with GH38 α-mannosidases

Jack bean α-mannosidase (*Canavalia ensiformis*) was obtained from Sigma-Aldrich. Both a 3.0 M ammonium sulphate suspension (Sigma-M7257) and a proteomics grade Jack bean α-mannosidase (Sigma-M5573) were used in the N-glycan analyses. Both batches gave identical results and are named JbMan in the further description. YlAms1 was expressed and purified as described in Example 4. JbMan and YlAMS1 were tested on a mixture of 8-amino-1,3,6,-pyrenetrisulfonic acid (APTS)-labeled sugars derived from an MNN4 overexpressing *Yarrowia lipolytica* strain, which contains $Man_8GlcNAc_2$ (M8), the monophosphorylated ManP-$Man_8GlcNAc_2$ (MP-M8) and/or the diphosphorylated $(ManP)_2$-$Man_8GlcNAc_2$ ($(MP)_2$-M8) sugars (referred to as MNN4 sugars or MNN4 N-glycans). In FIG. 4, the potential final hydrolysis products are schematically presented, assuming that the α-mannosidases also can fully trim the MNN4 N-glycans, including hydrolysis of the non-phosphorylated arm, hydrolysis of the terminal α-1,2-mannose if the underlying mannose is phosphorylated, and/or uncapping of the phosphate in the mannose-1-phospho-6-mannose linkage.

Unless otherwise stated all reactions with JbMan and YlAMS1 on APTS-labeled N-glycans were performed overnight at 37°C in an ammonium acetate buffer, 10 mM, pH 5.0 with 2 mM $CaCl_2$.

In FIG. 5, the DSA-FACE electroferograms are presented depicting the hydrolysis of the MNN4 N-glycans with JbMan. A sample was included with $Man_8GlcNAc_2$ as the substrate (Panel B) to be able to identify newly appearing peaks. JbMan sequentially hydrolyzed $Man_8GlcNAc_2$ (Panel C) till only $Man_1GlcNAc_2$ was obtained after overnight incubation (Panel D). The hydrolysis of a substrate solution containing $Man_8GlcNAc_2$ and ManP-$Man_8GlcNAc_2$ (Panel E) was more complex. Both the de-mannosylation and phosphate uncapping activities were responsible for the appearance of the fast-running

peak at the left hand side of the electropherogram when the substrate was incubated with JbMan during 2 hours (Panel F). The extra charge of a terminal phosphate together with the de-mannosylation reaction was responsible for the appearance of peaks displaying fast electrophoretic mobility. Nevertheless, after overnight incubation, only a peak identified as $Man_1GlcNAc_2$ was observed (Panel G). Phosphatase activity present in the commercial JbMan preparation is responsible for this result.

The digestion of MNN4 sugars with JbMan was repeated with a substrate solution containing ManP-$Man_8GlcNAc_2$ and $(ManP)_2$-$Man_8GlcNAc_2$ (Panel H). After incubating for 2 hours, a potential uncapped peak appeared and is indicated with "P-Mx" and "P2Mx" in panel I. In the fast electrophoretic mobility region, the peak resolution is smaller and it is possible that mono- and diphosphorylated uncapped structures, e.g., P-$Man_4GlcNAc_2$ and P2-$Man_6GlcNac_2$, ran together. The result after overnight digestion suggests a further de-mannosylation. The peaks indicated with P-My and P2-My in panel J could be P-$Man_3GlcNAc_2$ and P2-$Man_5GlcNAc_2$, but neutral $Man_1GlcNAc_2$, $Man_2GlcNAc_2$ and $Man_3GlcNAc_2$ also can be observed in panel J. As no $Man_8GlcNAc_2$ was present in the substrate solutions, these peaks are the result of a potential contaminating phosphatase activity and further mannose trimming.

To identify the uncapped peaks in panel J, the reaction mixture was treated with calf intestine phosphatase (CIP). Treatment of the uncapped glycans (thus containing a terminal phosphate) resulted in neutral oligosaccharides that ran much slower and appeared more to the right in the electropherogram. Indeed, $Man_3GlcNAc_2$ through $Man_6GlcNac_2$ appear in panel K. Although the activity was hampered by the presence of phosphatase activity in the commercial JbMan preparation, the presented data reveal that fully de-mannosylated and phosphate uncapped structures (i.e., P-$Man_3GlcNAc_2$ and P2-$Man_5GlcNAc_2$) can be obtained when treating APTS-labeled MNN4 sugars with JbMan.

De-mannosylation and phosphate uncapping activity also is observed with YlAMS1, as shown in FIG. 6. YlAMS1 can fully hydrolyze $Man_8GlcNAc_2$ through $Man_1GlcNAc_2$ (panel C). Incubation of YlAMS1 with a substrate solution containing $Man_8GlcNAc_2$ and ManP-$Man_8GlcNAc_2$ (Panel D) yields a product with a fast electrophoretic mobility, likely a phosphate uncapped glycan (Panel E). A series of

uncapped N-glycans were observed when the reaction was repeated with a diluted YlAMS1 sample during a 2 hour incubation (Panel F). The presence of phosphate uncapped glycans was confirmed by treating the reaction mixture with CIP, yielding a series of neutral N-glycans (Panel G). Thus, YlAMS1 can uncap $(ManP)_2$-$Man_8GlcNAc_2$ as observed in panel I, but it is still unclear which product is formed, P2- $Man_8GlcNAc_2$ or a further mannose trimmed glycan.

EXAMPLE 6

De-mannosylation and phosphate uncapping of glycoproteins expressed in a *Yarrowia lypolytica* strain with a higher degree of phosphorylated N-glycans with GH38 α-mannosidases

The human lysosomal α-glucosidase huGAA was expressed in *Y. lipolytica* strain OXYY1589 to yield a glycoprotein with a high degree of phosphorylated N-glycan structures. The huGAA was purified as described in Example 3.

Jack bean α-mannosidase (JbMan) was added to a solution of huGAA in 100 mM ammonium acetate, pH 5.0 with 2 mM $CaCl_2$. The reaction mixture was incubated overnight at room temperature. The N-glycans were released with PNGaseF, labelled with APTS and subsequently analyzed on DSA-FACE, essentially as described in Laroy, *et al.*, Nature Protocols, 1:397-405 (2006). The N-glycan profiles before and after the α-1,2-mannosidase treatment are shown in FIG. 7. The N-glycan mixture released from purified huGAA was mainly composed of ManP-$Man_8GlcNAc_2$ and $(ManP)_2$-$Man_8GlcNAc_2$ (panel B). A peak running slightly faster than ManP-$Man_8GlcNAc_2$ was assigned to ManP-$Man_7GlcNAc_2$. Only very minor amounts of $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ were present. Since JbMan is a glycoprotein, a control sample is presented in panel C in order to be able to correct for the Jack bean specific N-glycans. In panel D, the N-glycans obtained after incubating huGAA with JbMan are presented. The peaks corresponding to ManP-$Man_8GlcNAc_2$ and $(ManP)_2$-$Man_8GlcNAc_2$ were no longer present. Instead, a number of peaks appeared on the left hand side of the electropherogram (potentially phosphate uncapped N-glycans) together with $Man_1GlcNAc_2$. The latter mainly resulted from phosphatase activity present in the

commercial JbMan preparation and further de-mannosylation of the obtained neutral N-glycans.

EXAMPLE 7

Uncapping and de-mannosylation of recombinant human α-glucosidase (huGAA) with CcMan5 and CcMan4

Nucleic acids encoding *Cellulosimicrobium cellulans* mannosidase 4 (CcMan4) and *Cellulosimicrobium cellulans* mannosidase 5 (CcMan5) were cloned into vector pLSAH36, which contains a DsbA signal sequence and results in the expression of a protein with an N-terminal HIS tag. The nucleotide sequences of the open reading frame of DsbA-CcMan5 and DsbA-CcMan4 are provided in FIGs. 8 and 9, respectively. The proteins were expressed in *E.coli* B21 cells and proteins residing in the periplasm were isolated and purified using a Talon column. A graphical representation of the plasmids pLSAHCcMan5 and pLSAHCcMan4 is given in FIG. 10.

A series of CcMan5 uncapping and CcMan4 de-mannosylation experiments were performed with 100 μg batches of huGAA purified as described in Example 3. Thirty (30) μL of huGAA (3.7 mg/mL in 25 mM phosphate buffer, pH 6.0, with 100 mM mannitol) were added to 46 μL of 100 mM HEPES buffer, pH 7.0 with 3 mM $CaCl_2$. In one experiment (referred to as huGAA_CcMan4), a weight:weight (w:w) ratio of 100:1 of huGAA:CcMan4 was used in which 14 μL of CcMan4 (80 μg/ml formulated in PBS) was added to the huGAA solution. In another experiment (referred to as huGAA_CcMan5), a w:w ratio of 100:2 of huGAA:CcMan5 was used in which 14 μL CcMan5 (154 μg/mL formulated in PBS) was added to the huGAA solution. In a combined experiment (referred to as huGAA_CcMan4/5), a w:w ratio of 100:2:1 of huGAA:CcMan5:CcMan4 was used in which14 μL of CcMan5 and 14 μL of CcMan4 were added to 30 μL of huGAA and 32 μL of 100 mM HEPES buffer, pH 7.0 with 3 mM $CaCl_2$. In a control experiment (huGAA_control), 10 μL huGAA was diluted with 20 μL of 100 mM HEPES buffer, pH 7.0 with 3 mM $CaCl_2$. After incubating all of the samples for 16 hours at 30°C, the samples were kept at 4°C until used.

Two (2) μL of each sample were used for N-glycan analysis as described in Example 6. The DSA-FACE electropherograms of the huGAA treated samples are presented in FIG. 11. CcMan4 treatment resulted in the complete de-mannosylation of ManP-$Man_8GlcNAc_2$ and $(ManP)_2$-$Man_8GlcNAc_2$ with the formation of the products ManP-$Man_5GlcNAc_2$, ManP-$Man_6GlcNAc_2$ and $(ManP)_2$-$Man_6GlcNAc_2$ (FIG. 11, third panel). Under the above reaction conditions, the phosphate uncapping with CcMan5 was complete for the ManP-$Man_8GlcNAc_2$ N-glycan with the formation of P-$Man_8GlcNAc_2$. The diphosphorylated N-glycan $(ManP)_2$-$Man_8GlcNAc_2$ was hydrolyzed to the fully uncapped P2-$Man_8GlcNAc_2$, but also a slower running peak with comparable peak height was observed and corresponded to partially uncapped (ManP)-Man_8-(P)$GlcNAc_2$ (potentially with an uncapped phosphate on the α-1,6 arm and a capped phosphate on the α-1,3 arm of the N-glycan) (FIG. 11, fourth panel). Uncapped and de-mannosylated huGAA was obtained after treatment with CcMan5 and CcMan4, and resulted in an N-glycan profile with P_2-$Man_6GlcNAc_2$, (ManP)-Man_6-(P)$GlcNAc_2$, and P-$Man_5GlcNAc_2$. Minor peaks corresponding to Man_5 and P-$Man_6GlcNAc_2$, P-$Man_7GlcNAc_2$, ManP-$Man_7GlcNAc_2$ (the latter phosphorylated N-glycans potentially with the α-1,3 arm phosphorylated) were observed (FIG. 11, fifth panel. A schematic presentation of the uncapped N-glycans is shown in FIG. 12 (B).

Another CcMan5/CcMan4 uncapping and de-mannosylation experiment was performed with huGAA from the same purification batch. The experiment was performed essentially as described above, except that the formulation buffer for huGAA was 100 mM HEPES, pH 7.0 with 2 mM $CaCl_2$ and 100 mM mannitol (rather than 25 mM phosphate buffer, pH 6.0 with 100 mM mannitol). A w:w ratio of 100:3:0.5 for huGAA:CcMan5:CcMan4 was used. The reaction was incubated at 37°C for 24 hours. A sample of the commercial available human α-glucosidase, Myozyme® (alglucosidase alpha, Genzyme) was treated under identical conditions with CcMan4 at a w:w ratio of 100:0.5 for Myozyme:CcMan4. The N-glycan analysis of these samples was performed as discussed above. The N-glycan profile for huGAA purified in this manner and treated with CcMan5 and CcMan4 was similar to that presented in FIG. 11. The DSA-FACE electropherograms for Myozyme® treated with CcMan4 are presented in FIG. 13.

To follow intracellular huGAA processing (see example 10), a CcMan5/CcMan4 uncapping and de-mannosylation experiment was performed with huGAA from a different purification batch. The purification was performed under conditions similar to those described above, again using 100 mM HEPES, pH 7.0 with 2 mM $CaCl_2$ and 100 mM mannitol as the huGAA formulation buffer. The uncapping and de-mannosylation was performed at a w:w ratio of 100:3:0.5 for huGAA:CcMan5:CcMan4 and the reaction mixture was incubated for 24 hours at 30 C. The N-glycan profiles are shown in FIG. 14. In this experiment, the diphosphorylated N-glycans P_2-$Man_6GlcNAc_2$ and (ManP)-Man_6-(P)$GlcNAc_2$ were partially dephosphorylated to P-$Man_6GlcNAc_2$, (ManP)-$Man_6GlcNAc_2$ respectively. Phosphatase activity was detected in the huGAA sample using the general phosphatase substrate paranitrophenyl-phosphate (PNPP) in 100 mM HEPES buffer, pH 7.5 with 1 mM $MgCl_2$.

EXAMPLE 8

Uncapping and de-mannosylation of recombinant huGAA with Jack bean α-mannosidase

The uncapping and de-mannosylation experiments of Example 6 were repeated after the ammonium sulphate suspension of JbMan was further purified by gel filtration through a Superdex 200 column to remove contaminating phosphatase activities.

In one experiment referred to as huGAA_JbMan, a w:w ratio of 100:15 of huGAA:JbMan was used. Ten (10) µl of JbMan (1.5 mg/ml in PBS) was added to a solution containing thirty (30) µl of huGAA (3.7 mg/ml in 25 mM phosphate buffer, pH 6.0 with 100 mM mannitol) and 50 µl 100 mM sodium acetate buffer, pH 5.0. The control sample (huGAA_control) contained huGAA but no JbMan. After 16 hours incubation at 30 °C, the samples were maintained at 4 °C until further use. For N-glycan analysis, 2 µL of each sample was used to release and label the N-glycans as described in Example 6. The DSA-FACE electropherograms of the N-glycans from the huGAA treated with JbMan are presented in FIG. 15. Treatment with JbMan resulted in the partial uncapping and de-mannosylation of ManP-$Man_8GlcNAc_2$ and $(ManP)_2$-$Man_8GlcNAc_2$ on huGAA, with the formation of mainly P-$Man_5GlcNAc_2$ and (ManP)-Man_6-(P)$GlcNAc_2$. The latter N-glycan runs together with P-$Man_5GlcNAc_2$ on the electropherogram. A

minor amount of fully uncapped P2-$Man_6GlcNAc_2$ is also present. A peak running slower than P-$Man_5GlcNAc_2$ may be the neutral $Man_3GlcNAc_2$. P2-$Man_6GlcNAc_2$ and P-$Man_4GlcNAc_2$ are not further de-mannosylated by JbMan (FIG. 15, third panel).

A second JbMan uncapping and de-mannosylation experiment was performed with huGAA from the same purification batch. The experiment was performed, essentially as described above, 100 mM sodium acetate, pH 5.0 with 1 mM $ZnCl_2$ and 100 mM mannitol as the huGAA formulation buffer. A w:w ratio of 100:10 for huGAA:JbMan was used. The reaction was incubated at 37 °C for 24 hours. The N-glycan profile of these samples after JbMan treatment was similar to the N-glycan profile shown in FIG. 15.

To follow intracellular huGAA processing (see Example 10), an uncapping and de-mannosylation experiment with JbMan was performed with huGAA from a different purification batch. Similar reaction conditions as described above were used. The huGAA formulation buffer used was 100 mM sodium acetate, pH 5.0 with 1 mM $ZnCl_2$ and 100 mM mannitol, a w:w ratio of 100:10 for huGAA:JbMan was used, and the reaction mixture was incubated for 24 hours at 30 °C. The N-glycan profiles are shown in FIG. 16. The diphosphorylated N-glycan P_2-$Man_6GlcNAc_2$ is not observed in the electropherogram. Due to the presence of phosphatase activity in the huGAA sample, partial dephosphorylation occurred, resulting in the presence of the relatively high amounts of monophosphorylated P-$Man_6GlcNAc_2$ and ManP-$Man_6GlcNAc_2$, together with the neutral N-glycans $Man_3GlcNAc_2$ to $Man_6GlcNAc_2$.

EXAMPLE 9

Uptake of recombinant huGAA into Pompe fibroblasts

The uncapped and demannosylated huGAA and Myozyme® (non-treated and treated with CcMan4) from Example 7 and 8 were used in the cell uptake experiments. The specific enzyme activities of capped huGAA or huGAA treated with either CcMan5 (huGAA_CcMan5), Ccman4 (huGAA_CcMan4), a combination of CcMan4 and CcMan5 (huGAA_CcMan4/5), or Jack Bean mannosidase (huGAA_JBMan) (see Examples 7 and 8) were tested using the 4-MUG assay. Cleavage of the substrate 4-MUG by a

glucosidase leads to the generation of the fluorogenic product 4-MU, which can be visualized or detected by irradiation with UV light. See Example 3. The activity of huGAA was compared with that of Myozyme®. The enzymes were diluted to three different concentrations (125 ng/ml, 62.5 ng/ml, and 31.25 ng/ml) in 100 mM sodium acetate buffer pH 4.0 containing 0.1 % BSA (reaction buffer), and 50 µl of each dilution was added to a 96-well plate in triplicate. The 4-MUG substrate (Sigma) was diluted to 4 mM in reaction buffer and 50 µl of the diluted substrate was added to each well. The enzymatic reaction was incubated for 60 min at 37°C followed by the addition of 100 µl 150 mM EDTA-Na_2 salt, pH 11.5 to quench the reaction. The fluorescence was measured at excitation 360/40 nm and emission 460/40 nm. A standard curve with 4-methylumbelliferone (4-MU) was measured to calculate the specific activity. The activity of the various enzymes was reported as U/mg where 1 unit is defined as the amount of enzyme that catalyzes the hydrolysis of 1 nmol substrate per hour at 2 mM substrate concentration in 100 mM sodium acetate buffer, pH 4.0 + 0.1 % BSA. The specific activity of each of the enzymes was around 200 x 10^3 U/mg.

The uptake of huGAA treated with CcMan5 (huGAA_CcMan5), Ccman4 (huGAA_CcMan4), a combination of CcMan4 and CcMan5 (huGAA_CcMan4/5), or Jack Bean mannosidase was assessed in GM00248 fibroblasts, a human Pompe fibroblast cell line (Coriell Cell Repository, Camden, NJ). The GM00248 fibroblasts are deficient in acid alpha glucosidase activity (0.27% of normal) and have no detectable levels of GAA mRNA or protein. The GM00248 fibroblasts were seeded and grown to confluence in Minimum Essential Medium (MEM, Invitrogen) containing Earle's salts and nonessential amino acids supplemented with 15 % FCS and 2 mM glutamine. One day before administration of enzymes, cells were seeded in 24-well plates in Ham's F10 medium supplemented with 5% heat inactivated FCS (30 min at 56 °C).

On the day of the experiment, capped huGAA and uncapped huGAA were diluted in uptake medium to various enzyme activities followed by filtration through a 0.22 µm filter. The activity of each enzyme dilution in uptake medium was measured again using the 4-MUG assay to determine the actual enzyme activity that was added to the cells.

The GM00248 fibroblasts were incubated with the enzymes for 16 hours, washed twice with ice-cold PBS, and then lysed with 0.5 ml PBS + 0.5 % Triton X 100 (30 min, 4°C) supplemented with protease inhibitors. Cell lysates were spun at 10000xg to remove cell debris. The intracellular activity of huGAA was measured using the 4-MUG activity assay as described above. Protein concentrations were determined by the bicinchoninic acid method (microBCA kit, Pierce) following the manufacturer's protocol. The intracellular activity of huGAA is expressed as units per mg total protein (U/mg).

FIG. 17 shows the intracellular activity of huGAA in the GM00248 human Pompe fibroblasts. Capped huGAA that contains a mixture of ManP-$Man_8GlcNAc_2$ and $(ManP)_2$-$Man_8GlcNAc_2$ N-glycans (see FIG. 11, second panel) did not enter the cells. The intracellular activity of cells treated with capped huGAA was similar to non-treated cells (data not shown). HuGAA_CcMan4, which is completely de-mannosylated (see FIG. 11, third panel), also showed no uptake in Pompe fibroblasts. Although CcMan5 treatment resulted in the formation of uncapped monophosphorylated P-$Man_8GlcNAc_2$ and fully uncapped diphosphorylated P_2-$Man_8GlcNAc_2$, no cellular uptake was observed over the tested dose range (FIG. 17). Dose-dependent cellular uptake was observed for HuGAA that was uncapped and de-mannosylated huGAA with either the combination of CcMan4 and CcMan5 (huGAA_CcMan4/5) or with Jack Bean mannosidase (huGAA_JBMan). The intracellular activity of huGAA treated with either CcMan4/5 or JbMan reached a plateau level at around 500-1000 U/ml while the intracellular activity of Myozyme® did not reach a plateau at 2500 U/ml. Phosphate-uncapped and de-mannosylated huGAA was taken up approximately 2.5 times more efficiently than Myozyme®.

A second set of experiments was performed to investigate whether the uptake was due to binding to the mannose-6-phosphate (M6P) receptor. For these experiments, huGAA from the same purification batch used in the above experiments was treated with CcMan4 and CcMan5 mannosidases for uncapping the mannose-1-phosphate-6-mannose linked glycans as described in Example 7 or with Jack Bean mannosidase as described in Example 8. Myozyme® was used as a reference. To investigate the effect of terminal α-1,2 mannoses on the uptake efficiency of huGAA, Myozyme® was treated with CcMan4

mannosidase. The specific activity of the enzymes was determined using the 4-MUG assay as described above. The uptake assay was performed as described above. The enzymes were diluted to equal enzyme activities in uptake medium, filtered, and various doses were added to the GM00248 fibroblasts with or without the presence of 5 mM M6P (Sigma) and incubated for 16 hours. Each cell uptake experiment was performed in duplicate. After incubation, cells were washed with ice-cold PBS, lysed with 0.5 ml PBS + 0.5% Triton X 100 supplemented with protease inhibitors and assayed for intracellular huGAA activity using the 4-MUG assay.

FIG. 18 shows the uptake of huGAA enzymes in GM00248 fibroblasts. Treatment of Myozyme® with CcMan4 did not change the N-glycan profile of Myozyme® (see FIG. 13, third panel), nor did it change its uptake efficiency. The uptake of Myozyme® was inhibited by the addition of free M6P. The results in FIG. 18 show a dose-dependent uptake of uncapped and de-mannosylated huGAA (huGAA_CcMan4/5, huGAA_JBMan), which is inhibited by the addition of M6P. These results indicate that the uptake of uncapped and de-mannosylated huGAA is mediated via the M6P receptor.

EXAMPLE 10

Processing of huGAA in the lysosomes of Pompe fibroblasts.

HuGAA is produced in the endoplasmic reticulum as a 110 kDa precursor. It undergoes N-glycan processing in the Golgi apparatus and is further proteolytically processed in the lysosomes into active proteins of 76 kDa and 70kDa, through an intermediate molecular form of 95 kDa. The active proteins are responsible for degrading its natural substrate glycogen. In the following experiments, the intracellular processing of purified recombinant huGAA, produced as a 110 kDa protein in *Y. lipolytica*, was investigated. For these experiments, huGAA from a different purification batch than that used in Example 9, and in which the formulation buffer was exchanged to 100 mM HEPES, pH 7 with 2 mM $CaCl_2$ and 100 mM mannitol (see Example 7) was treated with the combination CcMan4 and CcMan5 or with Jack Bean mannosidase as described in Example 7. The specific activity of the uncapped enzymes was determined using the 4-MUG assay. One day before the experiment, GM00248 fibroblasts were seeded in 6-well

plates at a density of 5 x 10^5 cells/well in uptake medium as described above. The next day, the fibroblasts were incubated with 1000 U/ml huGAA_CcMan4/5 or huGAA_JBMan in 2 ml uptake medium for 14 hours or for 46 hours. As a reference, cells were incubated with Myozyme®; and cells that were not incubated with an enzyme were used as a negative control. Each cell uptake experiment was performed in duplicate. After incubation, the GM00248 fibroblasts were washed with ice-cold PBS and harvested by trypsinization (0.05% trypsin with 0.53 mM EDTA). Cells were centrifuged and lysed in 0.5 ml PBS + 0.5% TritonX100, supplemented with protease inhibitors. Cell lysates were centrifuged to remove cell debris and assayed for intracellular GAA activity with the 4-MUG assay as described above. Protein concentration was determined with the BCA method.

FIG. 19 shows the intracellular huGAA activity. Although huGAA_Ccman4/5 was partially dephosphorylated to P-Man_6GlcNA_2 and (ManP)-$Man_6GlcNac_2$ (FIG. 14, third panel), the enzyme was taken up 1.8 times better than Myozyme® at both tested incubation times. HuGAA_JBMan also was taken up better than Myozyme® but was less efficient compared to huGAA_CcMan4/5, probably due to the absence of the diphosphorylated N-glycan P2-$Man_6GlcNAc_2$ (FIG. 16, third panel).

The purpose of this experiment was to test whether huGAA taken up by the fibroblasts was processed to the active forms of 76 kDa and 70kDa. Therefore, cell samples were precipitated by the trichloroacetic acid (TCA)/ deoxycholate (DOC) method. Samples (500 µl, containing 160 µg protein) were mixed with 50 µl of 0.5% DOC and incubated on ice for 30 minutes. After adding TCA 100 % (100 µl) to obtain a final TCA concentration of 15%, samples were mixed and precipitated overnight at -20°C. The precipitate was centrifuged at 13000 rpm in a microcentrifuge for 30 min, followed by aspiration of TCA from the pellet. The pellet was washed with 500-700 µl of ice-cold acetone, mixed and centrifuged at 13000 rpm. The pellet was dried for 10 min at 50°C followed by re-solubilization in 1x NuPAGE® LDS sample buffer containing NuPAGE® sample reducing agent. After boiling the sample for 3 min at 100°C, 20 µg protein (10 µl) was loaded on a 4-12% NuPAGE® Bis-Tris gel (Invitrogen) with 1x MOPS SDS running buffer containing 500 µl of NuPAGE® antioxidant. Myozyme® (50

ng) were loaded on the gel as a reference. The samples were blotted overnight on a nitrocellulose membrane and the intracellular huGAA was detected using polyclonal rabbit anti-huGAA sera (1/2000 dilution) as primary antibody and a goat anti-rabbit IgG peroxidase conjugated antibody (1/5000 dilution, Sigma) as a secondary antibody. After washing the membrane with PBS/Tween, the membrane was developed using the ECL western blotting detection reagent (GeHealthcare). A 14h incubation period with the uncapped enzymes and with Myozyme® resulted in the presence of mainly the precursor protein. In the huGAA_Ccman4/5 treated cells, a minor amount of the 76 kDa protein was observed. After the 46h incubation, the uncapped enzymes were processed to the 76 kDa active polypeptide. Myozyme® also is processed to the active polypeptide but the bands were less intense.

EXAMPLE 11

Uncapping and de-mannosylation of recombinant huGAA with CcMan5 and Jack bean α-mannosidase

Recombinant huGAA was uncapped and demannosylated with CcMan5 and JBMan at a w:w ratio of 100:5:10 for huGAA:CcMan5:JbMan. To a solution of 1.08 ml huGAA (4.8 mg/ml in 10 mM sodium phosphate buffer, pH 6.0 with 40 mM NaCl), 1.69 ml of CcMan5 (0.154 mg/ml in PBS buffer) and 1.04 ml of JbMan (0.5 mg/ml in PBS buffer) were added. The total reaction volume was adjusted to 5.2 ml with 100 mM sodium acetate buffer, pH 5.0, containing 2 mM $CaCl_2$. The reaction mixture was incubated at 30°C for 15 hours. The uncapped and demannosylated huGAA was purified using a Hiload 16/60 superdex 200 gel filtration column (GE Healthcare) as described in Example 3.

The N-glycans were released from 10 µg of the final purified huGAA and labeled as described in Example 6. The DSA-FACE electropherogram of the N-glycans from the huGAA treated with both CcMan5 and JbMan is presented in FIG. 20. The main peaks observed after uncapping and demannosylation were the double phosphorylated P2-$Man_6GlcNAc_2$ and the monophosphorylated P-$Man_4GlcNAc_2$, P-$Man_5GlcNAc_2$ and P-$ManGlcNAc_2$.

EXAMPLE 12

Uptake of uncapped and demannosylated recombinant huGAA with CcMan 5 and JbMan into Pompe fibroblasts

The cellular uptake of uncapped, demannosylated, and purified huGAA (treated with JbMan and CcMan5 as described in Example 11) was compared to the cellular uptake of Myozyme® using the GM00248 fibroblast cell line as described in Example 9.

FIG. 21 shows the intracellular activity of purified uncapped and demannosylated huGAA versus the intracellular activity of Myozyme®. The amount of enzyme (expressed as enzyme activity units) added to the cells was converted to enzyme concentration (expressed as nM) and plotted versus the specific activity (expressed in U/mg) for the calculations of the K_{uptake}. K_{uptake} and the standard deviation were calculated in GraphPrism using non-linear regression through 14 data points (2 data points per concentration) for huGAA and through 12 data points for Myozyme®. Dose-dependent cellular uptake was observed for huGAA, reaching a plateau level at around 25 nM and a K_{uptake} of 1.7 ± 0.2 nM, while the intracellular activity of Myozyme did not reach a plateau at 200 nM and has a K_{uptake} of 64 ± 5 nM. Uncapped, demannosylated huGAA produced in *Yarrowia lipolytica* was taken up 30 times more efficiently than Myozyme® in Pompe fibroblasts.

EXAMPLE 13

Processing of uncapped and demannosylated recombinant huGAA with CcMan 5 and JbMan in the lysosomes of Pompe fibroblasts

A cell uptake assay was performed to determine if the *Yarrowia* produced huGAA that was treated with CcMan5 and JbMan as described in Example 11 was processed to its mature forms in the lysosomes. One day before the experiment, GM00248 fibroblasts were seeded in a 6-well plate at a density of $3x10^5$ cells/well in uptake medium. The next day, fibroblasts were stimulated with 2000 U/ml huGAA in 2 ml uptake medium for 8 hours or 24 hours, or stimulated for 24 hours ("pulse" period) then the cells were washed and 2 ml growth medium were added to the cells for a chase period of up to 100 hours. Cells not treated with enzyme were used as a negative control.

After incubation, cells were washed and cell lysates were precipitated using the DOC/TCA method as described in Example 10 and subjected to Western blotting. As a reference, purified huGAA (30 ng) was loaded on the gel. The samples were blotted overnight and the intracellular huGAA was detected using polyclonal rabbit anti-huGAA sera (1/2000 dilution) as primary antibody and a goat anti-rabbit IgG peroxidase conjugated antibody (1/8000 dilution, Abcam) as a secondary antibody. The membrane was developed using the ECL western blotting detection reagent (GeHealthcare).

An 8 hour incubation period with the uncapped and demannosylated enzyme resulted in the presence of the precursor protein (110 kDa). A 24 hour incubation period resulted in the presence of both the precursor protein and the processed protein (76 kD), while after a 24 hour pulse and up to 100 hour chase period, almost all protein was processed towards the 76 kD active polypeptide. These results demonstrate that the uncapped and demannosylated huGAA was taken up by the fibroblasts and processed to its active polypeptides in the lysosomes.

OTHER EMBODIMENTS

While the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method for uncapping mannose-1-phospho-6-mannose moieties and demannosylating phosphorylated N-glycans on a glycoprotein, said method comprising

   a) providing said glycoprotein having phosphorylated N-glycans containing said mannose-1-phospho-6-mannose moiety; and

   b) contacting said glycoprotein with a mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose moiety to mannose-6-phosphate and (ii) hydrolyzing terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkages.
2. A method of demannosylating phosphorylated N-glycans, said method comprising

   a) providing a glycoprotein comprising phosphorylated N-glycans; and

   b) contacting said glycoprotein with a mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose moeity to mannose-6-phosphate and (ii) hydrolyzing terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkages.
3. The method of claims 1 or 2, wherein said mannosidase is a family 38 glycosyl hydrolase.
4. The method of any one of claims 1-3, wherein said mannosidase is from *Canavalia ensiformis*.
5. The method of any one of claims 1-3, wherein said mannosidase is from *Yarrowia lipolytica*.
6. The method of any one of claims 1-5, said method further comprising, after steps (a) and (b), contacting a mammalian cell with said glycoprotein comprising said demannosylated phosphorylated N-glycans, wherein, after said contacting, said glycoprotein is transported to the interior of said mammalian cell.
7. The method of claim 6, wherein said mammalian cell is a human cell.
8. A method of directing a glycoprotein to the interior of a mammalian cell, the method comprising a) providing a glycoprotein wherein its mannose-6-phosphate moieties have been demannosylated, and b) contacting said cell with said demannosylated glycoprotein.

9. The method of claim 8, wherein said mannosidase is a family 47 glycosyl hydrolase.
10. The method of claim 8 or 9, wherein said glycoprotein is demannosylated with a mannosidase from *Aspergillus satoi*.
11. The method of claim 8, wherein said mannosidase is a family 92 glycosyl hydrolase.
12. The method of claim 8 or claim 11, wherein said glycoprotein is demannosylated with a mannosidase from *Cellulosimicrobium cellulans*.
13. The method of claim 8, wherein said mannosidase is a family 38 glycosyl hydrolase.
14. The method of claim 8 or claim 13, wherein said mannosidase is from *Canavalia ensiformis* or *Yarrowia lipolytica*.
15. A method of directing a glycoprotein to the interior of a mammalian cell, said method comprising

    a) providing a glycoprotein having a phosphorylated N-glycan, wherein said glycoprotein does not substantially bind to a mannose-6-phosphate receptor on said cell;

    b) contacting said glycoprotein with a mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated to produce a demannosylated glycoprotein, wherein said glycoprotein after said demannosylation, substantially binds to said mannose-6-phosphate receptor on said cell; and

    c) contacting said cell with said demannosylated glycoprotein.
16. The method of claim 15, wherein said mannosidase is a family 47 glycosyl hydrolase.
17. The method of claim 15 or claim 16, wherein said mannosidase is from *Aspergillus satoi*.
18. The method of claim 15, wherein said mannosidase is a family 92 glycosyl hydrolase.
19. The method of claim 15 or claim 18, wherein said mannosidase is from *Cellulosimicrobium cellulans*.
20. The method of claim 15, wherein said mannosidase is a family 38 glycosyl hydrolase.
21. The method of claim 15 or 20, wherein said mannosidase is from *Canavalia ensiformis* or *Yarrowia lipolytica*.

22. A method of converting a glycoprotein from a first form that does not substantially bind to a mannose-6-phosphate receptor on a mammalian cell to a second form that does substantially bind to a mannose-6-phosphate receptor on a mammalian cell, wherein in the first form, the glycoprotein comprises one or more N-glycans containing one or more mannose residues that are linked at the 1 position to a mannose residue that contains a phosphate residue at the 6 position, the method comprising contacting the first form of the glycoprotein with a mannosidase that demannosylates said terminal mannose residues.
23. The method of claim 22, wherein said mannosidase has uncapping and demannosylating activity.
24. The method of claim 22 or claim 23, wherein said mannosidase is a family 38 glycosyl hydrolase.
25. The method of any one of claims 22-24, wherein said mannosidase is from *Canavalia ensiformis* or *Yarrowia lipolytica*.
26. The method of claim 22, wherein said mannosidase does not have uncapping activity.
27. The method of claim 22 or claim 26, wherein said mannosidase is a family 47 or family 92 glycosyl hydrolase.
28. The method of claim 22, claim 26, or claim 27, wherein said mannosidase is from *Aspergillus satoi* or *Cellulosimicrobium cellulans*.
29. A method of directing a glycoprotein to the interior of a mammalian cell, the glycoprotein comprising one or more mannose-1-phospho-6-mannose moieties, the method comprising contacting the cell with the glycoprotein after (a) uncapping the one or more mannose-1-phospho-6-mannose moieties to mannose-6-phosphate on the glycoprotein, wherein, after uncapping, the glycoprotein does not substantially bind to a mannose-6-phosphate receptor on the cell and, after step (a), (b) demannosylating phosphorylated N-glycans on the glycoprotein, wherein after both the uncapping and the demannosylation, the glycoprotein does substantially bind to a mannose-6-phosphate receptor on the cell.
30. The method of claim 29, wherein step (a) and step (b) are catalyzed by two different enzymes.

31. The method of claim 29, wherein step (a) and step (b) are catalyzed by a single enzyme.
32. A method of directing a glycoprotein to the interior of a mammalian cell, said method comprising providing a glycoprotein having uncapped and demannosylated phosphorylated N-glycans, and contacting said mammalian cell with said glycoprotein.
33. The method of any one of claims 1-32, wherein said glycoprotein is a human protein.
34. The method of any one of claims 1-32, wherein said glycoprotein is a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein.
35. The method of claim 34, wherein said lysosomal protein is a lysosomal enzyme.
36. The method of claim 35, wherein said lysosomal enzyme is acid alpha glucosidase or alpha galactosidase.
37. The method of claim 33, wherein said glycoprotein is associated with a LSD.
38. The method of claim 37, wherein said LSD is Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.
39. A glycoprotein capable of being transported to the interior of a mammalian cell, wherein said glycoprotein has been treated with the method of any of claims 1-38.
40. A mammalian cell comprising said glycoprotein of claim 39.
41. The mammalian cell of claim 40, wherein said cell is a human cell.

42. A method of treatment, said method comprising administering the glycoprotein of claim 39 to a subject in need thereof.
43. An isolated fungal cell genetically engineered to produce glycoproteins comprising demannosylated phosphorylated N-glycans, said fungal cell comprising a nucleic acid encoding a mannosidase, said mannosidase capable of (i) hydrolyzing a mannose-1-phospho-6-mannose moiety to mannose-6-phosphate and (ii) hydrolyzing a terminal alpha-1,2 mannose, alpha-1,3 mannose and/or alpha-1,6 mannose linkage.
44. The fungal cell of claim 43, said fungal cell further comprising a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation.
45. The fungal cell of claim 43 or claim 44, wherein said fungal cell is genetically engineered to be deficient in OCH1 activity.
46. The fungal cell of any one of claims 43 to 45, said fungal cell further comprising a nucleic acid encoding a target protein, wherein said target protein is a glycoprotein.
47. The fungal cell of claim 46, wherein said target protein is a human protein.
48. The fungal cell of claim 46, wherein said target protein is a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein.
49. The fungal cell of claim 48, wherein said lysosomal protein is a lysosomal enzyme.
50. The fungal cell of claim 49, wherein said lysosomal enzyme is acid alpha glucosidase or alpha galactosidase.
51. The fungal cell of claim 47, wherein said glycoprotein is a protein associated with a LSD.
52. The fungal cell of claim 51, wherein said LSD is Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase

deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

53. The fungal cell of any one of claims 32 to 41, wherein said fungal cell is a *Yarrowia lipolytica* or *Arxula adeninivorans* cell.
54. The fungal cell of claim 33, wherein said polypeptide capable of promoting mannosyl phosphorylation is a MNN4 polypeptide.
55. The fungal cell of claim 43, wherein said MNN4 polypeptide is a *Yarrowia lipolytica*, *S. cerevisiae*, *Ogataea minuta*, *Pichia pastoris*, or *C. albicans* polypeptide.
56. The fungal cell of claim 44, wherein said polypeptide capable of promoting mannosyl phosphorylation is a *P. pastoris* PNO1 polypeptide.
57. The fungal cell of any one of claims 43 to 56, wherein said mannosidase comprises a secretion signal.
58. The fungal cell of any one of claims 43 to 56, wherein said mannosidase comprises a targeting signal to target said mannosidase to an intracellular compartment.

FIGURE 1A

ATGAAGCTTTCCACCATCCTCTTCACAGCCTGCGCTACCCTGGCTGCCGCCCAGCAGGGAGCCT
CTCGACCCGGACCCCGAGATGCCCAGGCTCACCCCGGACGACCTCGAGCTGTGCCCACCCAGTGTG
ACGTGCCCCCCAACTCTCGATTCGACTGTGCCCCCGACAAGGCCATCACCCAGGAGCAGTGCGAGG
CCCGAGGCTGTTGTTACATCCCCGCTAAGCAGGGCCTGCAGGGCGCTCAGATGGGCCAGCCCTGGT
GTTTCTTCCCCCCCTCTTACCCCTCCTACAAGCTGGAGAACCTGTCCTCTTCGGAGATGGGCTACAC
CGCCACCCTGACCCGAACCACCCCCACCTTTTTCCCCAAGGACATCCTGACCCTGCGACTGGACGTG
ATGATGGAGACCGAGAACCGACTGCACTTCACCATCAAGGACCCCGCCAACCGACGATACGAGGT
GCCCCTGGAGACCCCCCACGTGCACTCTCGAGCCCCTTCCCCCCTGTACTCTGTGGAGTTCTCTGAG
GAGCCCTTCGGCGTGATCGTGCGACGACAGCTGGACGGCCGAGTGCTGCTGAACACCACCGTGGCC
CCCCTGTTCTTCGCCGACCAGTTCCTGCAGCTGTCTACCTCTCTGCCCTCTCAGTACATCACCGGCCT
GGCCGAGCACCTGTCCCCCCTGATGCTGTCCACCTCTTGGACTCGAATCACCCTGTGGAACCGAGA
CCTGGCCCCCACCCCCGGTGCCAACCTGTACGGCTCTCACCCCTTCTACCTGGCCCTGGAGGACGGC
GGCTCTGCCCACGGCGTGTTTCTGCTGAACTCTAACGCCATGGACGTGGTGCTGCAGCCCTCTCCCG
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TGTGGTCCAGCAGTACCTGGACGTGGTCGGATACCCCTTCATGCCCCCCTACTGGGGCCTGGGCTTC
CACCTGTGTCGATGGGGCTACTCTTCTACCGCCATCACCCGACAGGTGGTGGAGAACATGACCCGA
GCCCACTTCCCCCTGGACGTGCAATGGAACGACCTGGACTACATGGACTCTCGACGAGACTTCACC
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AGCAGTTCCTGGTGTCTTGGTGTTAAC

FIGURE 1B

MKLSTILFTACATLAAAQQGASRPGPRDAQAHPGRPRAVPTQCDVPPNSRFDCAPDK
AITQEQCEARGCCYIPAKQGLQGAQMGQPWCFFPPSYPSYKLENLSSSEMGYTATLTRT
TPTFFPKDILTLRLDVMMETENRLHFTIKDPANRRYEVPLETPHVHSRAPSPLYSVEFSE
EPFGVIVRRQLDGRVLLNTTVAPLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTR
ITLWNRDLAPTPGANLYGSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRST
GGILDVYIFLGPEPKSVVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENM
TRAHFPLDVQWNDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISS
SGPAGSYRPYDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEF
HDQVPFDGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQF
LSTHYNLHNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGHWTGDVWSSWE
QLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMRNHNSLLSLP
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VDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPVEALGSLPPPPAAPREPAI
HSEGQWVTLPAPLDTINVHLRAGYIIPLQGPGLTTTESRQQPMALAVALTKGGEARGEL
FWDDGESLEVLERGAYTQVIFLARNNTIVNELVRVTSEGAGLQLQKVTVLGVATAPQQ
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FIGURE 2

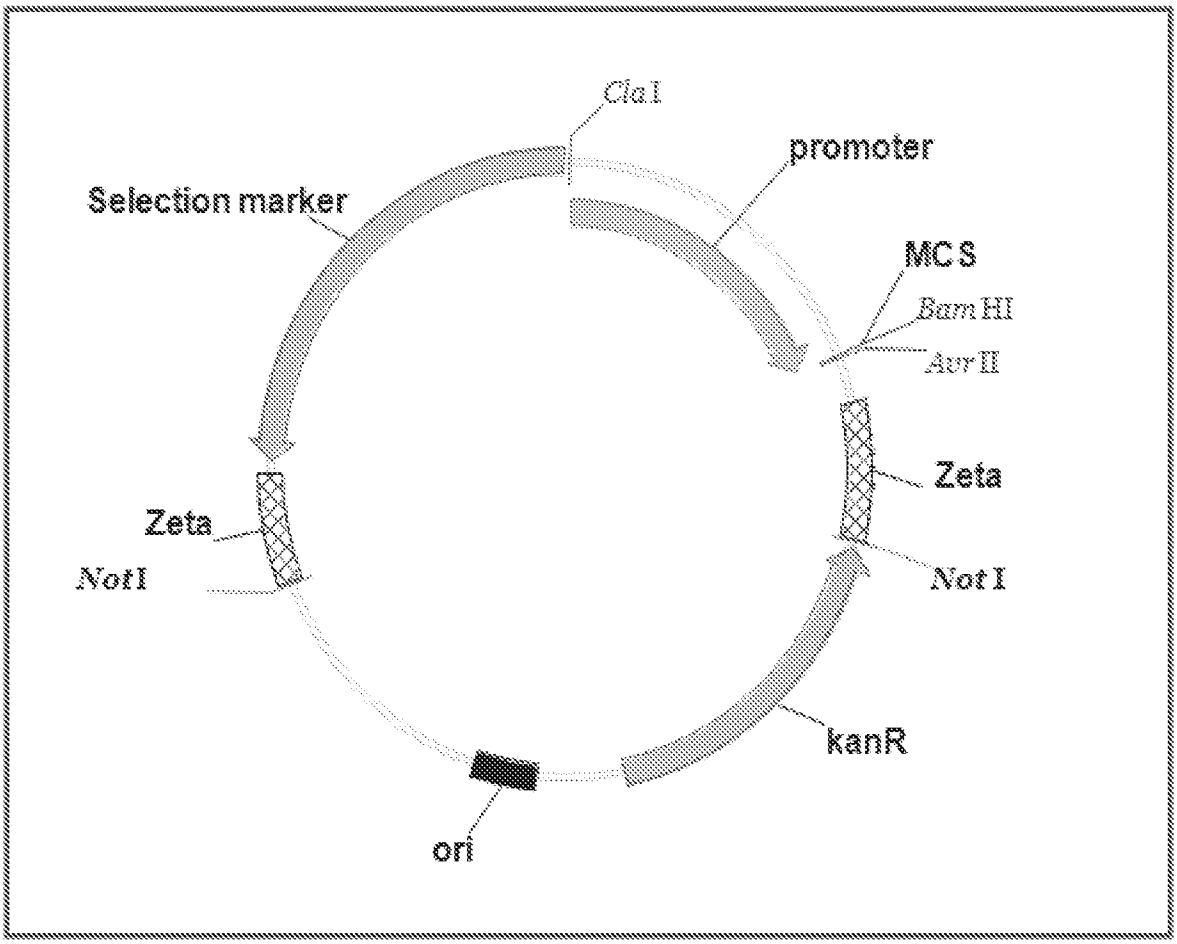
ClaI
promoter
Selection marker
MCS
BamHI
AvrII
Zeta
Zeta
NotI
NotI
kanR
ori

FIGURE 3A

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FIGURE 3B

atgcatcatcaccatcaccactattcgcacttcaacaacgagcctgtggcgaagcgggtgaacaacctgtttaccgaccgacttc
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FIGURE 3C

MYSHFNNEPVAKRVNNLFTDRLRQFTSDGEYRSLNLPAFYERERLDGKNHVAIETYAVSD
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NLNCEGLVFSESGEALIGLSGEERREWPIPDNWFDGKCHTFYIEASCNGMFGNATGSSIQ
PPSDNRYFRLDSADLVVINSEARHLFVDFWIIGDAAREFPGDSWQRGKALDVANKIMDAF
DPENPDESIAEGRKLAKEYLGDTTKAYKQQLPFADGLVYALGNCHIDTAWLWPFAETRRK
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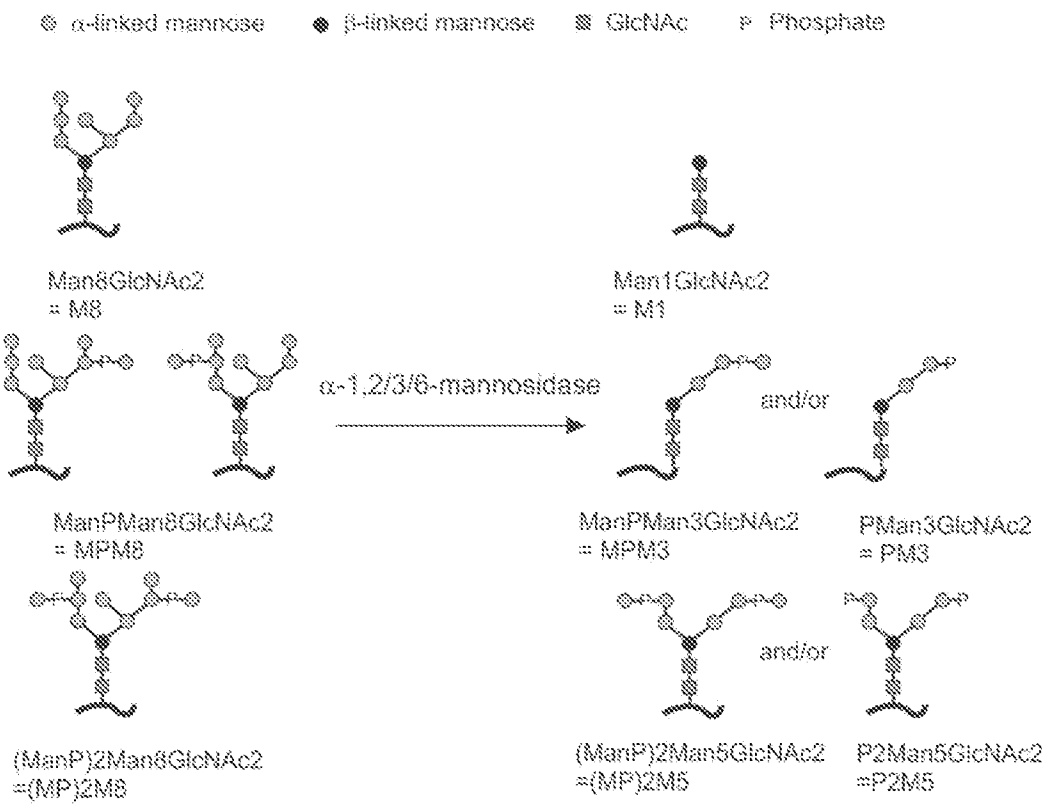
FIGURE 4


α-linked mannose
β-linked mannose
GlcNAc
P Phosphate
Man8GlcNAc2
= M8
Man1GlcNAc2
= M1
α-1,2/3/6-mannosidase
and/or
ManPMan8GlcNAc2
= MPM8
ManPMan3GlcNAc2
= MPM3
PMan3GlcNAc2
= PM3
and/or
(ManP)2Man8GlcNAc2
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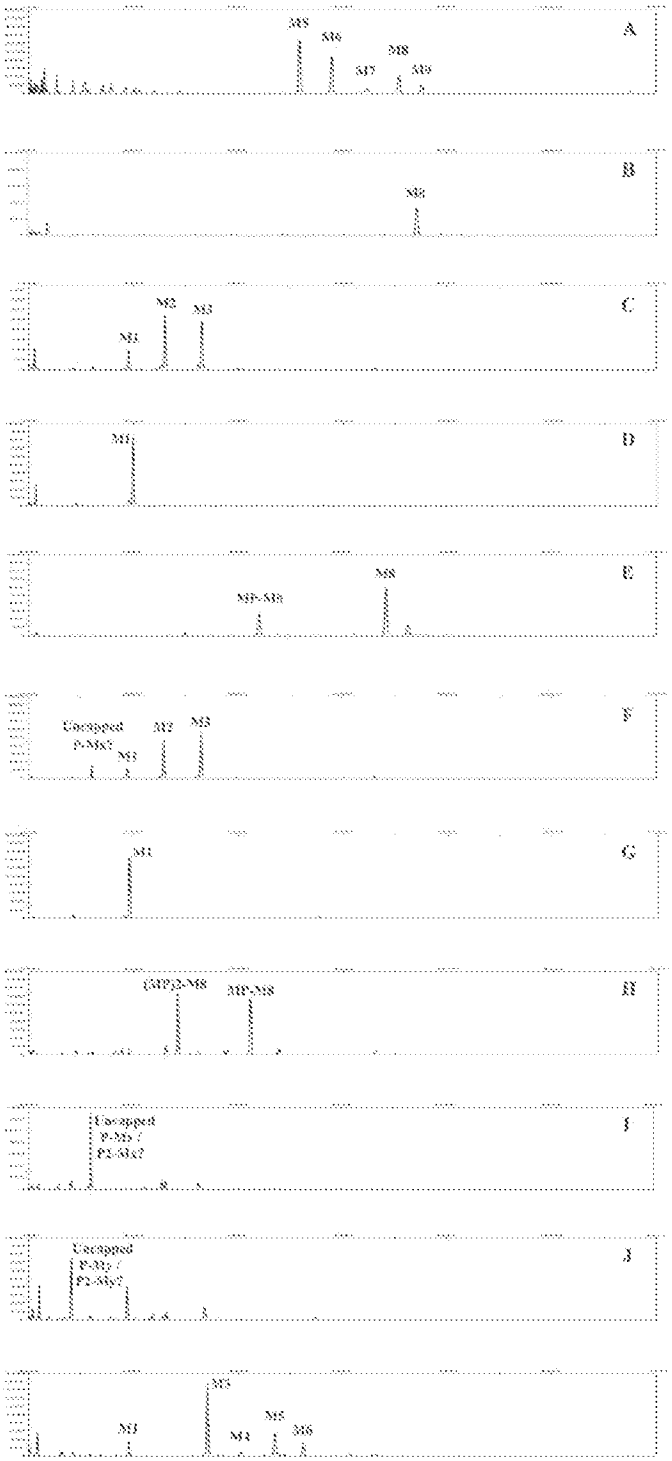
=P2M5

FIGURE 5



RNaseB

$Man_5GlcNAc_2$ control

$Man_5GlcNAc_2$ + JbMan
T = 120 min

$Man_5GlcNAc_2$ + JbMan
T = 24 h

Mnn4 N-glycans monophosphorylated control

Mnn4 N-glycans monophosphorylated +JbMan
T = 120 min

Mnn4 N-glycans monophosphorylated +JbMan
T = 24 h

Mnn4 N-glycans mono- and diphosphorylated control

Mnn4 N-glycans mono- and diphosphorylated + JbMan
T = 120 min

Mnn4 N-glycans mono- and diphosphorylated + JbMan
T = 24 h

Reaction J + CIP

FIGURE 6

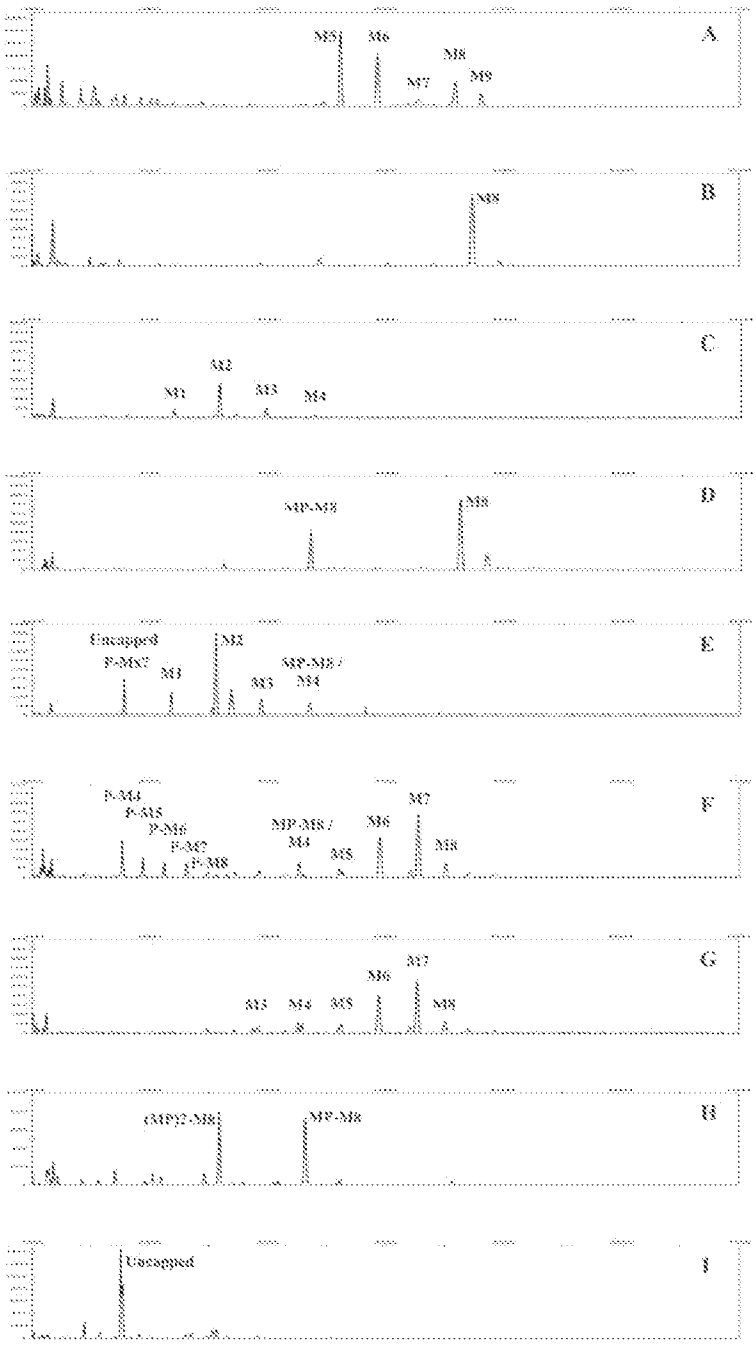

A
M5
M6
M7
M8
M9
RNaseB
B
M8
Man5GlcNAc2 control
C
M1
M2
M3
M4
Man5GlcNAc2 + Ams1
D
MP-M8
M8
Man4 N-glycans monophosphorylated control
E
Uncapped
P-M3
M2
M3
MP-M8 / M4
Man4 N-glycans monophosphorylated + Ams1
F
P-M4
P-M5
P-M6
P-M7
P-M8
MP-M8 / M4
M5
M6
M7
M8
Man4 N-glycans monophosphorylated + Ams1 (10x diluted), T = 120 min
G
M3
M4
M5
M6
M7
M8
Reaction F + CIP
H
(MP)2-M8
MP-M8
Man4 N-glycans mono- and diphosphorylated control
I
Uncapped
Man4 N-glycans mono- and diphosphorylated + Ams1

FIGURE 7

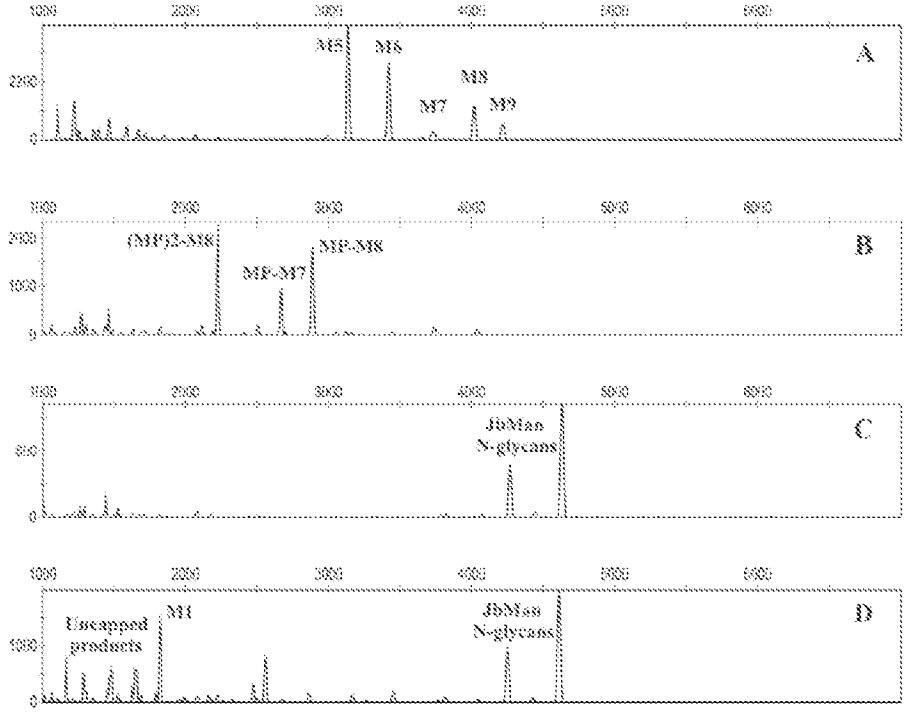

M5
M6
M8
M7
M9
A
RNaseB
(MP)2-M8
MP-M7
MP-M8
B
huGAA control
JbMan
N-glycans
C
JbMan control
Uncapped
products
M1
JbMan
N-glycans
D
huGAA + JbMan

FIGURE 8A

>DsbA-6xHis-CcMan5 (107bp - 5167bp, direct) 5061bp From pLSAHCcMan5

ATGAAAAAGATTTGGCTGGCGCTGGCTGGTTTAGTTTTAGCGTTTAGCGCATCGGCCGG
CCATCACCATCATCACCACGTGGGGCCCGGCTCGGACGAAGTGGATGCACCGGAACCTC
CGAGCGCAGATTATGCAAGCCTGGTTGATGTTTTTGTTGGCACCGAAGGTGATTTTGGT
AATGATATGCCTGCAGCACAGGCACCGAATGGTCTGGCAAAAGTTAATCCGCGTACCAC
ACCGGGTCGTAATAATACCGGTTATGATTATGCCCAGAGCAAAATTAGCGGTTTTACCC
ATACCAATCTGGATGGTGTTGGTGGTAGCGGTGGTGGTGGTGATCTGCTGGTTGTTCCG
ACCAGCGGTAGCTATACCGCACGTCCGGGTACAGGCACCTATGCACATCCGTTTAGCCA
TGATGATGAAGATGCAGGTCCGGGTTTTTATAGCGTTGGTCTGGGTAATGTTGCAGGCA
CCGATGGTGCAATTACCGGTGCTCCGGGTACAATTGAAGCAGAAGTTGCAGCAGCAACC
CGTAGCGGTGTTCATCGTTATGCATTTCCGGCAGGTAGCACCCCGAGCCTGGTTGTTGA
TCTGGAAACCAATAATACCAGCCGTCGTAGCAGCAGCGTTCAGGTTGAAACCCGTGCAG
ATGGCACCGTTGAACTGAGCGGTCAGGTTACCGGCTATTTTTATAATGCAGCCTATACC
CTGTATTATACCGCACGCACCCTGCAGCCTGCAACCGTTCAGACCTGGGGTGATGATGA
TCGTCTGGTTGATGCAACCGCACAGGATGGTGTTGATACCGGTGCAATTCTGACCTTTG
ATCCGGCAGATGCCGGTGAAATTGGTCTGCAGGTTACCCTGTCTCCGGTTAGCGTTGAA
CAGGCACGTATTGATCAGCAGGTTGAACTGGGTGATCTGAGCTTTGATGCAATTCGTGA
TCGTACCCGTGCAGAATGGAATGCAACCCTGGGTCGTGTTGCAATTGATGCAAGCACCG
CAACCGATCCGACCGGTGAACTGCAGCGTCTGTTTTATACCCATCTGTATCGCATGTTT
GCAATGCCGATGAATGCAACCAGCACCAGCGGCACCTATCGTGGTGTTGATGGTGCAGT
TCATGCAGCACAGGGCTTTACCTATTATGATAGCTGGGCAACCTGGGATGATTTTCGCA
AATTTAGCGTGATTGCCTATATTGATCCGGCACTGTATCGTGATATGGTTCAGAGCCTG
GTTTACCTGTTTGCAGATGCAGAAGCAACCGGTACAGGCGGTGGTCTGGGTGGTTTTGT
TCATAGCGTTCCGACCGTTCGTTGGGAACGTAGCAGCGTTGTTGTTGCAGATGCAATTG
CCAAAGGCTTTGATGGTTTTGATCGTCTGGATGAAGCATATCCGGCACTGCAGCGCCTG
GTTGGTCAGTATAGCGCAGATGAACTGCGTCGTGGTTATGTTGCAGGTAATCCGGGTGC
AAGCGTTCAGCGTGGTTATGATCAGTATGGTCTGAGCGTTATTGCCGATGAACTGGGTC
TGACCGAAGAAGCAGAAACCCTGCGCGAACAGGCAAGCTGGCCGATTGAAAAACTGACC
AAACCGGGTGCATGGACCGCAGCAGATGGTACACAGGTTGGTCTGCTGACACCGCGTGC
AGCCGATGGTAGCTGGCAGAGCGCAGATCATGCCAAATTTGAAGCAGCAGGTCTGTATC
AGGGCACCCTGTGGCAGTATCATTGGTATGATGCCTATGATATGGATGCACTGGTTGAA
GCAATGGGTGGTCATGAAGCAGCCCGTCTGGGTATGCGTCATATGTTTGGTGAACATGC
ACCGGATGATGGTAAAGCAATGCTGCATAGCAATGCCAATGAAATTGATCTGCAGGCAC
CGTACCTGTTTAATTATACCGGTGAACCGAGCCTGACCCAGAAATGGGCACGTGCAATT
TATACCAAAGAAACCTGGAATCGCTATATTGCAACCGGTAGCAGCTCTGCAGTTCCGTC
AGGTGGTGGTGAATTTACACCTCCGCTGAAAACCAAAGTTTATCGTCTGGACCCTCGTG
GTATGCTGCCGACCATGGATAATGATGCAGGTACAATGAGCACCATGTTTGTTGCAGCA
GCCGTTGGTCTGTTTCCGGTTACCGCAGGTAGCAGCCAGTTTCAGGTTGGTAGCCCGTT
TTTTGATAGCACCACCATTACCTATGATGATGGTAGCGCATTTACCGTTACCGCAGATG
GTGTTAGCGAAGATGCCTTTTATGTTCAGAGCGCAACCCTGGATGGTGCAACCTTTGGT
AATACCTGGGTTGATTATGCAACCGTTGTTGGTGGTGCAGATCTGGCATTTCGTATGGG
TGAACAGCCGAGCGATTGGGGCACCGATACCGCACCGGCATTTAGCATGAGCACCGCCA

FIGURE 8A (CONTINUED)

CCGATGAACCGGCAGAAGGTCCTCGCGTTAGCGCAGAACCGACCACCGTGCAGACCGGT
GATGGTGGTGCACTGGATGCAACCGTTACCCTGACACTGGATGGCGCACGTCTGGCAGC
ACCGGCAGGTACAGATCTGGTTACCAGCGGTGCAGCAAGCGTTGTTGGTCTGCCGGATG
GTGTTACCGCAGCAGTTACCGTTGCAAGCCCGACCGCACTGACCGTTAGCCTGACCGGC
ACCGCATCAGCAGATGCACGTTTTTTTGTGCATCTGCGTGATGCAGCACTGGCCGATGG
TGTTGCAGCCGCAAGCCTGCAGGGTCAGGGTGTTAGCGTTCGTTCTCCGCTGCGTCTGA
GCGTTGCAAGCGCAGAACGTGATGCACTGGCAGCACTGGTTGATGATGCCGTTCTGGTT
CGTCATGGTAATTATAGCAGCGTTACCTTTGATCGTTTAGCACCGCTCTGACAAAAGCA
CAGGAAGCACTGGGCGACGAAGCAGCAACCAGCATTGCACTGCGTTTTGCAGCAGATCG
TCTGGGTGCAGCAGCAGATGCACTGGATCTGACCGGTGGTGGTTATCGTACCCTGGAAG
CAGAACAGAGCGAAGCATGGTCTGGTGGTGAACTGAAAAATGAAGCCAATAGCAGCAGC
GGTAATCTGGGTGGTGTTCGTAGCGGTAGCTGGGTTCAGTATCGCGATATGACCTTTGA
AACCGCAGCCGGTGATACACCTCCGCGTTTTCTGACCGTTCGTTATGATACCAGCTTTG
CACCGACCGATACCCCGAGCACCGTTCGTGTTCATGCCGGTGATGTTTCTGGTCCGGTT
GTTGCAACCGTTGATCTGAAAGGCACCAGCGGTTGGGGTAAATATACCGAAGTTACCGC
AGAACTGGGTGATGTTCAGGCCCTGGTTGATGCCCAGGTTGTTACCTTTGAACTGCTGG
CACCGAGCGGTCGTAGCTGGGTTGGTAATTTTGATTGGTTTCGCTTTAGCGCAGAAGAT
CCGGCAGCACCGGGTCAGCCTGGTGAAAGCCCGACCGTTACCATTGAAGCCGAAGATTG
GACCGCAAGCAGCGGTCGTGGTCTGAAAAAAGAAAGCAGCACCTGGACCAGCGGTCCGG
TGACCAATGTTGGTGGTACAGCAGATGGTGATTGGATTGCCTATGGTGAAGTTGATCTG
GGTGAACTGCCGCTGGGCGAACTGAGCGTTCATTATGTGCATAATAGCAATCGCAGCGG
TAATAATAGCGCACTGAGCGTTTATCTGGATGCATTTGATCCGGCTAATCCGGGTGAAC
CGTTTGTTACCGTTCCGCTGCCGACCACCGGTAGCAGTTGGACCGCAGATGGCACAGCC
ACCGTTGTTCTGCCGGAAACCGTGCAGGGCACCCATGAAGTTTTTGTTCGTCTGAGCAC
CGAACCGTATGCAGATCATCCGTATGTTGCAAATCTGGATAGCCTGACCTTTGCACCGG
GTGGTCCGACCAGCGTTGTGGTTGAAAGCGAAGCCTGGACCAGCAATTCTGGTCGTGGC
CTGAAAAATGAATCTTCTACCTGGACCTCTGGTCCGGTTACAAATGTGGGTGGCACCGC
TGATGGCGATTGGCTGGCATATGGCGAAATTGATCTGGGCAGCGCAGCACTGGATCAGC
TGTCTGTGCATTATGTTCATAATTCTAATCGCTCTGGTCGTAATTCTGCACTGTCTGTG
TATCTGGATGCCTTTGATCCGGCAAATCCGGGTGAACCGTTTGTGACAGTGCCGCTGGC
AAATACCGGTAGCTCTTGGACCACCGATGGTACTGCAGTTGTGGATCTGCCGTCTACCG
TTCGTGGTAAACATCAGGTTTGGGTTCGTCTGTCTACCGAAGCATATGCCGATCATCCG
TATGTGGCCAATCTGGATTCTATGCGCTTTTTTACCGATGCATATGATGTTGAAGTTCC
TCCGACCGATACAGCAGCACTGGCAGCCGTTGTTGATGCAGCAGGTACACCGGAAGCAG
AAATTGCACGTTATGGTCGTATTGATGCCCGTGTTTTTACCCGTGAACTGGCAGCAGCA
CGTAGCGTTCTGGCCGATGCCGGTGCAACACAGGCACAGGCAGATGAACGTGCTCGTCG
TCTGGGTCTGGCAACCGATCAGCTGGTTCCGGCAGAACGTCGTCGTCTGGAAAATCTGG
TTGCCAGCGCAGAAGCACTGACCGACGAAGGTTATTCTCCGGAAAGCTGGCAGGCATTT
CGTACCGCACTGGCTGCTGCAACCGGCACCCTGGATGATGCAGCAGCATCTGATGAAGC
ACTGCATGATGCACGTCTGGCGCTGCAGGGTGCAGTTGATGCACTGGAAGAACCGGCAG
ATGTTGTTCTGGTTGAAGTTGAAGTTTCTCCGCGTTGTCTGGCAGGTAAACCGTATGTT
GCCGTTCGTGCAGTTAATGTTTCTGATGCAGCCGTTGATGTTGAACTGGCAAGCTCTCT
GGGCACCCGTAGCTTTGTTGGTGTGGCACCGGGTGCGAGCGCATATCAGAGCTTTGCAG
CCCGTAGCGCAACCGGTGATCTGGATGTTACCGTGACCGCAACCGGTGCAGATGGTACT
CAGACCGTTGAACAGGTTGTGACCGTTCCGAGCTGTAGCTAATAA

FIGURE 8B

ALAVVGLAPATAASAAPEPPSADYASLVDVFVGTEGDFGNDMPAAQAPNGLAKVNPRTT
PGRNNTGYDYAQSKISGFTHTNLDGVGGSGGGGDLLVVPTSGSYTARPGTGTYAHPFSH
DDEDAGPGFYSVGLGNVAGTDGAITGAPGTIEAEVAAATRSGVHRYAFPAGSTPSLVVD
LETNNTSRRSSSVQVETRADGTVELSGQVTGYFYNAAYTLYYTARTLQPATVQTWGDDD
RLVDATAQDGVDTGAILTFDPADAGEIGLQVTLSPVSVEQARIDQQVELGDLSFDAIRD
RTRAEWNATLGRVAIDASTATDPTGELQRLFYTHLYRMFAMPMNATSTSGTYRGVDGAV
HAAQGFTYYDSWATWDDFRKFSVIAYIDPALYRDMVQSLVYLFADAEATGTGGGLGGFV
HSVPTVRWERSSVVVADAIAKGFDGFDRLDEAYPALQRLVGQYSADELRRGYVAGNPGA
SVQRGYDQYGLSVIADELGLTEEAETLREQASWPIEKLTKPGAWTAADGTQVGLLTPRA
ADGSWQSADHAKFEAAGLYQGTLWQYHWYDAYDMDALVEAMGGHEAARLGMRHMFGEHA
PDDGKAMLHSNANEIDLQAPYLFNYTGEPSLTQKWARAIYTKETWNRYIATGSSSAVPS
GGGEFTPPLKTKVYRLDPRGMLPTMDNDAGTMSTMFVAAAVGLFPVTAGSSQFQVGSPF
FDSTTITYDDGSAFTVTADGVSEDAFYVQSATLDGATFGNTWVDYATVVGGADLAFRMG
EQPSDWGTDTAPAFSMSTATDEPAEGPRVSAEPTTVQTGDGGALDATVTLTLDGARLAA
PAGTDLVTSGAASVVGLPDGVTAAVTVASPTALTVSLTGTASADARFFVHLRDAALADG
VAAASLQGQGVSVRSPLRLSVASAERDALAALVDDAVLVRHGNYSSVTFDRFSTALTKA
QEALGDEAATSIALRFAADRLGAAADALDLTGGGYRTLEAEQSEAWSGGELKNEANSSS
GNLGGVRSGSWVQYRDMTFETAAGDTPPRFLTVRYDTSFAPTDTPSTVRVHAGDVSGPV
VATVDLKGTSGWGKYTEVTAELGDVQALVDAQVVTFELLAPSGRSWVGNFDWFRFSAED
PAAPGQPGESPTVTIEAEDWTASSGRGLKKESSTWTSGPVTNVGGTADGDWIAYGEVDL
GELPLGELSVHYVHNSNRSGNNSALSVYLDAFDPANPGEPFVTVPLPTTGSSWTADGTA
TVVLPETVQGTHEVFVRLSTEPYADHPYVANLDSLTFAPGGPTSVVVESEAWTSNSGRG
LKNESSTWTSGPVTNVGGTADGDWLAYGEIDLGSAALDQLSVHYVHNSNRSGRNSALSV
YLDAFDPANPGEPFVTVPLANTGSSWTTDGTAVVDLPSTVRGKHQVWVRLSTEAYADHP
YVANLDSMRFFTDAYDVEVPPTDTAALAAVVDAAGTPEAEIARYGRIDARVFTRELAAA
RSVLADAGATQAQADERARRLGLATDQLVPAERRRLENLVASAEALTDEGYSPESWQAF
RTALAAATGTLDDAAASDEALHDARLALQGAVDALEEPADVVLVEVEVSPRCLAGKPYV
AVRAVNVSDAAVDVELASSLGTRSFVGVAPGASAYQSFAARSATGDLDVTVTATGADGT
QTVEQVVTVPSCS (SEQ ID NO: 7)

FIGURE 8C

APEPPSADYASLVDVFVGTEGDFGNDMPAAQAPNGLAKVNPRTTPGRNNTGYDYAQSKISGF
THTNLDGVGGSGGGGDLLVVPTSGSYTARPGTGTYAHPFSHDDEDAGPGFYSVGLGNVAGTD
GAITGAPGTIEAEVAAATRSGVHRYAFPAGSTPSLVVDLETNNTSRRSSSVQVETRADGTVE
LSGQVTGYFYNAAYTLYYTARTLQPATVQTWGDDDRLVDATAQDGVDTGAILTFDPADAGEI
GLQVTLSPVSVEQARIDQQVELGDLSFDAIRDRTRAEWNATLGRVAIDASTATDPTGELQRL
FYTHLYRMFAMPMNATSTSGTYRGVDGAVHAAQGFTYYDSWATWDDFRKFSVIAYIDPALYR
DMVQSLVYLFADAEATGTGGGLGGFVHSVPTVRWERSSVVVADAIAKGFDGFDRLDEAYPAL
QRLVGQYSADELRRGYVAGNPGASVQRGYDQYGLSVIADELGLTEEAETLREQASWPIEKLT
KPGAWTAADGTQVGLLTPRAADGSWQSADHAKFEAAGLYQGTLWQYHWYDAYDMDALVEAMG
GHEAARLGMRHMFGEHAPDDGKAMLHSNANEIDLQAPYLFNYTGEPSLTQKWARAIYTKETW
NRYIATGSSSAVPSGGGEFTPPLKTKVYRLDPRGMLPTMDNDAGTMSTMFVAAAVGLFPVTA
GSSQFQVGSPFFDSTTITYDDGSAFTVTADGVSEDAFYVQSATLDGATFGNTWVDYATVVGG
ADLAFRMGEQPSDWGTDTAPAFSMSTATDEPAEGPRVSAEPTTVQTGDGGALDATVTLTLDG
ARLAAPAGTDLVTSGAASVVGLPDGVTAAVTVASPTALTVSLTGTASADARFFVHLRDAALA
DGVAAASLQGQGVSVRSPLRLSVASAERDALAALVDDAVLVRHGNYSSVTFDRFSTALTKAQ
EALGDEAATSIALRFAADRLGAAADALDLTGGGYRTLEAEQSEAWSGGELKNEANSSSGNLG
GVRSGSWVQYRDMTFETAAGDTPPRFLTVRYDTSFAPTDTPSTVRVHAGDVSGPVVATVDLK
GTSGWGKYTEVTAELGDVQALVDAQVVTFELLAPSGRSWVGNFDWFRFSAEDPAAPGQPGES
PTVTIEAEDWTASSGRGLKKESSTWTSGPVTNVGGTADGDWIAYGEVDLGELPLGELSVHYV
HNSNRSGNNSALSVYLDAFDPANPGEPFVTVPLPTTGSSWTADGTATVVLPETVQGTHEVFV
RLSTEPYADHPYVANLDSLTFAPGGPTSVVVESEAWTSNSGRGLKNESSTWTSGPVTNVGGT
ADGDWLAYGEIDLGSAALDQLSVHYVHNSNRSGRNSALSVYLDAFDPANPGEPFVTVPLANT
GSSWTTDGTAVVDLPSTVRGKHQVWVRLSTEAYADHPYVANLDSMRFFTDAYDVEVPPTDTA
ALAAVVDAAGTPEAEIARYGRIDARVFTRELAAARSVLADAGATQAQADERARRLGLATDQL
VPAERRRLENLVASAEALTDEGYSPESWQAFRTALAAATGTLDDAAASDEALHDARLALQGA
VDALEEPADVVLVEVEVSPRCLAGKPYVAVRAVNVSDAAVDVELASSLGTRSFVGVAPGASA
YQSFAARSATGDLDVTVTATGADGTQTVEQVVTVPSCS (SEQ ID NO:8)

FIGURE 9A

DsbA-6xHis-CcMan4(107bp - 5494bp, direct) 5388bp from pLSAHCcMan4

ATGAAAAAGATTTGGCTGGCGCTGGCTGGTTTAGTTTTAGCGTTTAGCGCATCGGCCGG
CCATCACCATCATCACCACGTGGGGCCCGGCTCGGACGAAGTGGATGCAGAACCGGGTG
ATTTTAGCAGCAGCTTTGAATCTGGCGATCCGGCAGCACTGCCGACCACCGTTGCAGAA
CGTGATGGTGCACCGTGGCAGGCAAATGTTGGTAGCTTTACCGCAGGTCTGCCTGGTAG
CGTTCTGGGTCAGCTGAAAGGTGTTACCGCAAGCGCACAGAATCTGCCGAATGAAGGTG
CAGCAAATCTGGCAGATGGTAGCAGCGGCACCAAATGGCTGGCATTTGCAAGCACCGGT
TGGGTTCGTTATGAATTTGCAGAACCGGTTAGCTTTGTTGCATATACCATGACCAGCGG
TGATGATGCCGCAGGTCGTGATCCGAAAACCTGGACCGTTGAAGGTAGCAATGATGGTT
CTACCTGGGCAGCACTGGATCGTCGTACCGATGAAGATTTTCCGAATCGTCAGCAGACC
CGTACCTTTGAACTGGAAGCACCGACCGCAGCATATACCTATCTGCGTCTGAATGTTAC
CGCAAATAGCGGTGATAGCATTGTTCAGCTGGCAGGTTGGGATCTGAGCGCAGATCTGT
CTGCAGGTCCGAGCGCAGCACCGATGACCACCAAAGTTGGCACCGGTCCGCGTGTTAGC
TTTACCAATAAAGCCGGTGTTGGTTTTAGCGGTCTGCATAGCCTGCGTTATGATGGTAG
CCATCTGGCCGATGGTGAAACCTATGCAACCAATGTGCTGTATGATGATGTTGATGTTG
TGGTTGGTGAAGATACCCGTCTGAGCTATACCATTTTTCCGGAACTGCTGGATGATCTG
CAGTATCCGAGCACCTATGCAGCAGTTGATGTTCTGTTTACCGATGGCACCTATCTGAG
CGATCTGGGTGCACGTGATGCACATGAAACCGTTGCAACCGCACAGGCACAGGGTGAAG
GTAAAATTCTGTATGCCGATCAGTGGAATAGCGTTCGTGTTGATCTGGGTGATGTTGCA
GAAGGTAAAACCGTTGATCAGGTTCTGCTGGGTTATGATAATCCGGGTGGTCATGCAGG
CACCAAATTTGCAGGTTGGCTGGATGATGTTGAAATTACCGCAGAACCGGCAACCATTG
ATGGTAGCTCACTGGCAAATTATGTTGATACCCGTCGTGGCACCCTGGCAAGCGGTAGC
TTTAGCCGTGGTAATAATATTCCGGCAACCGCAACCCCGAATGGTTTTAATTTTTGGAC
CCCGTATACCAATGCAAGCAGCCAGAGCTGGCTGTATGAATATCATAAAGCCAATAATG
CGAATAATAAACCGGTTCTGCAGGGTTTTGGTATTAGCCATGAACCGAGCCCGTGGATG
GGTGATCGTAATCAGCTGACCTTTCTGCCGAGCACCGCAAGCGGTACACCGGATGCAAC
CCTGAGCACCCGTGGTCTGGAATTTGATCATGCAGATGAAACCGCACGTCCGGATTATT
ATGGTGTGACCTTTACCAATGGTAGCGCAATTGAAGCAACCCCGACCGATCATGGTGCA
GTTCTGCGTTTTAGCTATCCGGGTGCAAAAGGTCATGTTCTGGTGGATAAAGTTGATGG
TAGCAGTAAACTGACCTATGATCAGGCAACCGGCACCATTAGCGGTTGGGTTGAAAATG
GTAGCGGTCTGAGCGTTGGTCGTACCCGTATGTTTGTTGCAGGCACCTTTGATCGTAGC
CCGACCGCAGTTGGCACAGCAGCAGGTAATCGTGCAGATGCACGTTTTGCAACCTTTGA
AACCAGCAGCGATAAAACCGTGGAACTGCGTGTTGCAACCAGCTTTATTAGCCTGGATC
AGGCACGTAAAAATCTGGATCTGGAAGTTACCGGTAAAACCTTTACCGAAGTTAAAGCA
GCAGCAGCACAGGCATGGAATGATCGTCTGGGTGTTATTGAAGTTGAAGGTGCAAGCGA
AGATCAGCTGGTTACCCTGTATAGCAATCTGTATCGCCTGAATCTGTATCCGAATAGCC
AGTTTGAAAATACCGGCACCGCACAGGAACCGGTTTATCGTTACGCATCTCCGGTTAGC
GCAACCACCGGTAGCGCAACCGATACCCAGACCAATGCCAAAATTGTGGATGGCAAAAT
TTATGTGAATAATGGCTTTTGGGATACCTATCGTACCGCATGGCCTGCATATAGCCTGC
TGTATCCGGAACTGGCAGCAGAACTGGTTGATGGTTTTGTTCAGCAGTATCGTGATGGT
GGTTGGATTGCACGTTGGAGCAGTCCGGGTTATGCAGATCTGATGACCGGTACAAGCTC
TGATGTTGCATTTGCAGATGCCTATCTGAAAGGTAGCCTGCCGACCGGTACAGCACTGG
AAGCATATGATGCAGCACTGCGTAATGCAACCGTTGCACCTCCGAGCAATGCAGTTGGT

FIGURE 9A (CONTINUED)

CGTAAAGGTCTGCAGACAAGCCCGTTTCTGGGTTTTACACCGGAAAGCACCCATGAAAG
CGTTAGCTGGGGTCTGGAAGGTCTGGTTAATGATTTTGGCATTGGCAATATGGCTGCAG
CACTGGCAGAAGATCCGGCAACACCGGAAGAACGTCGTGAAACCCTGCGTGAAGAAAGC
GCATATTTTCTGGAACGTGCCACCCATTATGTTGAACTGTTTGATCCGGAAGTGGATTT
TTTTGTTCCGCGTCATGAAGATGGTACATGGGCAGTTGATCCGGAAACCTATGATCCGG
AAGCATGGGGTGGTGGTTATACCGAAACCAATGGCTGGAATTTTGCATTTCATGCACCG
CAGGATGGTCAGGGTCTGGCAAATCTGTATGGTGGTAAACAGGGTCTGGAAGATAAACT
GGATGAATTTTTTAGCACACCGGAAAAAGGTGCAGGTAATGGTGGTATTCATGAACAGC
GTGAAGCACGTGATGTTCGTATGGGTCAGTGGGGTATGAGCAATCAGGTTAGCCATCAT
ATTCCGTGGCTGTATGATGCAGCCGGTGCTCCGAGCAAAGCACAGGAAAAAGTTCGCGA
AGTTACCCGTCGTCTGTTTGTTGGTAGCGAAATTGGTCAGGGTTATCCGGGTGATGAAG
ATAATGGTGAAATGTCCTCCTGGTGGATTTTTGCAAGCCTGGGTTTTTATCCGCTGCAG
GTTGGTAGCGATCAGTATGCAGTTGGTTCTCCGCTGTTTGATAAAGCAACCGTTCATCT
GCCGGATGGTGATCTGGTTGTTAATGCCGAAAATAATAGCGTGGATAATGTGTATGTTC
AGAGCCTGGCAGTTGATGGTGAAGCACGTACCAGCACCAGCCTGAGCCAGGCAGATCTG
AGCGGTGGCACCACCCTGGATTTTGTTATGGGTCCGGAACCGAGCGATTGGGGCACCGG
TGAAGATGATGCACCTCCGTCACTGACCGAAGGTGATGAACCTCCGACACCGGTTCAGG
ATGCAACCACCGCAGGCCTGGGCACCACCACCGTTGCCGATGGTGATGCCACCACCTCT
GCAGCAGCCCTGACCGATAATACCAGCGGCACCCGTACCACCTTTGCAACCACCACCCC
GAGCATTACATGGGCAGGTAATGGCATTCGTCCGACCGTTGGTAGCTATACCCTGACCT
CTGGTGCAAGCGGCACCGCAAGCCCGTCTGCATGGACCCTGGAAGGTTCTGATGATGGC
GAAACCTGGACCACACTGGATGAACGTAGCGGTGAACAGTTTCGTTGGGCACTGCAGAC
CCGTCCGTTTACCGTTGCCGAACCGACCGCATTTGCACGTTATCGTGTTACCGTTACCG
CAACCAGCGGTTCTGGTGCACTGAGCCTGGCAGAAGTTGAACTGCTGGCAGATCCGAAA
GAAAGCGGTGCAGAAGAACTGACCCTGTCTGCAGCACCGGATCGTGATGGCGTTACCGG
TCGTGAAGTTAGCGGTTCTTTTGCAACCCTGACCGGTGTTGAAGGTGATGTTGCCGCAC
TGGATGTTCAGGTTGCATTTGGTGATGGTAGCGAACCGGTTGCAGGTACACTGCGTGCC
GGTGCATTTGGTGGTTATGCAGTTGATGCAGCACATACCTGGACCGCACCGGGTGTTTA
TCCGGTTACCGTGACCGTTAGCGGTGAAGGTATTGAAACCGTTAGCGCAAGCAGCTATG
TTAGCGTTAGCCTGCTGCGTGAAGGTTCTCTGCTGGCAGCATATGATAATGTGTGCATT
GGTGATGCAGGTACAACCGTTGGTTCTTGTGATGGTCAGGGCGTTTTTTTTGATCGTGC
ACAGCTGGCAGCAAAAGGTTTTGTGCAGGGTGAACGTGCAACCGTTCCGGGTACAGATC
TGGCATTTGATGTTCCGGCAGTTCCGGCTGGTCAGCCTGATAATGCAACCGGTGATGGT
CAGACCATTGAACTGGATGTTCCGGCTGATGCAGAACAGCTGAGCGTTATTGGCACCGG
CACCGAAAAAAATCAGCAGGCAACCGGTACACTGACCTTTGATGATGGTTCTACCCAGC
CGATTGATCTGAGCTTTGGTGATTGGAGCGGTGCAGCACGTAATCCGGTGTTTGGTAAT
ATTCCGGTTGCAGTTACCGATAGCCGTCTGCGTGGTGGTTCTCCGCAGACCGGTACACC
GGCAGCATTTTTTGCCACCGCACCGATTACCCTGCCGGAAGGTAAACGTCCGGTTAGCC
TGACCCTGCCGGATCAGCCTGGTGAACTGAGCCGTGATGGTCGTATTCATGTTGTTGCA
GTTGCACATGATGGCACCTTTGCAGAACATCCTGCACTGGAAGTGACCGCAGCAGAAGG
TGTTACCCTGGCAGTTGGTCAGACCTCAGATGTTGCACTGGCACAGGTTGCCGGTGGTC
GTGAAGGTGCAGATCTGCGTGCCGCAGTTACCTGGGGTGATGGTTCTGATGTGGCAGCC
GGTGCCGTTACCGATGGTAGCGTTAGCGGTAGCCATGCATATACCGCAGCAGGCACCTA

FIGURE 9A (CONTINUED)

TACCGCATATGTTGTTGTGGATGATGGTTGGACCAGCCAGGTTGTTGAAGTTCCGGTGA
CCGTTACAGAAGCCGAACCGGCACTGGCCGTTGATGTCACCGTTAGCACCCGTTGCCTG
GCAGGTAAAGCATATGTTGCAGTGCGTGCAGAAAATGGTGAAGATGTTCCGCTGGCAAT
TCGTCTGGTTACCCCGTTTGGCACCAAAGAAGTTGCAGCAGTTGCTCCGGGAGCCAATG
CATATCAGAGCTTTGCAACCCGTGTTACCGCAGTTGAAGCAGGCACCGTTACCGTTGAA
GCCACCCGTGGCACCGGTGATGAAGAAGTTACCGCCAGCATTCAGGCAGATTATGCAGC
CGTTACCTGCGGTTAATAA

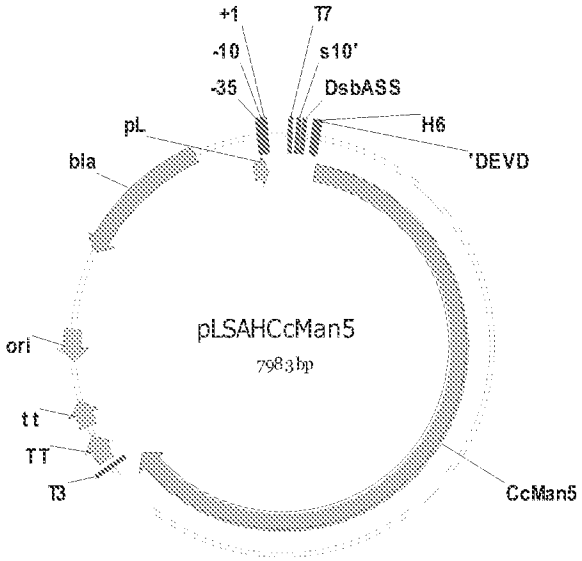
FIGURE 9B

MTRPLPPGRAVARSGSGRARPLGLVLAAALAVPLGVPLAAPAGALAAAPAAAAEPGDFS
SSFESGDPAALPTTVAERDGAPWQANVGSFTAGLPGSVLGQLKGVTASAQNLPNEGAAN
LADGSSGTKWLAFASTGWVRYEFAEPVSFVAYTMTSGDDAAGRDPKTWTVEGSNDGSTW
AALDRRTDEDFPNRQQTRTFELEAPTAAYTYLRLNVTANSGDSIVQLAGWDLSADLSAG
PSAAPMTTKVGTGPRVSFTNKAGVGFSGLHSLRYDGSHLADGETYATNVLYDDVDVVVG
EDTRLSYTIFPELLDDLQYPSTYAAVDVLFTDGTYLSDLGARDAHETVATAQAQGEGKI
LYADQWNSVRVDLGDVAEGKTVDQVLLGYDNPGGHAGTKFAGWLDDVEITAEPATIDGS
SLANYVDTRRGTLASGSFSRGNNIPATATPNGFNFWTPYTNASSQSWLYEYHKANNANN
KPVLQGFGISHEPSPWMGDRNQLTFLPSTASGTPDATLSTRGLEFDHADETARPDYYGV
TFTNGSAIEATPTDHGAVLRFSYPGAKGHVLVDKVDGSSKLTYDQATGTISGWVENGSG
LSVGRTRMFVAGTFDRSPTAVGTAAGNRADARFATFETSSDKTVELRVATSFISLDQAR
KNLDLEVTGKTFTEVKAAAAQAWNDRLGVIEVEGASEDQLVTLYSNLYRLNLYPNSQFE
NTGTAQEPVYRYASPVSATTGSATDTQTNAKIVDGKIYVNNGFWDTYRTAWPAYSLLYP
ELAAELVDGFVQQYRDGGWIARWSSPGYADLMTGTSSDVAFADAYLKGSLPTGTALEAY
DAALRNATVAPPSNAVGRKGLQTSPFLGFTPESTHESVSWGLEGLVNDFGIGNMAAALA
EDPATPEERRETLREESAYFLERATHYVELFDPEVDFFVPRHEDGTWAVDPETYDPEAW
GGGYTETNGWNFAFHAPQDGQGLANLYGGKQGLEDKLDEFFSTPEKGAGNGGIHEQREA
RDVRMGQWGMSNQVSHHIPWLYDAAGAPSKAQEKVREVTRRLFVGSEIGQGYPGDEDNG
EMSSWWIFASLGFYPLQVGSDQYAVGSPLFDKATVHLPDGDLVVNAENNSVDNVYVQSL
AVDGEARTSTSLSQADLSGGTTLDFVMGPEPSDWGTGEDDAPPSLTEGDEPPTPVQDAT
TAGLGTTTVADGDATTSAAALTDNTSGTRTTFATTTPSITWAGNGIRPTVGSYTLTSGA
SGTASPSAWTLEGSDDGETWTTLDERSGEQFRWALQTRPFTVAEPTAFARYRVTVTATS
GSGALSLAEVELLADPKESGAEELTLSAAPDRDGVTGREVSGSFATLTGVEGDVAALDV
QVAFGDGSEPVAGTLRAGAFGGYAVDAAHTWTAPGVYPVTVTVSGEGIETVSASSYVSV
SLLREGSLLAAYDNVCIGDAGTTVGSCDGQGVFFDRAQLAAKGFVQGERATVPGTDLAF
DVPAVPAGQPDNATGDGQTIELDVPADAEQLSVIGTGTEKNQQATGTLTFDDGSTQPID
LSFGDWSGAARNPVFGNIPVAVTDSRLRGGSPQTGTPAAFFATAPITLPEGKRPVSLTL
PDQPGELSRDGRIHVVAVAHDGTFAEHPALEVTAAEGVTLAVGQTSDVALAQVAGGREG
ADLRAAVTWGDGSDVAAGAVTDGSVSGSHAYTAAGTYTAYVVVDDGWTSQVVEVPVTVT
EAEPALAVDVTVSTRCLAGKAYVAVRAENGEDVPLAIRLVTPFGTKEVAAVAPGANAYQ
SFATRVTAVEAGTVTVEATRGTGDEEVTASIQADYAAVTCG

FIGURE 9B (CONTINUED)

MTRPLPPGRAVARSGSGRARPLGLVLAAALAVPLGVPLAAPAGALAAAPAAAAEPGDFS
SSFESGDPAALPTTVAERDGAPWQANVGSFTAGLPGSVLGQLKGVTASAQNLPNEGAAN
LADGSSGTKWLAFASTGWVRYEFAEPVSFVAYTMTSGDDAAGRDPKTWTVEGSNDGSTW
AALDRRTDEDFPNRQQTRTFELEAPTAAYTYLRLNVTANSGDSIVQLAGWDLSADLSAG
PSAAPMTTKVGTGPRVSFTNKAGVGFSGLHSLRYDGSHLADGETYATNVLYDDVDVVVG
EDTRLSYTIFPELLDDLQYPSTYAAVDVLFTDGTYLSDLGARDAHETVATAQAQGEGKI
LYADQWNSVRVDLGDVAEGKTVDQVLLGYDNPGGHAGTKFAGWLDDVEITAEPATIDGS
SLANYVDTRRGTLASGSFSRGNNIPATATPNGFNFWTPYTNASSQSWLYEYHKANNANN
KPVLQGFGISHEPSPWMGDRNQLTFLPSTASGTPDATLSTRGLEFDHADETARPDYYGV
TFTNGSAIEATPTDHGAVLRFSYPGAKGHVLVDKVDGSSKLTYDQATGTISGWVENGSG
LSVGRTRMFVAGTFDRSPTAVGTAAGNRADARFATFETSSDKTVELRVATSFISLDQAR
KNLDLEVTGKTFTEVKAAAAQAWNDRLGVIEVEGASEDQLVTLYSNLYRLNLYPNSQFE
NTGTAQEPVYRYASPVSATTGSATDTQTNAKIVDGKIYVNNGFWDTYRTAWPAYSLLYP
ELAAELVDGFVQQYRDGGWIARWSSPGYADLMTGTSSDVAFADAYLKGSLPTGTALEAY
DAALRNATVAPPSNAVGRKGLQTSPFLGFTPESTHESVSWGLEGLVNDFGIGNMAAALA
EDPATPEERRETLREESAYFLERATHYVELFDPEVDFFVPRHEDGTWAVDPETYDPEAW
GGGYTETNGWNFAFHAPQDGQGLANLYGGKQGLEDKLDEFFSTPEKGAGNGGIHEQREA
RDVRMGQWGMSNQVSHHIPWLYDAAGAPSKAQEKVREVTRRLFVGSEIGQGYPGDEDNG
EMSSWWIFASLGFYPLQVGSDQYAVGSPLFDKATVHLPDGDLVVNAENNSVDNVYVQSL
AVDGEARTSTSLSQADLSGGTTLDFVMGPEPSDWGTGEDDAPPSLTEGDEPPTPVQDAT
TAGLGTTTVADGDATTSAAALTDNTSGTRTTFATTTPSITWAGNGIRPTVGSYTLTSGA
SGTASPSAWTLEGSDDGETWTTLDERSGEQFRWALQTRPFTVAEPTAFARYRVTVTATS
GSGALSLAEVELLADPKESGAEELTLSAAPDRDGVTGREVSGSFATLTGVEGDVAALDV
QVAFGDGSEPVAGTLRAGAFGGYAVDAAHTWTAPGVYPVTVTVSGEGIETVSASSYVSV
SLLREGSLLAAYDNVCIGDAGTTVGSCDGQGVFFDRAQLAAKGFVQGERATVPGTDLAF
DVPAVPAGQPDNATGDGQTIELDVPADAEQLSVIGTGTEKNQQATGTLTFDDGSTQPID
LSFGDWSGAARNPVFGNIPVAVTDSRLRGGSPQTGTPAAFFATAPITLPEGKRPVSLTL
PDQPGELSRDGRIHVVAVAHDGTFAEHPALEVTAAEGVTLAVGQTSDVALAQVAGGREG
ADLRAAVTWGDGSDVAAGAVTDGSVSGSHAYTAAGTYTAYVVVDDGWTSQVVEVPVTVT
EAEPALAVDVTVSTRCLAGKAYVAVRAENGEDVPLAIRLVTPFGTKEVAAVAPGANAYQ
SFATRVTAVEAGTVTVEATRGTGDEEVTASIQADYAAVTCG (SEQ ID NO:10)

FIGURE 10


+1
T7
-10
s10'
-35
DsbASS
pL
H6
bla
'DEVD
pLSAHCcMan5
7983 bp
ori
t t
T T
T3
CcMan5

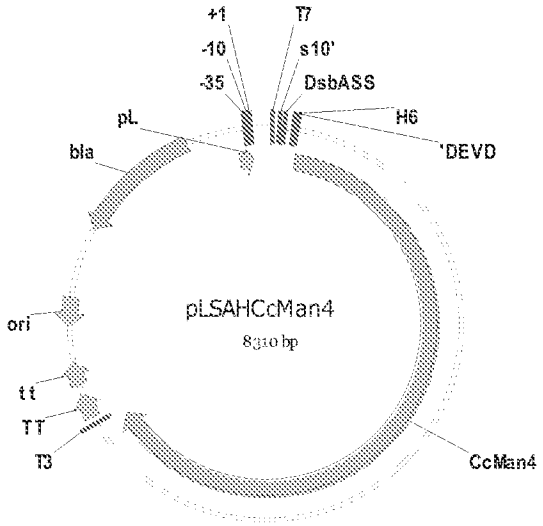

+1
T7
-10
s10'
-35
DsbASS
pL
H6
bla
'DEVD
pLSAHCcMan4
8310 bp
ori
t t
T T
T3
CcMan4

FIGURE 11

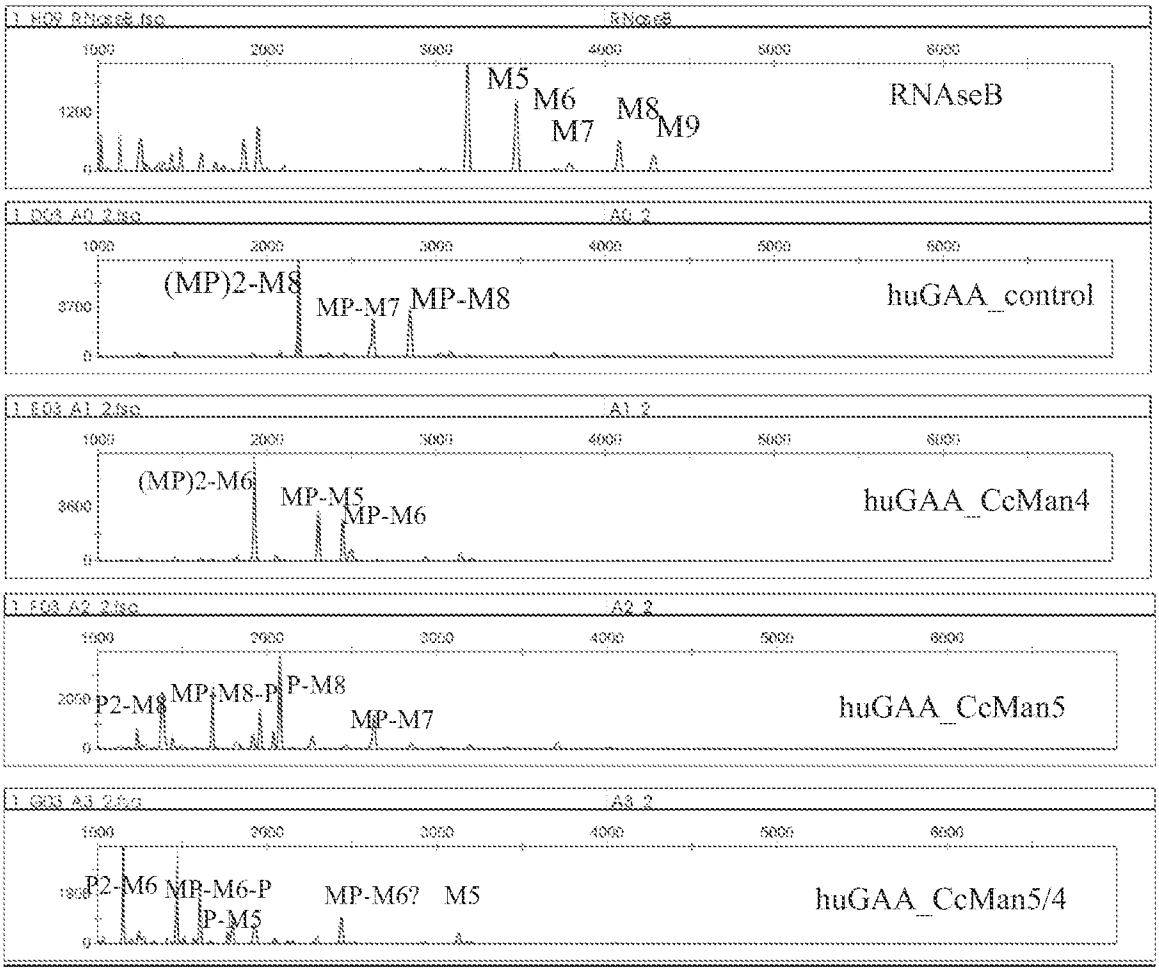

RNaseB
M5
M6
M7
M8
M9
huGAA_control
(MP)2-M8
MP-M7
MP-M8
huGAA_CcMan4
(MP)2-M6
MP-M5
MP-M6
huGAA_CcMan5
P2-M8
MP-M8-P
P-M8
MP-M7
huGAA_CcMan5/4
P2-M6
MP-M6-P
P-M5
MP-M6?
M5

FIGURE 12

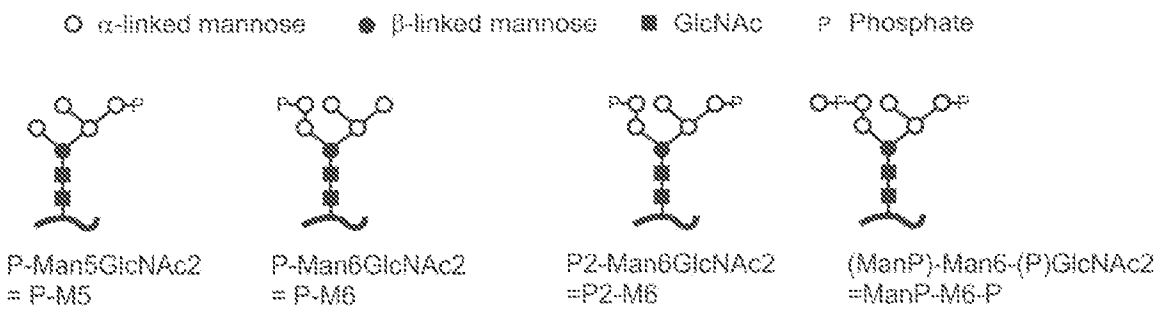

α-linked mannose
β-linked mannose
GlcNAc
P Phosphate
P-Man5GlcNAc2
= P-M5
P-Man6GlcNAc2
= P-M6
P2-Man6GlcNAc2
=P2-M6
(ManP)-Man6-(P)GlcNAc2
=ManP-M6-P

FIGURE 13

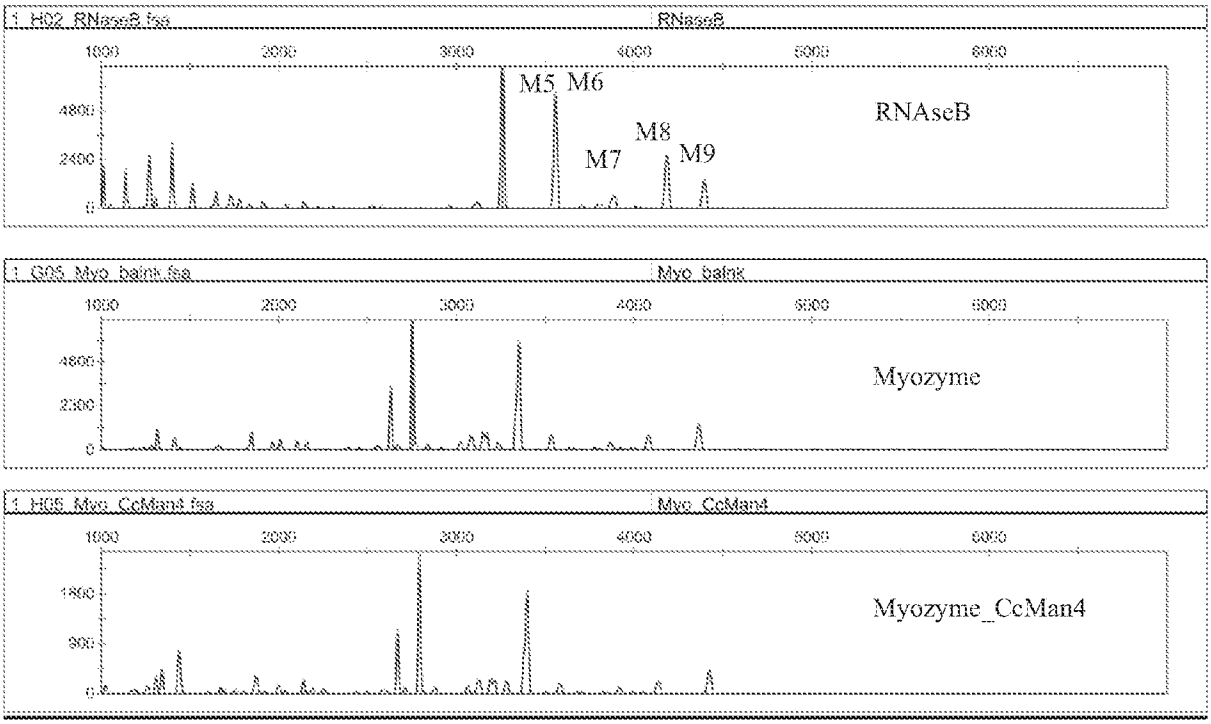

1_H02_RNaseB.fsa
RNaseB
1000
2000
3000
4000
5000
6000
4800
2400
0
M5
M6
M7
M8
M9
RNAseB
1_G05_Myo_balnk.fsa
Myo_balnk
1000
2000
3000
4000
5000
6000
4800
2400
0
Myozyme
1_H05_Myo_CcMan4.fsa
Myo_CcMan4
1000
2000
3000
4000
5000
6000
1800
900
0
Myozyme_CcMan4

FIGURE 14

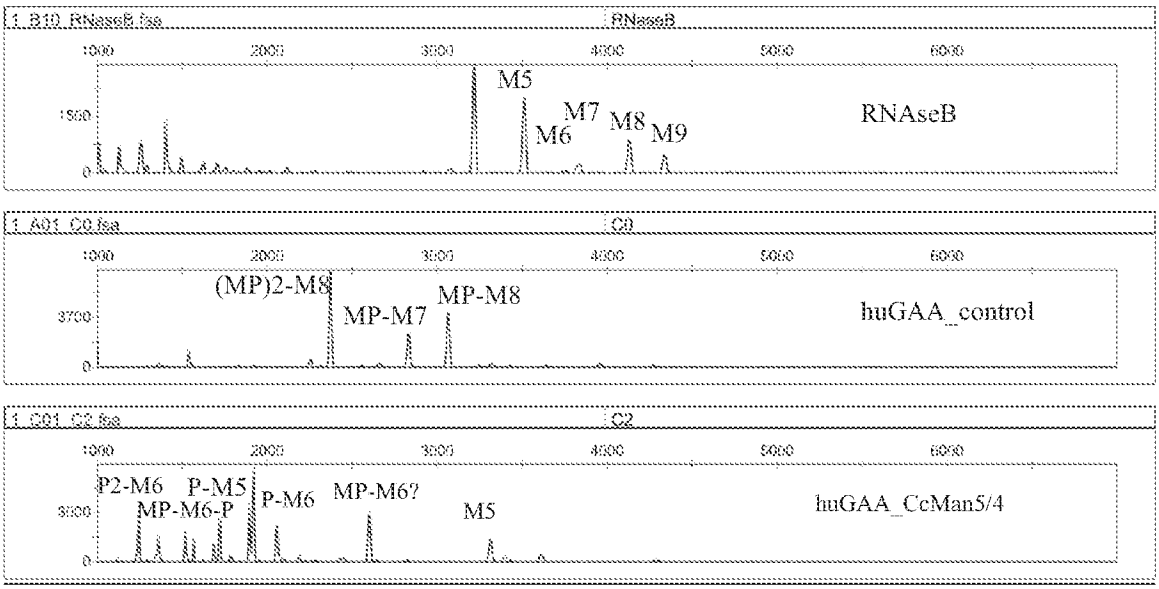

1_B10_RNaseB.fsa
RNaseB
M5
M6
M7
M8
M9
RNAseB
1_A01_C0.fsa
C0
(MP)2-M8
MP-M7
MP-M8
huGAA_control
1_C01_C2.fsa
C2
P2-M6
P-M5
MP-M6-P
P-M6
MP-M6?
M5
huGAA_CcMan5/4

FIGURE 15

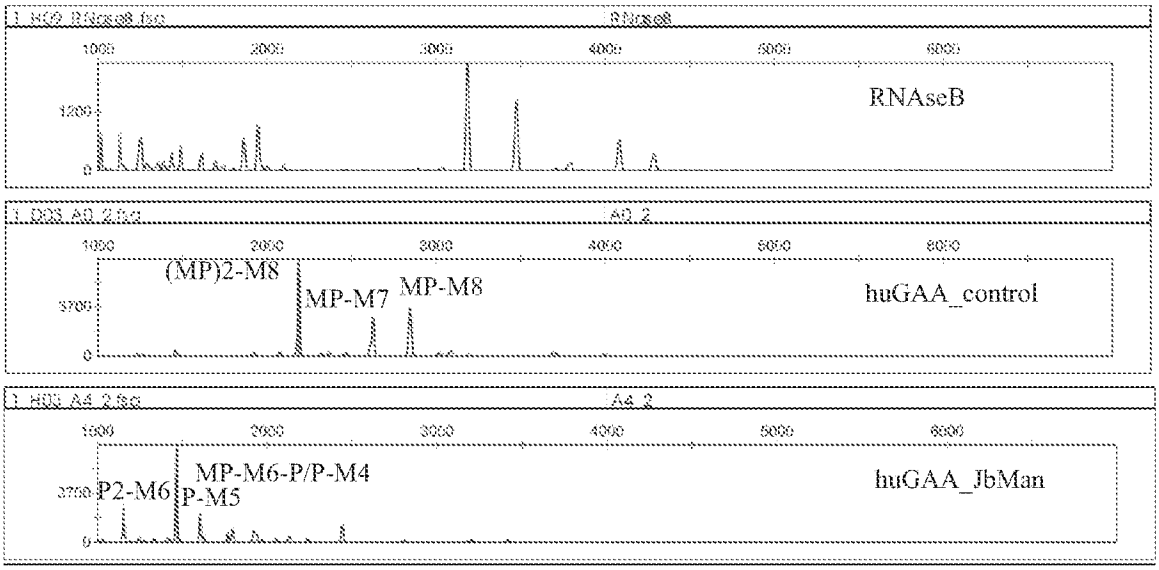

RNAseB
(MP)2-M8
MP-M7
MP-M8
huGAA_control
P2-M6
MP-M6-P/P-M4
P-M5
huGAA_JbMan

FIGURE 16

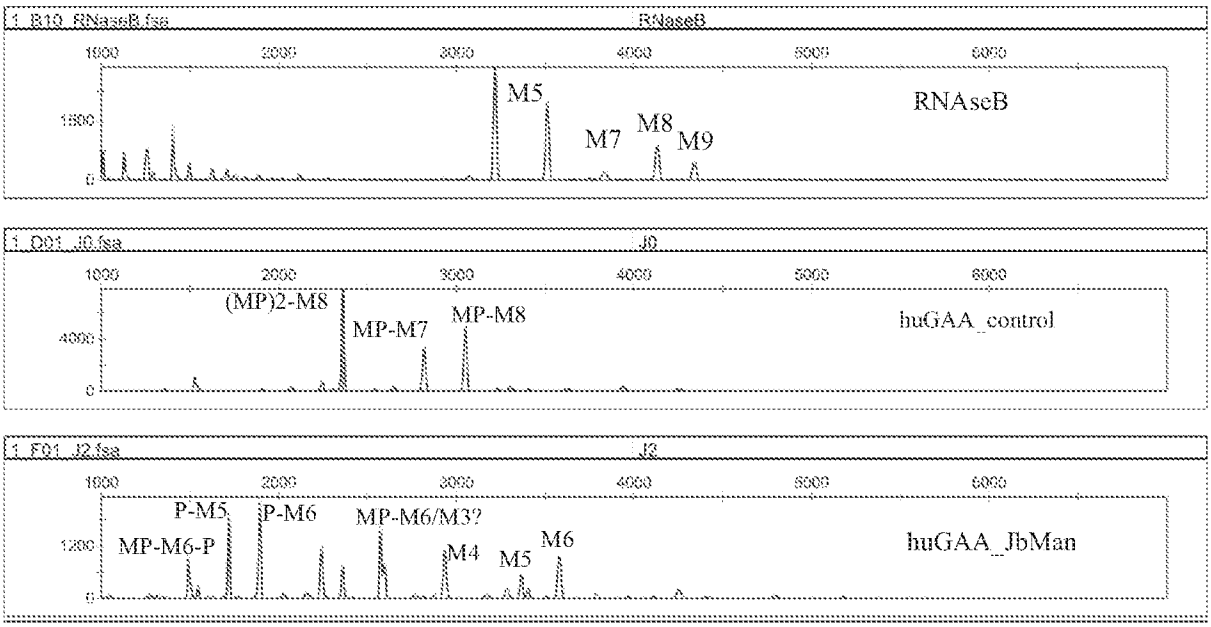

1_B10_RNaseB.fsa
RNaseB
1000
2000
3000
4000
5000
6000
1500
0
M5
M7
M8
M9
RNAseB
1_D01_J0.fsa
J0
4000
(MP)2-M8
MP-M7
MP-M8
huGAA_control
1_F01_J2.fsa
J2
1200
P-M5
P-M6
MP-M6/M3?
MP-M6-P
M4
M5
M6
huGAA_JbMan

FIGURE 17

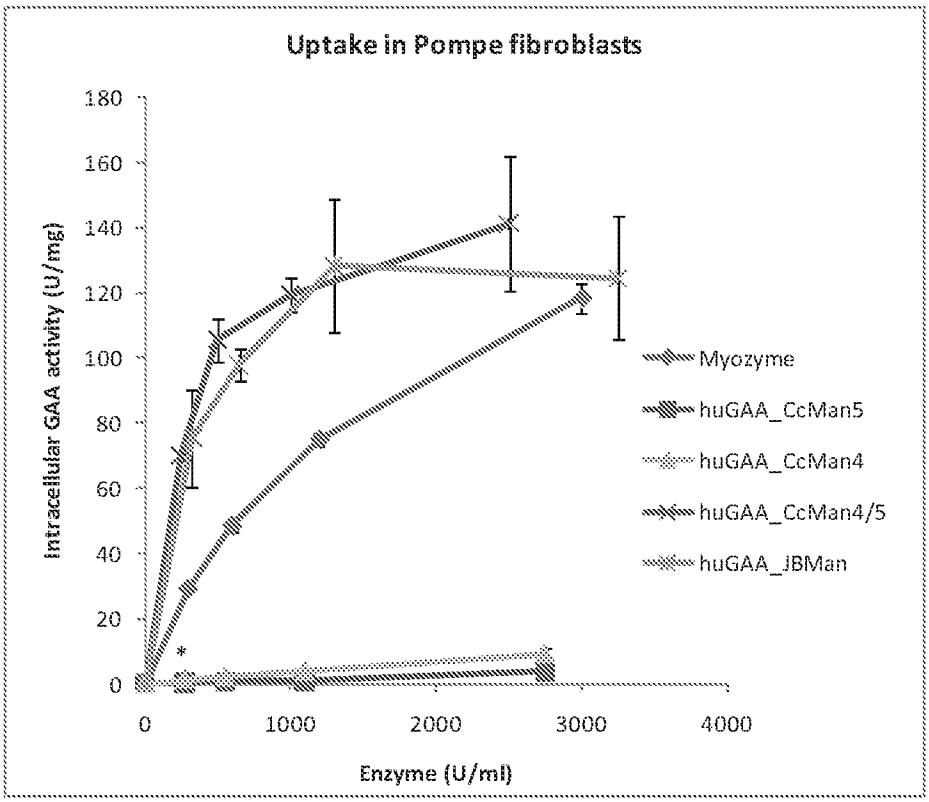

Uptake in Pompe fibroblasts
Intracellular GAA activity (U/mg)
180
160
140
120
100
80
60
40
20
0
0
1000
2000
3000
4000
Enzyme (U/ml)
Myozyme
huGAA_CcMan5
huGAA_CcMan4
huGAA_CcMan4/5
huGAA_JBMan
*

FIGURE 18

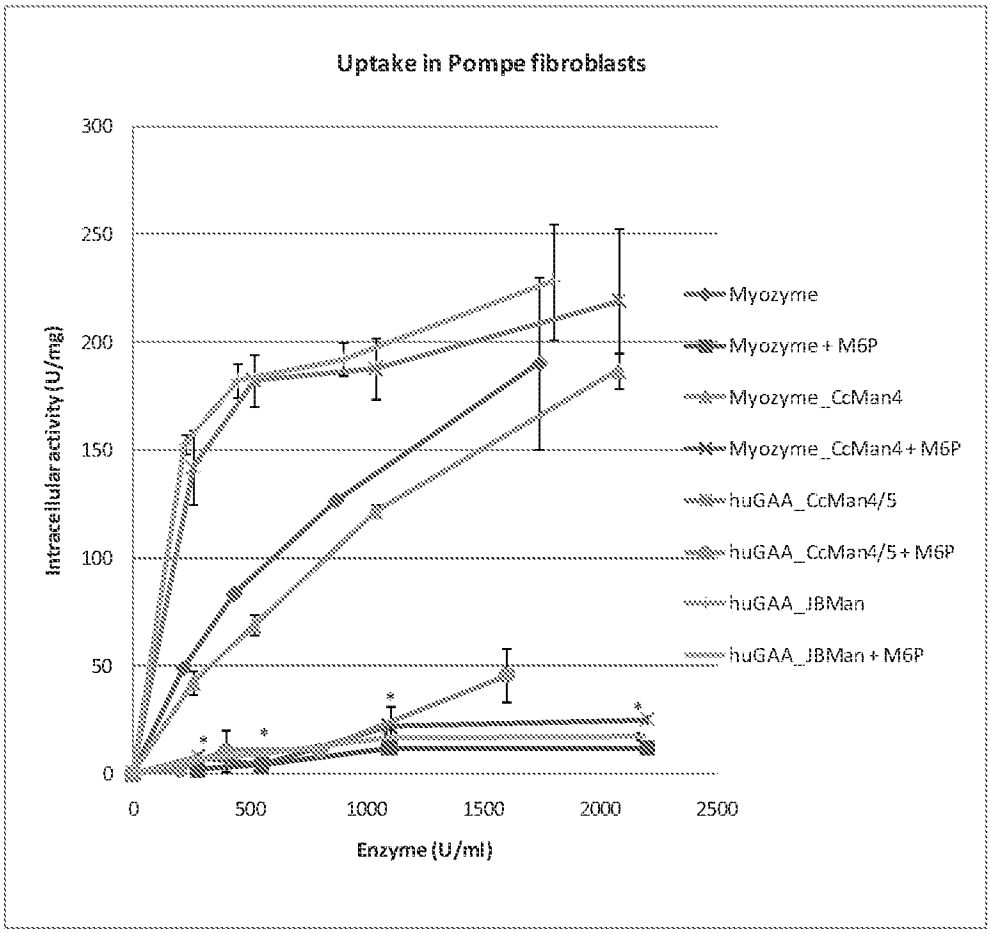

Uptake in Pompe fibroblasts
Intracellular activity (U/mg)
Enzyme (U/ml)
0
50
100
150
200
250
300
0
500
1000
1500
2000
2500
Myozyme
Myozyme + M6P
Myozyme_CcMan4
Myozyme_CcMan4 + M6P
huGAA_CcMan4/5
huGAA_CcMan4/5 + M6P
huGAA_JBMan
huGAA_JBMan + M6P

FIGURE 19

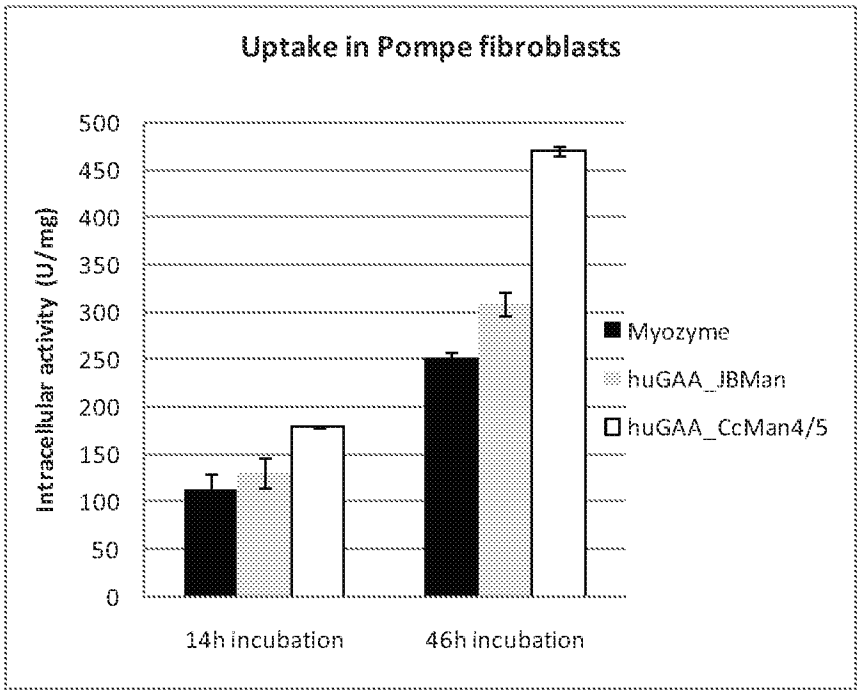

Uptake in Pompe fibroblasts
Intracellular activity (U/mg)
500
450
400
350
300
250
200
150
100
50
0
14h incubation
46h incubation
Myozyme
huGAA_JBMan
huGAA_CcMan4/5

FIGURE 20

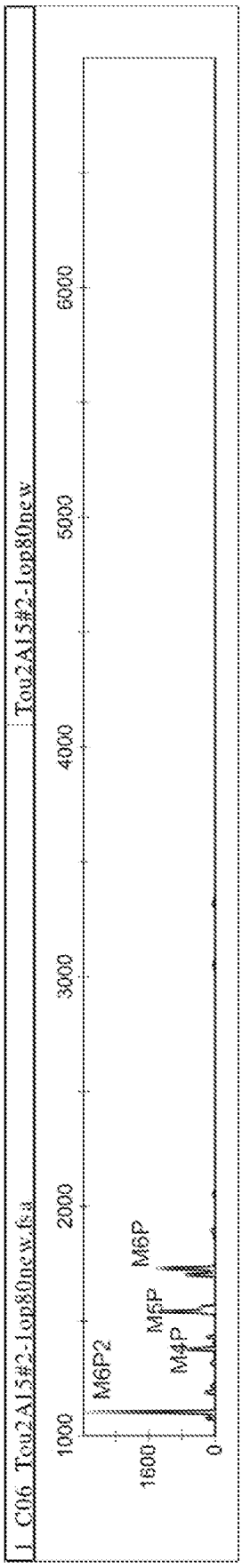

1_C06_Tou2A15#2-1op80new.fca
Tou2A15#2-1op80new
1000
2000
3000
4000
5000
6000
1600
0
M6P2
M4P
M5P
M6P

FIGURE 21

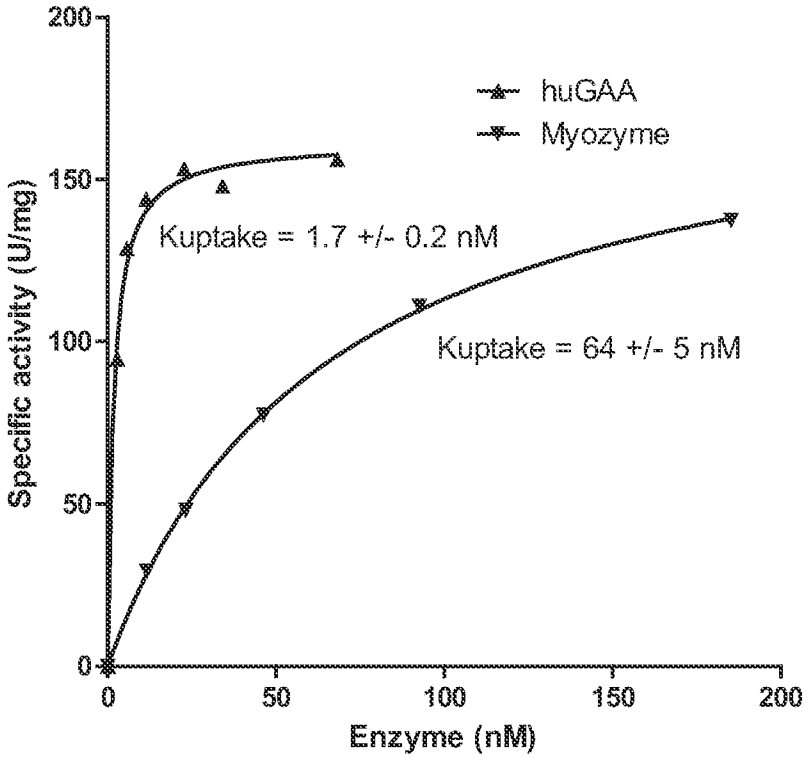

200
150
100
50
0
Specific activity (U/mg)
huGAA
Myozyme
Kuptake = 1.7 +/- 0.2 nM
Kuptake = 64 +/- 5 nM
0
50
100
150
200
Enzyme (nM)

FIGURE 22

```
1 mhlpslslsl talaiaspsa ayphfgssqp vlhsssdttq sradaikaaf shawdgylqy
61 afphdelhpv sngygdsrng wgasavdals tavimrnati vnqildhvgk idysktnttv
121 slfettiryl ggmlsgydll kgpvsdlvqn sskidvlltq sknladvlkf afdtpsgvpy
181 nnlnitsggn dgaktnglav tgtlalewtr lsdltgdtty adlsqkaesy llnpqpksae
241 pfpglvgsni nisngqftda qvswnggdds yyeylikmyv ydpkrfglyk drwvaaaqst
301 mqhlashpss rpdltflasy nngtlglssq hltcfdggsf llggtvlnrt dfinfgldlv
361 sgchdtynst ltgigpesfs wdtsdipssq qslyekagfy itsgayilrp eviesfyyaw
421 rvtgqetyrd wiwsafsavn dycrtssgfs gltdvnaang gsrydnqesf lfaevmkysy
481 mafaedaawq vqpgsgnqfv fnteahpvrv sst
```