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(71) Applicants *(for all designated States except US)*: **OXYRANE UK LIMITED** [GB/GB]; Greenheys House, Manchester Science Park, 10 Pencroft Way, Manchester M15 6JJ (GB). **VIB VZW** [BE/BE]; Rijvisschestraat 120, B-9052 Gent (BE). **UNIVERSITEIT GENT** [BE/BE]; Sint-Pietersnieuwstraat 25, B-9000 Gent (BE).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **PIENS, Kathleen, Camilla, Telesphore, Alida, Maria** [BE/BE]; Citadellaan 54, B-9000 Gent (BE). **VERVECKEN, Wouter** [BE/BE]; Edmond Van Hoorebekestraat 90, B-9050 Gent-ledeberg (BE).

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(54) **Title**: DE-MANNOSYLATION OF PHOSPHORYLATED N-GLYCANS

(57) **Abstract**: Methods for demannosylating phosphorylated N-glycans on a glycoprotein are described that use a mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated.

De-Mannosylation of Phosphorylated N-Glycans

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Application Serial No. 61/387,924, filed on September 29, 2010. The disclosure of the prior application is incorporated by reference in its entirety.

TECHNICAL FIELD

This invention relates to alpha-mannosidases that can hydrolyze the terminal α-1,2-mannose when the underlying mannose is phosphorylated.

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 the discovery of a mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated.

In one aspect, this document features a method for demannosylating phosphorylated N-glycans on a glycoprotein. The method includes providing the glycoprotein having phosphorylated N-glycans; and contacting the glycoprotein with a

mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated. The mannosidase can be from *Aspergillus satoi* or *Cellulosimicrobium cellulans*. The method further can include isolating the glycoprotein containing the demannosylated phosphorylated N-glycan. 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 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 capable of hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated; 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 demannosylated phosphorylated N-glycans. 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 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.

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 demannosylated phosphorylated N-glycans. 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.

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 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. 3 is a depiction of the nucleotide sequence of the open reading frame (ORF) of DsbA-CcMan5 (SEQ ID NO:3).

FIG. 4 is a depiction of the nucleotide sequence of the ORF of DsbA-CcMan4 (SEQ ID NO: 4)

FIG. 5 is a schematic of the plasmids pLSAHCcMan5 and pLSAHCcMan4.

FIG. 6 is a schematic the potential final hydrolysis products, assuming that the α-1,2-mannosidases can also hydrolyze the terminal α-1,2-mannose if the underlying mannose is phosphorylated.

FIG. 7 is a schematic of the reaction products obtained using a phosphate uncapping enzyme.

FIG. 8 contains the DSA-FACE electroferograms for the hydrolysis of a N-glycan preparation containing $Man_8GlcNAc_2$ and the monophosphorylated sugar ManP-$Man_8GlcNAc_2$ (Panel B) with HjMan and AsMan.

FIG. 9A and 9B are DSA-FACE electroferograms for the hydrolysis of a N-glycan preparation containing $Man_8GlcNAc_2$ and the monophosphorylated sugar ManP-$Man_8GlcNAc_2$ (Panel B) with HjMan (9A) and AsMan (9B), where the MNN4 sugars were first treated with the phosphate uncapping enzyme CcMan5.

FIG. 10 contains DSA-FACE electroferograms for the hydrolysis of a MNN4 preparation using either AsMan or HjMan.

FIG. 11 contains the N-glycan profiles before and after the α -1,2-mannosidase treatment.

FIG. 12A and 12B are DSA-FACE electroferograms of the activity of the periplasmic solution on (APTS)-labeled N-glycans derived from a MNN4 overexpressing strain.

FIG. 13 is the DSA-FACE electroferogram analysis of the activity of CcMan4 and CcMan5 after incubating with huGAA expressed in *Y. lipolytica*.

FIG. 14 is the DSA-FACE electroferogram analysis of the activity of recombinantly expressed ERManI or GolgiManIA after incubating with huGAA expressed in *Y. lipolytica*.

FIG. 15 is a depiction of the amino acid sequence of a mannosidase from *Aspergillus saitoi* (SEQ ID NO: 5).

DETAILED DESCRIPTION

In general, this document provides methods and materials for hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the immediately underlying mannose is phosphorylated. 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 hydrolyze a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated. 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 a 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 or SEQ ID NO:4. 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. The percent identity between a particular amino acid sequence and the amino acid sequence set forth in SEQ ID NO:5 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 an *Aspergillus saitoi* nucleotide sequence) to DNA or RNA from a test source is an indication of the presence of DNA or RNA (e.g., an *Aspergillus saitoi* 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 amino acid sequence set forth in SEQ ID NOs: 5 or the amino acid sequence encoded by the nucleotide sequences set forth in SEQ ID NOs: 3 and 4. 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 encoded by the nucleic acid sequences set forth in SEQ ID NOs: 3 or 4 or the mannosidase having the amino acid sequence set forth in SEQ ID NOs: 5 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., demannosylating) of the wild-type, full-length, mature protein.

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

Methods of Demannosylating Glycoproteins

As described herein, glycoproteins containing phosphorylated N-glycans can be demannosylated using a mannosidase that can hydrolyze a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated. Non-limiting examples of such mannosidases include a mannosidase from *Aspergillus satoi* (As) (also known as *Aspergillus phoenicis*) or a mannosidase from *Cellulosimicrobium cellulans* (e.g., CcMan4). The amino acid sequence of the *Aspergillus satoi* mannosidase is set forth in SEQ ID NO:5 (see FIG. 15) and in GenBank Accession No. BAA08634. A CcMan4 polypeptide is encoded by the nucleotide sequence set forth in SEQ ID NO: 4 (see FIG. 4).

The *Aspergillus satoi* mannosidase and a *Cellulosimicrobium cellulans* mannosidase (e.g., CcMan4) can be recombinantly produced. 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)).

To produce demannosylated glycoproteins, a target molecule containing a terminal alpha-1,2 mannose linkage or moiety where the underlying mannose is phosphorylated is contacted under suitable conditions with a mannosidase or a cell lysate containing a recombinantly produced mannosidase. 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 mannosidase or cell lysate, the terminal alpha-1,2 mannose linkage can be hydrolyzed to produces a demannosylated target molecule.

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 any molecule containing a terminal alpha-1,2 mannose linkage or moiety where the underlying mannose is phosphorylated. 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.

In Vivo Methods of Demannosylating Glycoproteins

Genetically engineered cells described herein can be used to produce 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 terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated, a nucleic acid encoding a target molecule, wherein the cell produces the target molecule containing demannosylated phosphorylated N-glycans. 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 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 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

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 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 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

A 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 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.

A 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, a 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 such a molecule in a tissue can be monitored using appropriate screening assays as part of a clinical testing procedure, *e.g.*, to determine the efficacy of a given treatment regimen.

The frequency of dosing for a 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 a 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.

EXAMPLES

EXAMPLE 1

Generation of an huGAA expression strain

Y. lipolytica strain OXYY1589 was constructed that contained three copies of the human alpha glucosidase (also known as acid alpha glucosidase (GAA) or acid maltase EC3.2.1.3) and two copies of the *Y. lipolytica* MNN4 gene. The genotype of strain OXYl589 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. In all cases (unless otherwise specified), a huGAA integration fragment was obtained by NotI restriction digestion in order to remove the kanamycin resistance gene from the expression plasmids. The resulting fragments were all separated by agarose gel electrophoresis followed by Qiagen column purification of the correct huGAA fragment. Strain OXYYl589 was constructed by first cloning human GAA (huGAA) into a *Y. lipolytica* expression vector and constructing a *Y. lipolytica* MNN4 tandem expression vector. Three stable integrative transformations were then performed in order to obtain the final huGAA production strain OXYY1589.

<u>*Y. lipolytica* codon optimized huGAA expression vector:</u> The nucleotide sequence encoding the 110 kDA human GAA (huGAA) precursor was chemically synthesized and codon optimized for *Y. lipolytica* expression. 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 ORF of huGAA (FIG. 1A) is 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 in expression vector. The construct is under the control of the inducible POX2 promoter. The complete amino acid sequence of the fusion protein is shown on FIG. 1B.

A general scheme of an expression vector is presented in FIG. 2. The bacterial moiety was derived from the plasmid pHSS6, and comprises a bacterial origin of replication (ori) and the kanamycin-resistant gene conferring resistance to kanamycin (KanR). The integration cassette comprised 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 was 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.

<u>Tandem YlMNN4 expression vector:</u> The YlMNN4 gene was cloned under control of the inducible pPOX2 promoter and the (semi)constitutive hp4d promoter. These two expression cassettes of YlMNN4 were subcloned into 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 the expression cassette purified from pRAN058 and pRAN059 vectors using URA3 and LEU2 marker to produce intermediate recombinant strain OXYY1569. 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. PCR screening of genomic DNA was performed in order to confirm the integration of the foreign huGAA DNA into the genome of *Y. lipolytica*. Primers were designed to amplify a fragment of 2552bp from 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 huGAA DIG labeled specific probe.

In order to select a clone secreting high levels of huGAA, several randomly selected clones that were identified as positive in the PCR screening and Southern blot were grown in shake flasks under POX2 inducing conditions according to a standard procedure. In all cases, the culture supernatant was collected 72h post-induction and screened in a standard Western blot and enzyme activity assay analysis. N-Glycan analysis of OXYY1569 indicated the predominant structure in OXYY1569 is $Man_8GlcNAc_2$.

Intermediate Strain OXYYl584: Recombinant strain OXYYl569 was transformed in order to integrate two copies of the *Y. lipolytica* MNN4 gene into its genome to produce OXYYl584. The transformation was performed with a SacII/XmaI derived expression cassette excised from plasmid OXYP1479B. The expression cassette was designed for targeted integration into the ADE2 locus of *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 arbitrary chosen transformants was SpeI digested and probed with MNN4 specific DIG labeled probe. Correct targeted integration of MNN4 expression cassette into the ADE2 locus of *Y. lipolytica* genome should give 4207bp and 5683bp bands. Southern blot 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 are $Man_8GlcNAc_2(PMan)_1$ and $Man_8GlcNAc_2$ $(PMan)_2$.

Production strain OXYYl589: 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 the Not I excised expression cassette from pRAN069. Transformants were first screened by PCR on gDNA for presence of the additional copy of huGAA. To evaluate huGAA production arbitrary 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. 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 the GAA (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 1200rpm 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. In case of foam detection, 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 was then concentrated 20 times via TFF and diafiltered against 20 mM sodium phosphate pH 6 and 100 mM NaCl on 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 contain rhGAA were then pooled and subjected to 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.

Protein concentration was done on 15 ml Amicon Ultra centrifugal devices (Millipore) with a molecular weight cut-off of 10 kDa.

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 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 pH11 was added to stop the reaction and the release of the fluorogenic reaction product 4-methylumbelliferone 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 150mM citrate-phosphate buffer pH4, 1% BSA) in reaction wells of a microtiter plate and were subsequently incubated at 37°C. After 1 to 2 hours an equal volume of stop buffer, 10% sodium carbonate pH 12, was added to quench the reaction and convert the released p-nitrophenol (PNP) to 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 with 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

Expression of CcMan5 and CcMan4

CcMan4 and CcMan5 ORFs were cloned into the vector pLSAH36, which contains a DsbA signal sequence and results in the expression of a protein with an N-terminal polyhistidine tag. Expression of the proteins was performed in *E. coli* BL21 cells. Proteins residing in the periplasm were isolated and purified using a Talon column. The nucleotide sequences of the ORF of DsbA-CcMan5 and DsbA-CcMan4 are provided in FIG. 3 and FIG.4. A graphical representation of the plasmids pLSAHCcMan5 and pLSAHCcMan4 is given in FIG. 5.

EXAMPLE 5

De-mannosylation of APTS-labeled phosphorylated N-glycans with GH47 α-mannosidases

The GH47 α-1,2-mannosidase from *Hypocrea jecorina* (Hj) (anamorph: *Trichoderma reesei*) can sequentially cleave all 4 α-1,2 linked mannose sugars from a Man9GlcNAc2 oligosaccharide (Maras, M. *et al.*, J. Biotechnol, 77: 255-263 (2000)). A similar activity is described for the α-1,2-mannosidase from *Aspergillus satoi* (As) (also known as *Aspergillus phoenicis*)(Ichishima E. *et al.*, Biochem. J., 339: 589-597).

HjMan (Genbank nr AAF34579) was expressed in *Pichia pastoris* and purified as described in Maras, M. *et al.*, J. Biotechnol, 77: 255-263 (2000). A commercial enzyme preparation of AsMan (Genbank nr BAA08634) is available from Prozyme-Glyco.

The α-1,2-mannosidases from *H. jecorina* and *A. satoi* were tested on a mixture of 8-amino-1,3,6,-pyrenetrisulfonic acid (APTS)-labeled sugars derived from *Yarrowia lipolytica* cells overexpressing the MNN4 gene, containing $Man_8GlcNAc_2$ (M8), the monophosphorylated ManP-$Man_8GlcNAc_2$ (MP-M8) and/or the diphosphorylated $(ManP)_2$-$Man_8GlcNAc_2$ ((MP)2-M8) sugars. In FIG. 6, the potential final hydrolysis products are presented in a schematic presentation, assuming that the α-1,2-mannosidases can also hydrolyze the terminal α -1,2-mannose if the underlying mannose is phosphorylated. To be able to test the activity of the α -1,2-mannosidases on phosphorylated N-glycans with the phosphate residue uncapped (thus an oligosaccharide with a terminal phosphate present), the MNN4 sugars were treated with CcMan5, a GH92 enzyme from *Cellulosimicrobium cellulans* with phosphate uncapping activity (FIG. 7). CcMan5 removes the terminal mannose in a mannose-phospho-mannose diester linkage.

Unless otherwise stated all reactions with AsMan and HjMan on APTS-labeled N-glycans were performed overnight at 37°C in an ammonium acetate buffer, 10 mM, pH 5.0 with 2 mM $CaCl_2$. The CcMan5 reaction was done at room temperature and pH 7.0, using a 10 mM HEPES buffer with 2 mM $CaCl_2$ added. To confirm the presence of phosphate uncapped glycans, or to be able to identify the structure of the fast-running glycans with a terminal phosphate, calf intestine phosphatase (CIP) was subsequently added to the reaction mixture. After phosphate hydrolysis neutral N-glycans are obtained which can be identified through the comparison with the electroferogram from the APTS-labeled N-glycans released from RNAseB (Man9-5GlcNAc2).

In FIG. 8, the DSA-FACE electroherograms are presented for the hydrolysis of a N-glycan preparation containing $Man_8GlcNAc_2$ and the monophosphorylated sugar ManP-$Man_8GlcNAc_2$ (Panel B) with HjMan and AsMan. $Man_8GlcNAc_2$ was hydrolyzed to $Man_5GlcNAc_2$ by both α-1,2-mannosidases, while a difference was observed for the hydrolysis of ManP-$Man_8GlcNAc_2$. HjMan most likely released two α-1,2-linked mannoses (ManP-$Man_6GlcNAc_2$ in panel C). The slightly faster running peak that appeared after AsMan treatment suggested the release of three α-1,2-linked mannoses and thus the formation of ManP-$Man_5GlcNAc_2$ (Panel D). The newly formed products did not disappear after treatment with calf intestine phosphatase (CIP), confirming that the phosphates are still mannose-capped (Panel E and F).

To confirm that the fast running peak in panel E is ManP-Man6GlcNAc2 and the one in panel D is ManP-$Man_5GlcNAc_2$ (FIG. 8), the experiment with HjMan and AsMan was repeated on MNN4 sugars which were first treated with the phosphate uncapping enzyme CcMan5 (yielding P-$Man_8GlcNAc_2$, panel C in Fig. 8). After incubation with the α-1,2-mannosidases for 20, 40 and 180 minutes at 37 °C the reaction was stopped by heating the reaction mixture at 100 °C for 3 minutes. Next a CIP treatment was performed. The results with HjMan are presented in FIG.9A. HjMan sequentially cleaved P-$Man_8GlcNAc_2$ to P-$Man_7GlcNAc_2$ and P-$Man_6GlcNAc_2$ (Panel D to F). AsMan can sequentially removed three α-1,2-linked mannoses, resulting in the formation of P-$Man_7GlcNAc_2$, P-$Man_6GlcNAc_2$ and P-$Man_5GlcNAc_2$. See FIG. 9B.

The above results (Fig. 9A and 9B), together with the overnight α-1,2-mannosidase digestion of MNN4 glycans (Fig. 8) could only be explained if only the α-1,6 arm (the right arm of the N-glycans (schematically presented in Fig. 6A and 6B) is phosphorylated. Only the *A. satoi* α-1,2-mannosidase is capable of hydrolyzing the terminal α-1,2-mannose if the underlying mannose is phosphorylated (phosphate residue capped with a mannose residue or uncapped). HjMan only removes the two α-1,2-linked mannoses from the non-phosphorylated α-1,3 arm.

The fact that AsMan can remove a terminal α -1,2-mannose if the underlying mannose is phosphorylated and HjMan cannot, is also confirmed when a MNN4 preparation composed of monophosphorylated ManP-$Man_8GlcNAc_2$ and diphosphorylated $(ManP)_2$-$Man_8GlcNAc_2$ (Fig. 10, panel C) is used. AsMan can hydrolyze $(ManP)_2$-$Man_8GlcNAc_2$, as was observed from the appearance of two extra fast-running peaks in panel D (most likely $(ManP)_2$-$Man_7GlcNAc_2$ and $(ManP)_2$-$Man_6GlcNAc_2$). HjMan did not hydrolyze $(ManP)_2$-$Man_8GlcNAc_2$ (Fig. 10, panel E).

EXAMPLE 6

<u>De-mannosylation with GH47 α -mannosidases of glycoproteins with a high degree of phosphorylated N-glycans expressed in a *Yarrowia lypolytica* strain</u>

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.

HjMan and AsMan were added to a solution of huGAA in 100 mM ammonium acetate, pH 5.0 with 2 mM CaCl2. The reaction mixture was incubated overnight at room temperature. The N-glycans were released with PNGaseF, labelled with APTS and subsequently analysed on DSA-FACE, essentially as described in Laroy W. *et al.*, Nature Protocols, 1: 397-405 (2006). The N-glycan profiles before and after the α -1,2-mannosidase treatment are shown in Figure 11. The N-glycan mixture released from purified huGAA was composed mainly of ManP-Man_8GlcNAc2 and $(ManP)_2$-$Man_8GlcNAc_2$ (Fig. 11, panels B and E). A peak running slightly faster than ManP-$Man_8GlcNAc_2$ could be assigned to ManP-$Man_7GlcNAc_2$. Only very minor amounts of $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ were present. After incubation of huGAA with HjMan the conversion of ManP-$Man_8GlcNAc_2$ to ManP-$Man_7GlcNAc_2$ and ManP-$Man_6GlcNAc_2$ was observed, while $(ManP)_2$-$Man_8GlcNAc_2$ was not hydrolyzed (Fig. 11, panel C). The electroferogram in panel D shows the sugars obtained after treatment of huGAA with AsMan. AsMan hydrolyzed $(ManP)_2$-$Man_8GlcNAc_2$ with the formation of

$(ManP)_2$-$Man_7GlcNAc_2$ and $(ManP)_2$-$Man_6GlcNAc_2$ at the left side of the electroferogram and next to ManP-$Man_7GlcNAc_2$ and ManP-$Man_6GlcNAc_2$. ManP-$Man_5GlcNAc_2$ was also formed from the hydrolysis of ManP-$Man_8GlcNAc_2$. These data confirm that the α -1,6 arm is phosphorylated in the ManP-$Man_8GlcNAc_2$ structure and that also on the glycoprotein level AsMan can hydrolyze the terminal α -1,2-mannose if the underlying mannose is phosphorylated. The HjMan activity was limited to the release of α -1,2-linked mannoses from the neutral Man8GlcNAc2 N-glycans or to the removal of the two α -1,2-linked mannoses on the non-phosphorylated α -1,3 arm in ManP-$Man_8GlcNAc_2$.

EXAMPLE 7

De-mannosylation of APTS-labeled phosphorylated N-glycans with GH92 α -mannosidases

CcMan4 and CcMan5 were expressed in *E. coli* and different cell fractions were isolated as described in Example 4. The activity of the periplasmic solution was tested on (APTS)-labeled N-glycans derived from a MNN4 overexpressing strain and analyzed on DSA-FACE (Fig. 12). The N-glycans were incubated overnight at room temperature with CcMan4, CcMan5 or with a mixture of both enzymes in a 10 mM HEPES buffer, pH 7.0 with 2 mM $CaCl_2$. A control experiment with AsMan was included, and was performed as described in example 5. In the experiment shown in Figure 12A, a MNN4 fraction was used containing mainly $Man_8GlcNAc_2$ (M8) and monophosphorylated ManP-$Man_8GlcNAc_2$ (MP-M8) (Panel B). CcMan4 hydrolyzed $Man_8GlcNAc_2$ and ManP-$Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ and ManP-$Man_5GlcNAc_2$, respectively (panel C). The same reaction products were obtained with AsMan (panel D and example 5). CcMan5 did not hydrolyze the glycosidic linkage between two α-1,2 mannoses (thus no shift of the $Man_8GlcNAc_2$ peak), but uncapped the phosphate in ManP-$Man_8GlcNAc_2$, yielding the fast-running peak P-$Man_8GlcNAc_2$ (Panel E). After incubation of this reaction mixture with CIP only a peak corresponding with $Man_8GlcNAc_2$ is observed (Panel F). A mixture of CcMan4 and CcMan5 hydrolyzed $Man_8GlcNAc_2$ and ManP-$Man_8GlcNAc_2$ to $Man_5GlcNAc_2$ and P-$Man_5GlcNAc_2$, respectively (Panel G). Therefore only a peak corresponding with $Man_5GlcNAc_2$ is observed after CIP treatment (Panel H). CcMan4 also hydrolyzed the diphosphorylated $(ManP)_2$-$Man_8GlcNAc_2$ to $(ManP)_2$-$Man_6GlcNAc_2$ (Fig 12B, panel J), as was also observed with AsMan (panel K and example 5). A mixture of CcMan4 and CcMan5 produced P2-$Man_6GlcNAc_2$ and P-$Man_5GlcNAc_2$ (Panel N).

CcMan4 is thus an α-1,2-mannosidase capable of also hydrolyzing the terminal α -1,2-mannose if the underlying mannose is phosphorylated. It can be used in combination with the phosphate uncapping enzyme CcMan5.

EXAMPLE 8

De-mannosylation with GH92 α -mannosidases of glycoproteins with a high degree of phosphorylated N-glycans expressed in a *Yarrowia lypolytica* strain

CcMan4 and CcMan5 were incubated with huGAA expressed in *Y. lipolytica*. The analysis was performed as described in Example 6. A 100 mM HEPES buffer, pH 7.0 with 2 mM $CaCl_2$ was used for both CcMan4 and CcMan5 in an overnight assay at room temperature. The DSA-FACE analysis is presented in FIG. 13. 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-$Man_8GlcNAc_2$ could be assigned to ManP-$Man_7GlcNAc_2$.

CcMan4 de-mannosylated the huGAA glycoprotein. In the electroferogram (Panel C) peaks corresponding with $(ManP)_2$-$Man_6GlcNAc_2$, ManP-$Man_5GlcNAc_2$ and ManP-$Man_6GlcNAc_2$ were observed. In combination with CcMan5 the phosphate uncapped products P2-$Man_6GlcNAc_2$, P-$Man_5GlcNAc_2$ and P-$Man_6GlcNAc_2$ were formed as shown in panel E.

EXAMPLE 9

Additional examples of de-mannosylation with GH47 α -mannosidases of glycoproteins with a high degree of phosphorylated N-glycans expressed in a *Yarrowia lypolytica* strain

ERManI and GolgiManIA are two class I α -1,2-mannosidases belonging to family GH47. Recombinantly expressed ERManI and GolgiManIA were incubated overnight at room temperature with huGAA expressed in *Y. lipolytica*. The analysis was performed as described in Example 6 and FIG. 10. A 100 mM HEPES buffer, pH 7.0 with 2 mM $CaCl_2$ was used for ERManI, while the incubation with GolgiManIA is performed in a 100 mM MES buffer, pH 6.0 with 2 mM $CaCl_2$. The DSA-FACE analysis is presented in FIG. 14.

Both ERManI and GolgiManIA could de-mannosylate the huGAA glycoprotein, but their activity was limited to the hydrolysis of ManP-$Man_8GlcNAc_2$ to ManP-$Man_6GlcNAc_2$ (Panels D and E respectively). This result was also obtained with the GH 47 α -1,2-mannosidase from *H. Jecorina* (HjMan, Fig.11, panel C), while the GH47 α -1,2-mannosidase from *A. satoi* (AsMan, Fig. 11, panel F) and the GH92 CcMan4 (Fig. 12, panel C) trimmed $(ManP)_2$-$Man_8GlcNAc_2$ and ManP-$Man_8GlcNAc_2$ to $(ManP)_2$-$Man_6GlcNAc_2$ and ManP-$Man_5GlcNAc_2$, respectively.

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 demannosylating phosphorylated N-glycans on a glycoprotein, said method comprising
a) providing said glycoprotein having phosphorylated N-glycans; and
b) contacting said glycoprotein with a mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated.

2. The method of claim 1, wherein said mannosidase is from *Aspergillus satoi*.

3. The method of claim 1, wherein said mannosidase is from *Cellulosimicrobium cellulans*.

FIGURE 1A

ATGAAGCTTTCCACCATCCTCTTCACAGCCTGCGCTACCCTGGCTGCCGCCCAGCAGGGAGCCT
CTCGACCCGGACCCCGAGATGCCCAGGCTCACCCCGGACGACCTCGAGCTGTGCCCACCCAGTGTG
ACGTGCCCCCCAACTCTCGATTCGACTGTGCCCCCGACAAGGCCATCACCCAGGAGCAGTGCGAGG
CCCGAGGCTGTTGTTACATCCCCGCTAAGCAGGGCCTGCAGGGCGCTCAGATGGGCCAGCCCTGGT
GTTTCTTCCCCCCCTCTTACCCCTCCTACAAGCTGGAGAACCTGTCCTCTTCGGAGATGGGCTACAC
CGCCACCCTGACCCGAACCACCCCCACCTTTTTCCCCAAGGACATCCTGACCCTGCGACTGGACGTG
ATGATGGAGACCGAGAACCGACTGCACTTCACCATCAAGGACCCCGCCAACCGACGATACGAGGT
GCCCCTGGAGACCCCCCACGTGCACTCTCGAGCCCCTTCCCCCCTGTACTCTGTGGAGTTCTCTGAG
GAGCCCTTCGGCGTGATCGTGCGACGACAGCTGGACGGCCGAGTGCTGCTGAACACCACCGTGGCC
CCCCTGTTCTTCGCCGACCAGTTCCTGCAGCTGTCTACCTCTCTGCCCTCTCAGTACATCACCGGCCT
GGCCGAGCACCTGTCCCCCCTGATGCTGTCCACCTCTTGGACTCGAATCACCCTGTGGAACCGAGA
CCTGGCCCCCACCCCCGGTGCCAACCTGTACGGCTCTCACCCCTTCTACCTGGCCCTGGAGGACGGC
GGCTCTGCCCACGGCGTGTTTCTGCTGAACTCTAACGCCATGGACGTGGTGCTGCAGCCCTCTCCCG
CCCTGTCTTGGCGATCTACCGGCGGCATCCTGGACGTGTACATCTTCCTGGGCCCTGAGCCCAAGTC
TGTGGTCCAGCAGTACCTGGACGTGGTCGGATACCCCTTCATGCCCCCCTACTGGGGCCTGGGCTTC
CACCTGTGTCGATGGGGCTACTCTTCTACCGCCATCACCCGACAGGTGGTGGAGAACATGACCCGA
GCCCACTTCCCCCTGGACGTGCAATGGAACGACCTGGACTACATGGACTCTCGACGAGACTTCACC
TTCAACAAGGACGGCTTCCGAGACTTCCCCGCCATGGTCCAGGAGCTGCACCAGGGAGGACGACG
ATACATGATGATCGTGGACCCCGCCATCTCTTCTTCCGGACCCGCCGGATCTTACCGACCCTACGAC
GAGGGCCTGCGACGAGGCGTGTTCATCACCAACGAGACCGGCCAGCCCCTGATCGGCAAGGTGTG
GCCCGGCTCTACCGCCTTCCCCGACTTCACCAACCCCACCGCCCTGGCTTGGTGGGAGGACATGGT
GGCCGAGTTCCACGACCAGGTGCCCTTCGACGGCATGTGGATCGACATGAACGAGCCCTCTAACTT
CATCCGAGGCTCTGAGGACGGCTGTCCCAACAACGAGCTGGAGAACCCCCCCTACGTGCCCGGCGT
GGTGGGCGGAACCCTGCAGGCCGCCACCATCTGTGCCTCTTCGCACCAGTTTCTGTCTACCCACTAC
AACCTGCACAACCTGTACGGACTGACCGAGGCCATTGCCTCTCACCGAGCCCTGGTGAAGGCCCGA
GGCACCCGACCCTTCGTGATCTCTCGATCTACCTTCGCCGGCCACGGCCGATACGCCGGACACTGG
ACCGGCGATGTGTGGTCCTCTTGGGAGCAGCTGGCCTCTTCTGTGCCCGAGATCCTGCAGTTCAACC
TGCTGGGCGTGCCCCTGGTGGGCGCCGACGTGTGTGGCTTCCTGGGCAACACCTCTGAGGAGCTGT
GTGTTCGATGGACCCAGCTCGGCGCCTTCTACCCTTTCATGCGAAACCACAACTCCCTGCTGTCTCT
GCCCCAGGAGCCCTACTCGTTCTCTGAGCCCGCTCAGCAGGCCATGCGAAAGGCTCTGACCCTGCG
ATACGCCCTGCTGCCCCACCTGTACACCCTGTTCCACCAGGCCCACGTGGCTGGAGAGACCGTGGC
CCGACCCCTGTTCCTGGAGTTCCCTAAGGACTCTTCTACCTGGACCGTGGACCATCAGCTGCTGTGG
GGCGAGGCCCTCCTGATCACCCCCGTGCTGCAGGCCGGCAAGGCTGAGGTGACCGGCTACTTCCCT
CTGGGCACCTGGTACGACCTGCAGACCGTGCCTGTGGAGGCCCTGGGATCTCTGCCCCCTCCTCCCG
CCGCTCCCCGAGAGCCCGCCATCCACTCTGAGGGCCAGTGGGTGACCCTGCCCGCTCCCCTGGACA
CCATCAACGTGCACCTGCGAGCCGGCTACATCATCCCTCTGCAGGGACCCGGCCTGACCACCACCG
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CTGTTCTGGGACGATGGCGAGTCTCTGGAGGTGCTGGAGCGAGGCGCCTACACCCAGGTGATCTTT
CTGGCCCGAAACAACACCATCGTGAACGAGCTGGTGCGAGTGACCTCTGAGGGCGCTGGTCTGCAG
CTCCAGAAGGTGACCGTCCTGGGCGTGGCCACCGCTCCCCAGCAGGTCCTGTCTAACGGCGTGCCC
GTGTCTAACTTCACCTACTCTCCCGACACCAAGGTGCTGGACATCTGTGTGTCTCTGCTGATGGGCG
AGCAGTTCCTGGTGTCTTGGTGTTAAC

FIGURE 1B

MKLSTILFTACATLAAAQQGASRPGPRDAQAHPGRPRAVPTQCDVPPNSRFDCAPDK
AITQEQCEARGCCYIPAKQGLQGAQMGQPWCFFPPSYPSYKLENLSSSEMGYTATLTRT
TPTFFPKDILTLRLDVMMETENRLHFTIKDPANRRYEVPLETPHVHSRAPSPLYSVEFSE
EPFGVIVRRQLDGRVLLNTTVAPLFFADQFLQLSTSLPSQYITGLAEHLSPLMLSTSWTR
ITLWNRDLAPTPGANLYGSHPFYLALEDGGSAHGVFLLNSNAMDVVLQPSPALSWRST
GGILDVYIFLGPEPKSVVQQYLDVVGYPFMPPYWGLGFHLCRWGYSSTAITRQVVENM
TRAHFPLDVQWNDLDYMDSRRDFTFNKDGFRDFPAMVQELHQGGRRYMMIVDPAISS
SGPAGSYRPYDEGLRRGVFITNETGQPLIGKVWPGSTAFPDFTNPTALAWWEDMVAEF
HDQVPFDGMWIDMNEPSNFIRGSEDGCPNNELENPPYVPGVVGGTLQAATICASSHQF
LSTHYNLHNLYGLTEAIASHRALVKARGTRPFVISRSTFAGHGRYAGHWTGDVWSSWE
QLASSVPEILQFNLLGVPLVGADVCGFLGNTSEELCVRWTQLGAFYPFMRNHNSLLSLP
QEPYSFSEPAQQAMRKALTLRYALLPHLYTLFHQAHVAGETVARPLFLEFPKDSSTWT
VDHQLLWGEALLITPVLQAGKAEVTGYFPLGTWYDLQTVPVEALGSLPPPPAAPREPAI
HSEGQWVTLPAPLDTINVHLRAGYIIPLQGPGLTTTESRQQPMALAVALTKGGEARGEL
FWDDGESLEVLERGAYTQVIFLARNNTIVNELVRVTSEGAGLQLQKVTVLGVATAPQQ
VLSNGVPVSNFTYSPDTKVLDICVSLLMGEQFLVSWC*

FIGURE 2

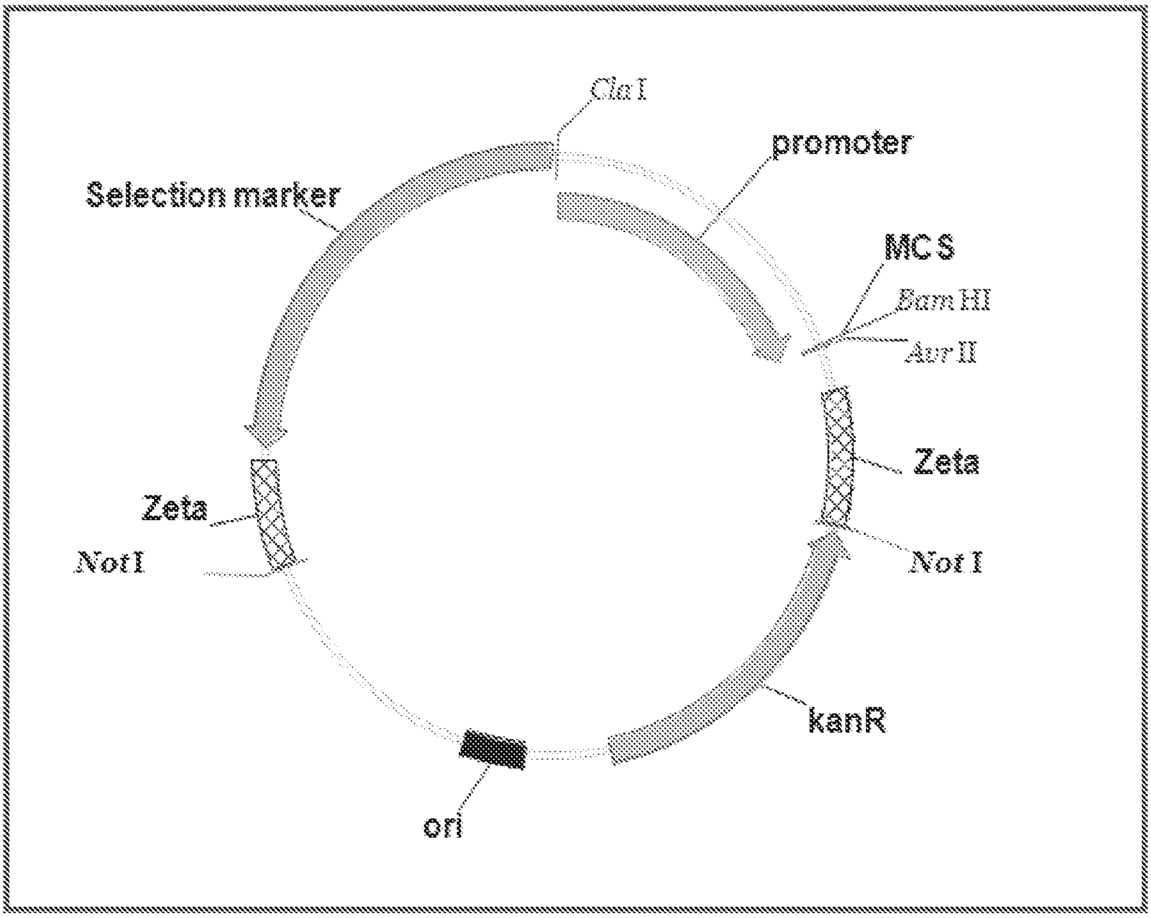
Cla I
promoter
Selection marker
MCS
Bam HI
Avr II
Zeta
Zeta
Not I
Not I
kanR
ori

FIGURE 3

>DsbA-6xHis-CcMan5 (107bp - 5167bp, direct) 5061bp From pLSAHCcMan5
ATGAAAAAGATTTGGCTGGCGCTGGCTGGTTTAGTTTTAGCGTTTAGCGCATCGGCCGG
CCATCACCATCATCACCACGTGGGGCCCGGCTCGGACGAAGTGGATGCACCGGAACCTC
CGAGCGCAGATTATGCAAGCCTGGTTGATGTTTTTGTTGGCACCGAAGGTGATTTTGGT
AATGATATGCCTGCAGCACAGGCACCGAATGGTCTGGCAAAAGTTAATCCGCGTACCAC
ACCGGGTCGTAATAATACCGGTTATGATTATGCCCAGAGCAAAATTAGCGGTTTTACCC
ATACCAATCTGGATGGTGTTGGTGGTAGCGGTGGTGGTGATCTGCTGGTTGTTCCG
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
CCGATGAACCGGCAGAAGGTCCTCGCGTTAGCGCAGAACCGACCACCGTGCAGACCGGT
GATGGTGGTGCACTGGATGCAACCGTTACCCTGACACTGGATGGCGCACGTCTGGCAGC

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

FIGURE 4

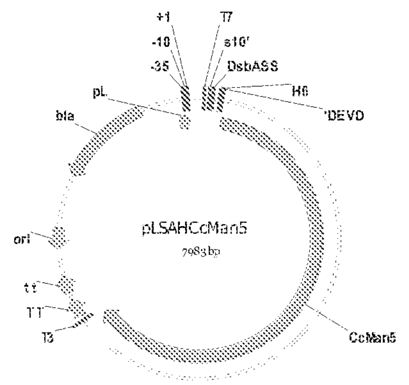
DsbA-6xHis-CcMan4 (107bp - 5494bp, direct) 5388bp from pLSAHCcMan4

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
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
TACCGCATATGTTGTTGTGGATGATGGTTGGACCAGCCAGGTTGTTGAAGTTCCGGTGA
CCGTTACAGAAGCCGAACCGGCACTGGCCGTTGATGTCACCGTTAGCACCCGTTGCCTG
GCAGGTAAAGCATATGTTGCAGTGCGTGCAGAAAATGGTGAAGATGTTCCGCTGGCAAT
TCGTCTGGTTACCCCGTTTGGCACCAAAGAAGTTGCAGCAGTTGCTCCGGGAGCCAATG
CATATCAGAGCTTTGCAACCCGTGTTACCGCAGTTGAAGCAGGCACCGTTACCGTTGAA

GCCACCCGTGGCACCGGTGATGAAGAAGTTACCGCCAGCATTCAGGCAGATTATGCAGC
CGTTACCTGCGGTTAATAA

FIGURE 5


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

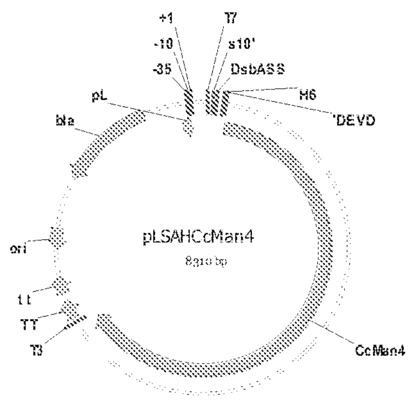

+1
-10
-35
pL
bla
T7
s10'
DsbASS
H6
'DEVD
ori
pLSAHCcMan4
8310 bp
t t
TT
T3
CcMan4

FIGURE 6

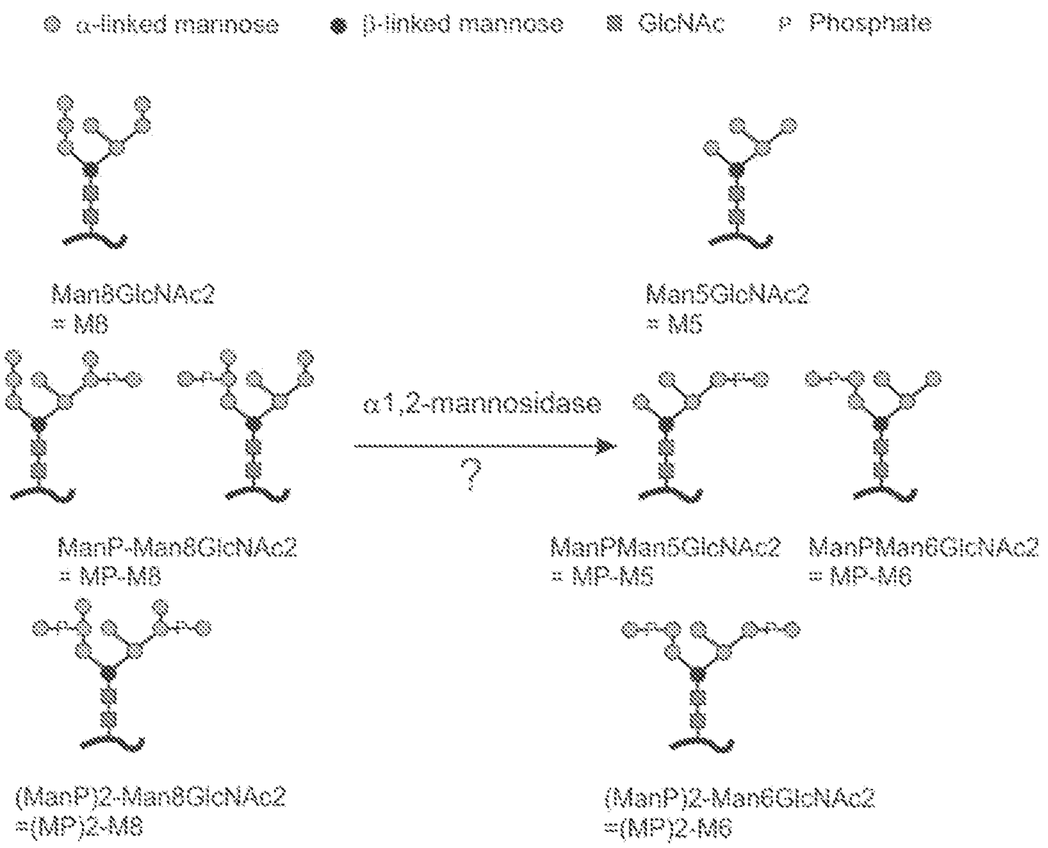

α-linked mannose
β-linked mannose
GlcNAc
P Phosphate
Man8GlcNAc2
= M8
Man5GlcNAc2
= M5
α1,2-mannosidase
?
ManP-Man8GlcNAc2
= MP-M8
ManPMan5GlcNAc2
= MP-M5
ManPMan6GlcNAc2
= MP-M6
(ManP)2-Man8GlcNAc2
=(MP)2-M8
(ManP)2-Man6GlcNAc2
=(MP)2-M6

FIGURE 7

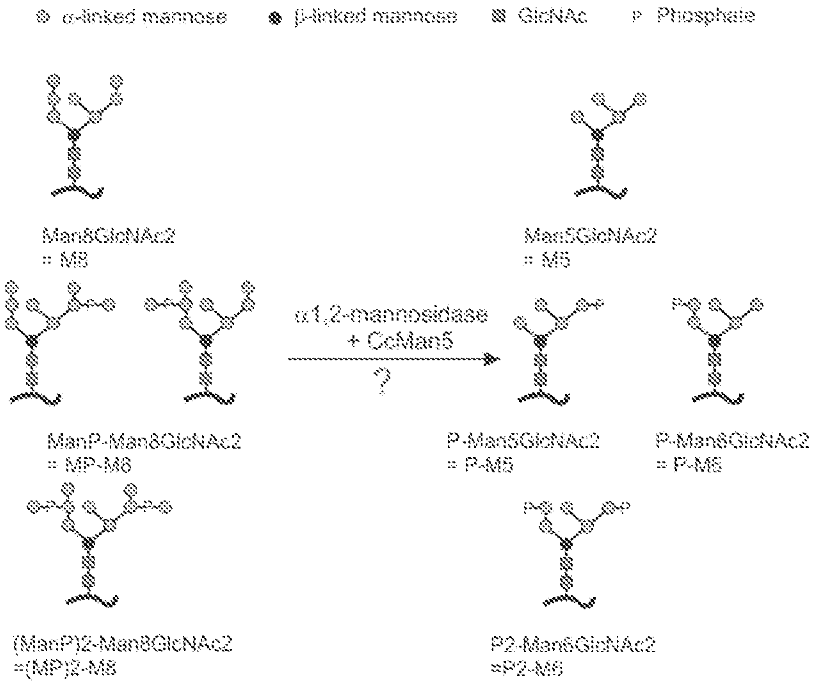

α-linked mannose
β-linked mannose
GlcNAc
P Phosphate
Man8GlcNAc2
= M8
Man5GlcNAc2
= M5
ManP-Man8GlcNAc2
= MP-M8
α1,2-mannosidase
+ CcMan5
?
P-Man5GlcNAc2
= P-M5
P-Man6GlcNAc2
= P-M6
(ManP)2-Man8GlcNAc2
=(MP)2-M8
P2-Man6GlcNAc2
=P2-M6

FIGURE 8

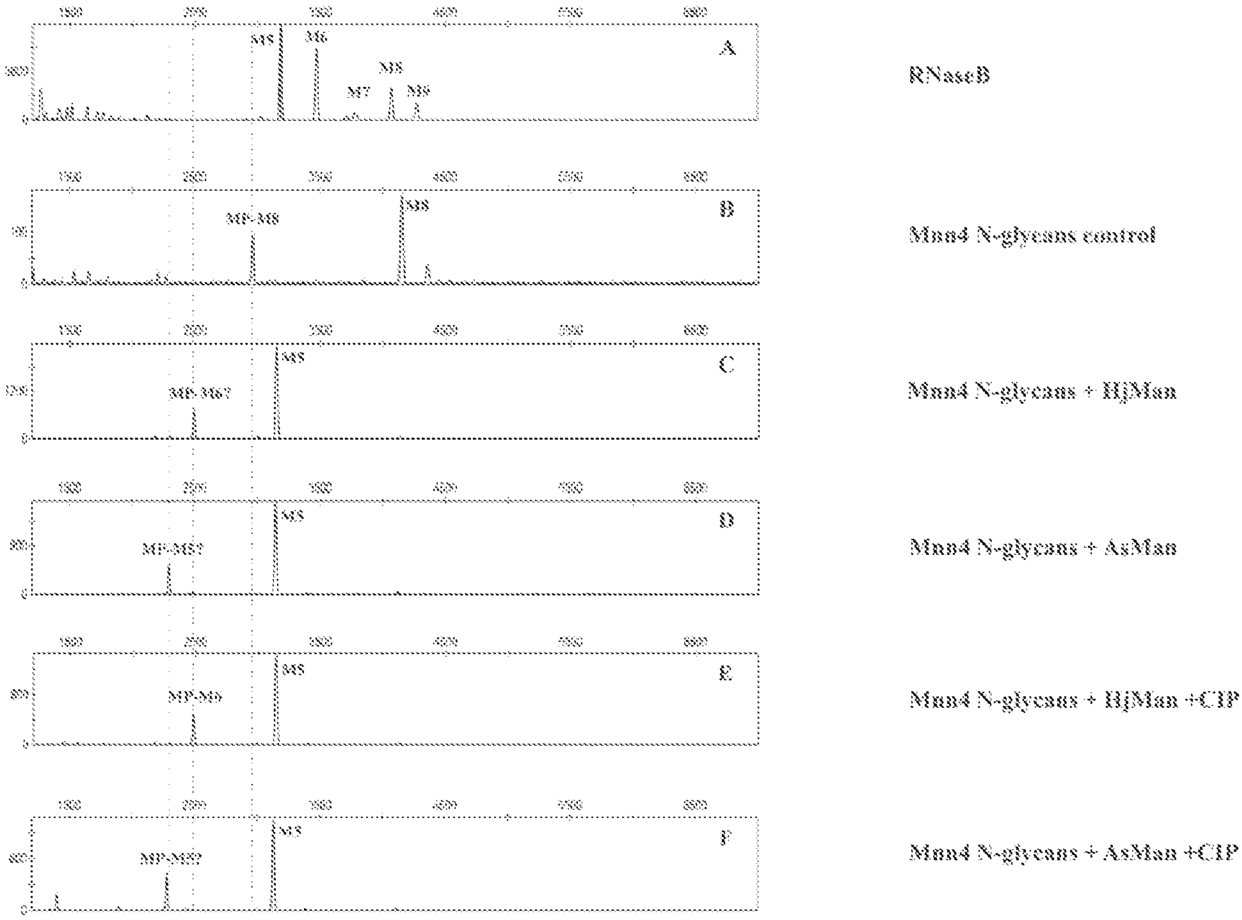
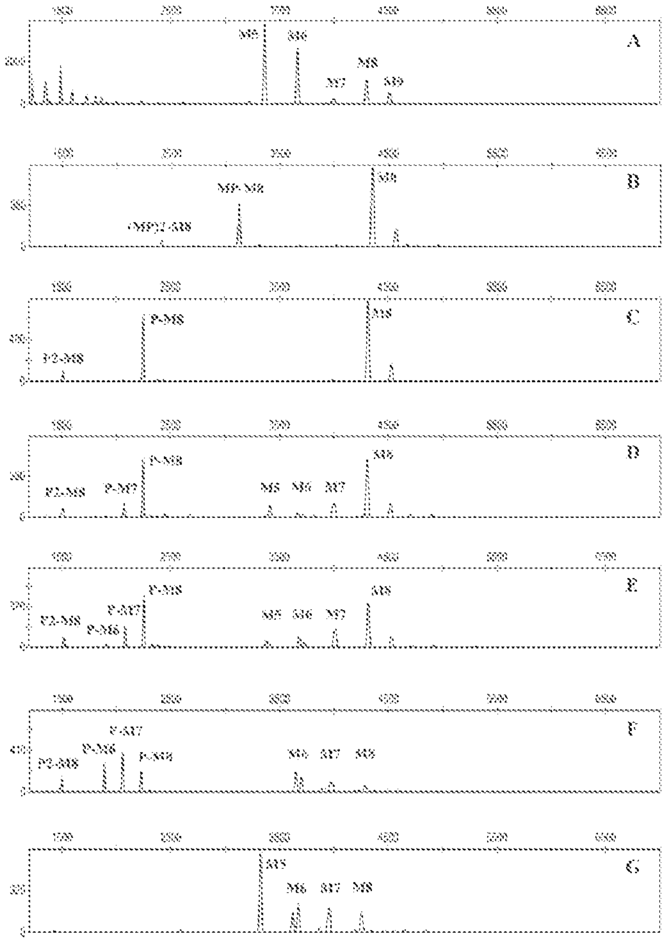

A
B
C
D
E
F
M5
M6
M7
M8
M9
MP-M8
MP-M6?
MP-M5?
MP-M6
RNaseB
Mnn4 N-glycans control
Mnn4 N-glycans + HjMan
Mnn4 N-glycans + AsMan
Mnn4 N-glycans + HjMan +CIP
Mnn4 N-glycans + AsMan +CIP

FIGURE 9A



RNaseB

Mnn4 N-glycans control

Mnn4 N-glycans + CcMan5

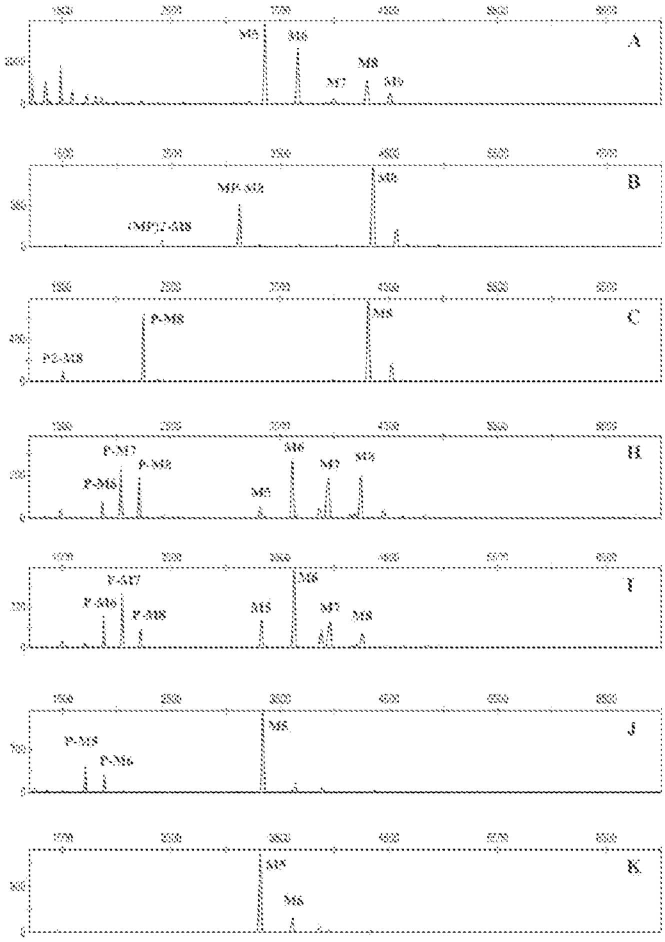
Mnn4 N-glycans + CcMan5 + RjMan
T1 = 20 min

Mnn4 N-glycans + CcMan5 + RjMan
T2 = 40 min

Mnn4 N-glycans + CcMan5 + RjMan
T3 = 180 min

Reaction F + CIP

FIGURE 9B



RNaseB

Man4 N-glycans control

Man4 N-glycans + CcMan5

Man4 N-glycans + CcMan5 + AsMan
T1 = 20 min

Man4 N-glycans + CcMan5 + AsMan
T2 = 40 min

Man4 N-glycans + CcMan5 + AsMan
T3 = 180 min

Reaction J + CIP

FIGURE 10

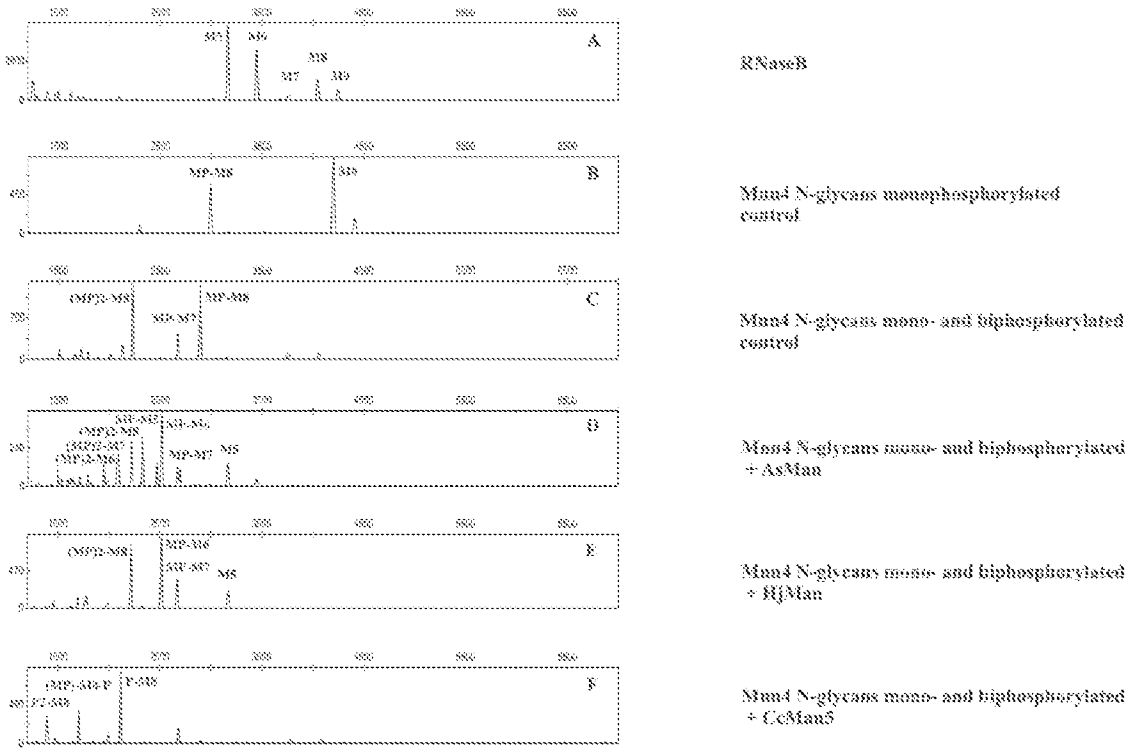

A
B
C
D
E
F
RNaseB
Mnn4 N-glycans monophosphorylated control
Mnn4 N-glycans mono- and biphosphorylated control
Mnn4 N-glycans mono- and biphosphorylated + AsMan
Mnn4 N-glycans mono- and biphosphorylated + HjMan
Mnn4 N-glycans mono- and biphosphorylated + CcMan5

FIGURE 11

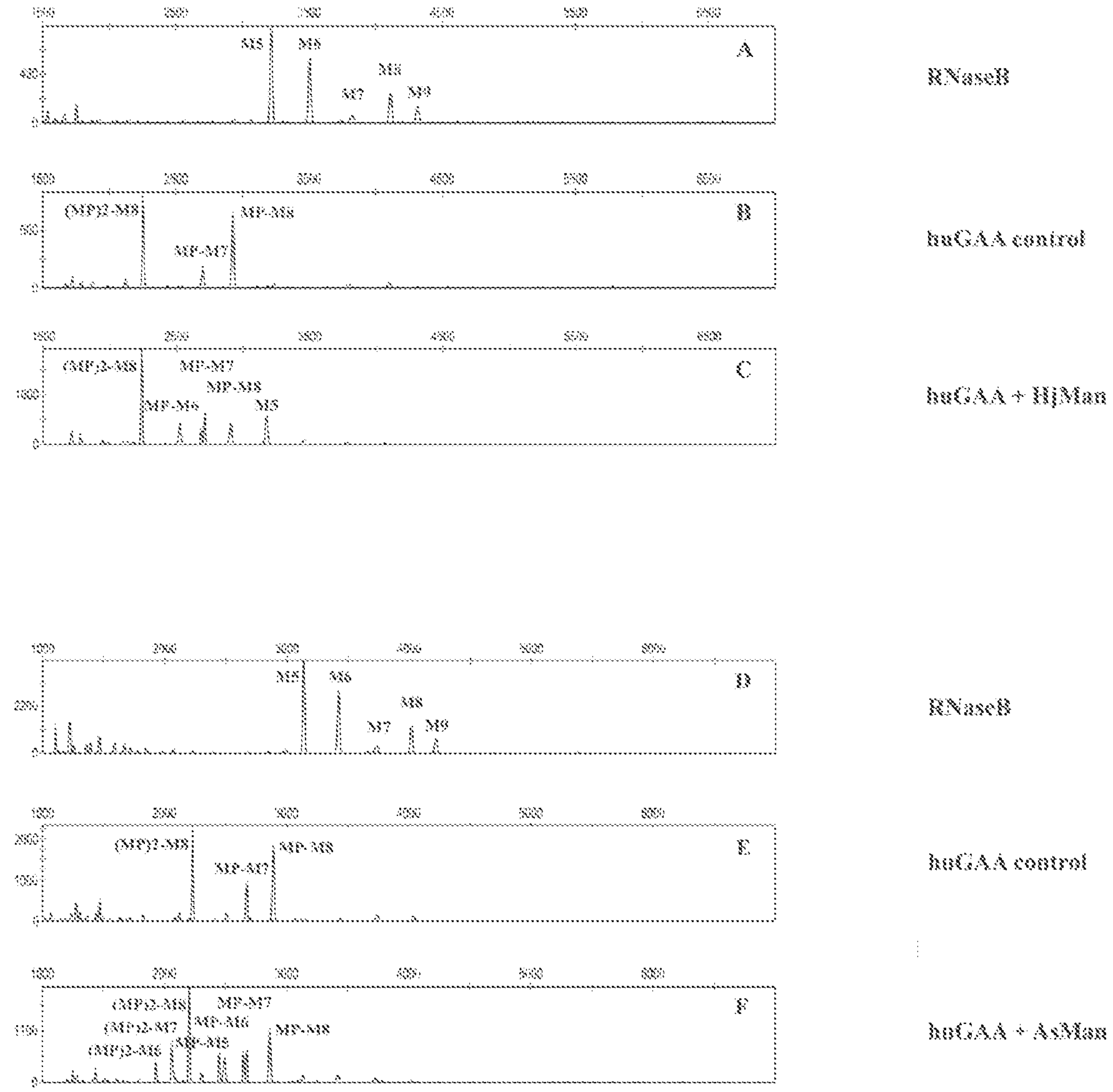

M5
M6
M7
M8
M9
A
RNaseB
(MP)2-M8
MP-M7
MP-M8
B
huGAA control
(MP)2-M8
MP-M7
MP-M8
MP-M6
M5
C
huGAA + HjMan
M5
M6
M7
M8
M9
D
RNaseB
(MP)2-M8
MP-M7
MP-M8
E
huGAA control
(MP)2-M8
(MP)2-M7
(MP)2-M6
MP-M7
MP-M6
MP-M5
MP-M8
F
huGAA + AsMan

FIGURE 12A

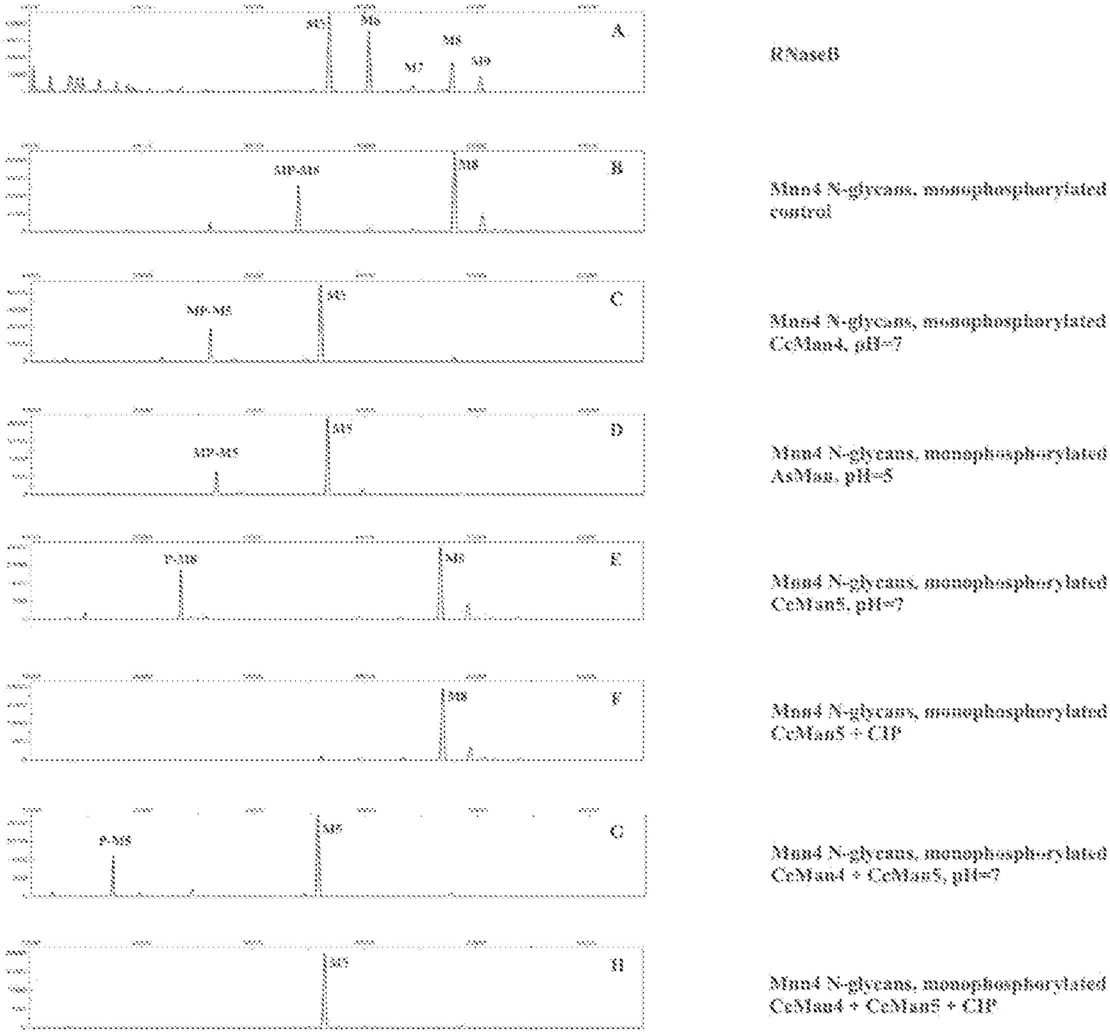
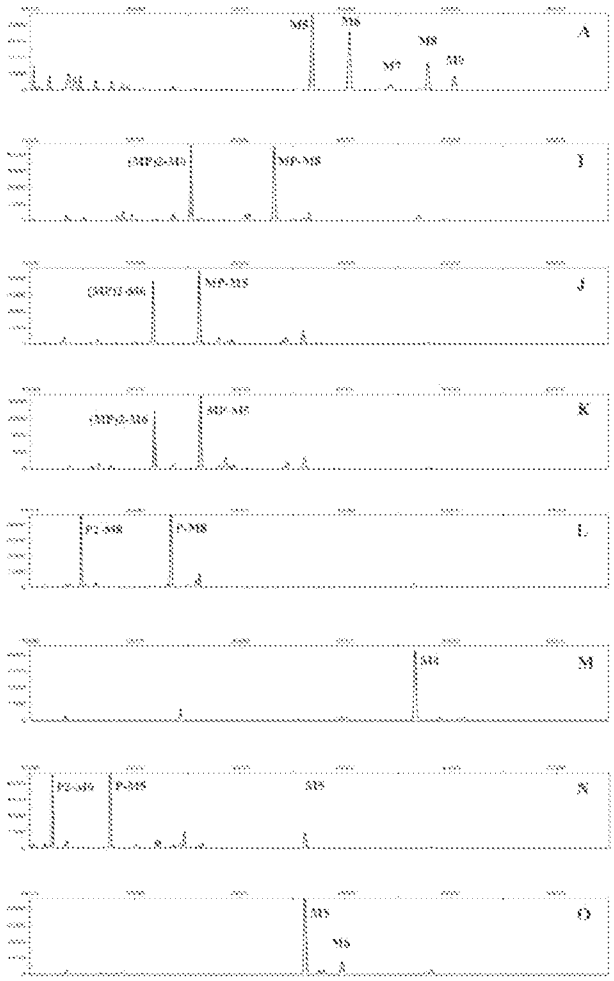

RNaseB
Man4 N-glycans, monophosphorylated control
Man4 N-glycans, monophosphorylated CcMan4, pH=7
Man4 N-glycans, monophosphorylated AsMan, pH=5
Man4 N-glycans, monophosphorylated CcMan5, pH=7
Man4 N-glycans, monophosphorylated CcMan5 + CIP
Man4 N-glycans, monophosphorylated CcMan4 + CcMan5, pH=7
Man4 N-glycans, monophosphorylated CcMan4 + CcMan5 + CIP

FIGURE 12B



RNaseB

Mnn4 N-glycans, mono- and diphosphorylated control

Mnn4 N-glycans, mono- and diphosphorylated CcMan4, pH=7

Mnn4 N-glycans, mono- and diphosphorylated AsMan, pH=5

Mnn4 N-glycans, mono- and diphosphorylated CcMan5, pH=7

Mnn4 N-glycans, mono- and diphosphorylated CcMan5 + CIP

Mnn4 N-glycans, mono- and diphosphorylated CcMan4 + CcMan5, pH=7

Mnn4 N-glycans, mono- and diphosphorylated CcMan4 + CcMan5 + CIP

FIGURE 13

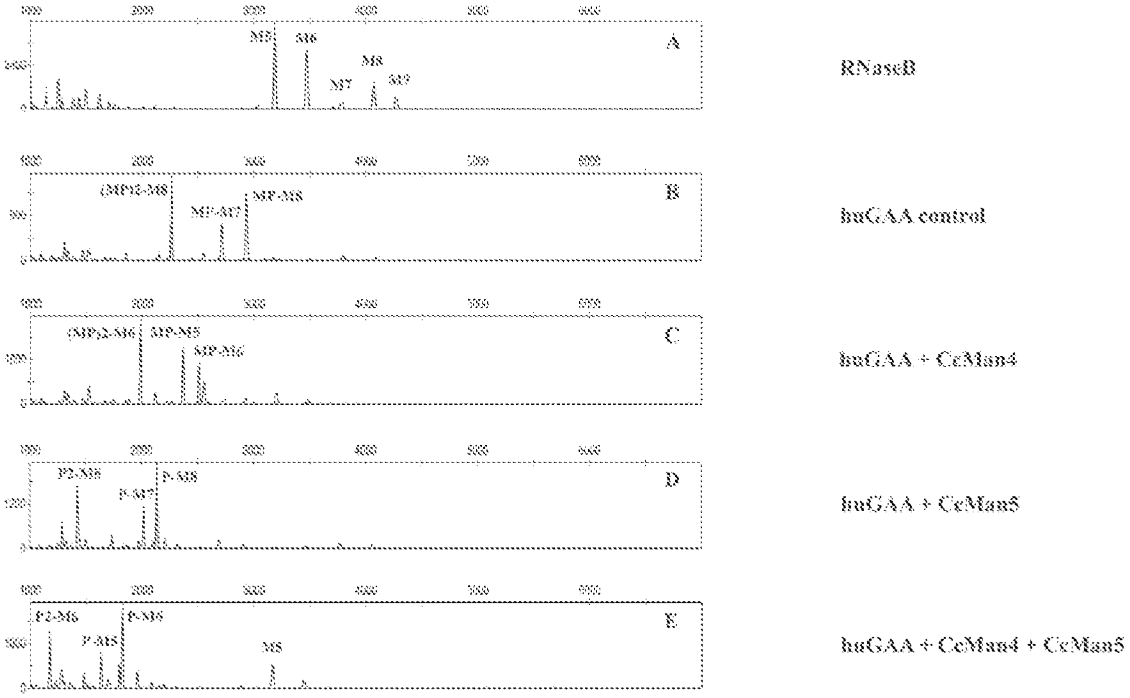

A
M8
B
MP-M8
C
D
P-M8
E
P2-M6
M5
RNaseB
huGAA control
huGAA + CcMan4
huGAA + CcMan5
huGAA + CcMan4 + CcMan5

FIGURE 14

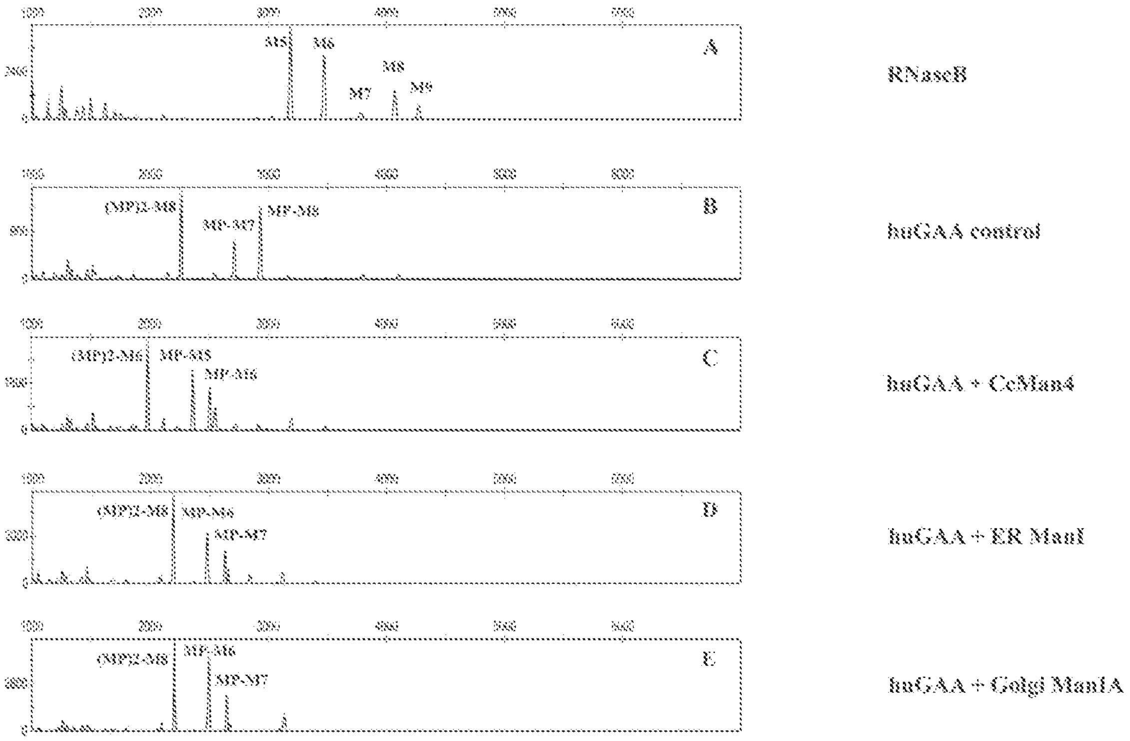

A
M5
M6
M7
M8
M9
RNaseB
B
(MP)2-M8
MP-M7
MP-M8
huGAA control
C
(MP)2-M6
MP-M5
MP-M6
huGAA + CeMan4
D
(MP)2-M8
MP-M6
MP-M7
huGAA + ER ManI
E
(MP)2-M8
MP-M6
MP-M7
huGAA + Golgi ManIA

FIGURE 15

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