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(54) Title: METHOD FOR REDUCING THE EXTENT OF O-MANNOSYLATION OF GLYCOPROTEINS

(57) Abstract: The present invention provides in vitro and in vivo methods using lysosomal mannosidases to remove O-linked mannose residues from intact glycoproteins, including hydrolyzing the Man-α-O-Ser/Thr glycosidic bond.

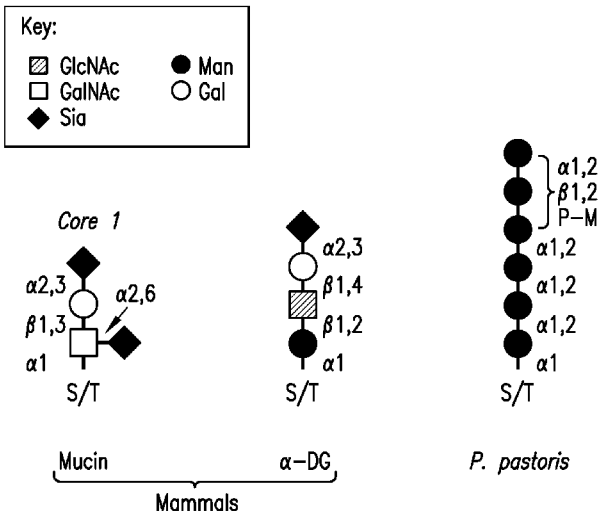

FIG.1A

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TITLE OF THE INVENTION

METHOD FOR REDUCING THE EXTENT OF O-MANNOSYLATION OF GLYCOPROTEINS

FIELD OF THE INVENTION

The present invention relates to the field of glycoengineering, and provides methods for removing O-linked mannose residues from intact glycoproteins, including hydrolyzing the Man-α-O-Ser/Thr glycosidic bond.

BACKGROUND OF THE INVENTION

The therapeutic properties of glycoproteins are strongly dependent on the composition of their glycans. Glycosylation is highly dependent on the cellular production system, the particular production clone and the culture process. The methylotrophic yeast *Pichia pastoris* is an attractive expression system for heterologous protein production due to its ability to secrete large amounts of protein and to perform post-translational modifications, including glycosylation. The nature of the oligosaccharides that are present on a recombinant glycoprotein can impact protein folding, stability, trafficking, functional activity and immunogenicity.

Even though *P. pastoris* has the advantage of post-translational modifications and folding pathways, its N- and O-linked glycosylation patterns differ significantly from those of humans. In yeast production strains, the modification of N-linked glycans in the golgi typically involves a series of additions of mannose residues by different mannosyltransferases, which results in "outer chain" glycosylations. Such modifications are generally undesired because it can lead to heterogeneity of a recombinant protein product, both from the perspective of molecular weight and composition, thereby complicating protein purification.

In *P. pastoris*, O-glycosylation is also referred to as O-mannosylation since it is primarily composed of two, three, or four α-1,2-linked mannose (Man) residues, which are attached to an initiating α-linked mannose residue attached to either a serine or threonine on the protein backbone (Duman *et al.* 1998). The α-1,2-Man polymers can be further capped by a β-1,2-Man disaccharide or phosphomannose (Figure 1A) (Trimble *et al.* 2004). The presence of β-linked mannose on glycans on recombinant proteins is of concern since it has been associated with increased immunogenicity. Also of concern is the presence of linear chains of α-1,2-linked mannose residues which may produce off-target interactions with *in vivo* receptors, such as Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (Cukan *et al.* 2012).

Over the last decade the N-and O-linked glycosylation pathways of *P. pastoris* have been engineered to produce recombinant proteins with human like N-linked glycosylation (Choi et al. 2003; Hamilton et al. 2003; Hamilton et al. 2006). Current technologies allow for the manufacturing of glycan-defined glycoproteins devoid of non-desirable yeast-type glycosylation, including phosphomannose and β-mannose additions (Hopkins *et al.* 2011). Depending on the degree of engineering, glycoproteins possessing residual amounts of single mannose residues (GSO2.0) linked to the protein backbone or O-linked mannose chains composed of α-1,2-linked mannose polymers (GSO1.0) may remain (Figure 1B) (Hamilton *et al* 2013, manuscript in press).

Having the ability to trim down extended α-mannose structures, and/or hydrolyze the Man-α-*O*-Ser/Thr glycosidic bond on intact glycoproteins secreted from glycoengineered *P. pastoris* would enhance the value and the utility of yeast expression systems in general, and *P. pastoris* expression systems in particular, for the production of therapeutic glycoproteins.

SUMMARY OF THE INVENTION

The invention provides methods to reduce the extent of O-mannosylation on intact glycoproteins. In particular embodiments the extent of O-mannosylation is reduced on intact glycoproteins produced in yeast or fungal host cells. In particular embodiments the invention also provides methods to hydrolyze the Man-α-*O*-Ser/Thr glycosidic bond on intact glycoproteins, including proteins expressed in glycoengineered *P. pastoris.* In general, the disclosed invention provides a strategy to modify the O-linked glycosylation pattern or O-glycoprofile of recombinant proteins that possess O-linked mannose residues, exemplified by proteins that are expressed in *P. pastoris* and *S. cerevisiae.* In practice, the disclosed methods can be used to facilitate the removal of O-mannosylation from therapeutic proteins produced in any expression system that is characterized by an O-glycoprofile that adds O-linked alpha-mannose residues.

In one aspect, the invention provides a method for reducing the extent of O-mannosylation of a recombinant glycoprotein comprising the steps of contacting a secreted recombinant glycoprotein comprising O-linked mannose residues with lysosomal mannosidase under conditions which allow for the enzymatic activity of mannosidase to produce a glycoprotein composition which comprises a reduced level of O-mannosylation compared to the O-mannosylation of the recombinant glycoprotein that is not treated with lysosomal mannosidase. In a particular embodiment, at least one α1,2 mannose, α1,3 mannose and/or α1,6 mannose residue in an O-linked mannose polymer is hydrolyzed. In an alternative embodiment

the disclosed methods remove O-mannose residues from intact glycoproteins by hydrolyzing the Man-α-O-Ser/Thr glycosidic bond.

The methods of the invention can be practiced using a host cell capable of producing the Man-α-O-Ser/Thr glycosidic bond, such as, but not limited to yeast or fungal host cells. For example, the methods of the invention can be practiced using wild-type *S.cerevisiae* or glycoengineered *Pichia pastoris* host cells. Other suitable host cells include *Pichia finlandica, Pichia trehalophila, Pichia koclamae, Pichia membranaefaciens, Pichia opuntiae, Pichia thermotolerans, Pichia salictaria, Pichia guercuum, Pichia pijperi, Pichia stiptis, Pichia methanolica, Pichia sp., Saccharomyces sp., Hansenula polymorpha, Kluyveromyces sp., Candida albicans, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Trichoderma reesei, Chrysosporium lucknowense, Fusarium sp., Fusarium gramineum, Fusarium venenatum* and *Neurospora crassa.*

As shown herein the methods of the invention can be practiced using a therapeutic glycoprotein selected from an antibody, a hormone, a cytokine, an enzyme or a bioactive peptide, which is produced in an expression system which performs O-glycosylation in a manner which produces single O-linked mannose or oligomannosyl-glycans that differ from the mucin-type glycans produced by human cells. Heterologous protein products having reduced O-glycosylation produced using the disclosed methods are also part of the present invention. In some embodiments the treated glycoprotein is optionally isolated/purified and its O-glyoprofile is characterized using an appropriate analytical protocol (i.e., high-performance anion exchange chromatorgraphy with pulsed amperometric detection (HPAEC-PAD or quadruple time of flight (Q-TOF) mass spectrometry). Alternatively, a functional assay, or a PK/PD determination can be performed to evaluate the effect of the reduced level of O-mannosylation on the activity profile or half-life of the treated glycoprotein.

The methods can be practiced on any recombinant glycoprotein possessing at least one O-linked glycan, possessing at least one α-linked mannose residue. More specifically, the method can be used to modify the O-glycoprofile of recombinant glycoprotein possessing an O-linked glycan comprising a mannose residue directly linked to a serine or threonine residue of the glycoprotein by an α-bond. For example, recombinant glycoprotein characterized by an O-linked glycan comprising mannose polymers, wherein at least one α1,2 mannose, α1,3 mannose and/or α1,6 mannose is linked to the initiating serine or threonine linked mannose residue can be modified using the disclosed methods. As shown herein, the disclosed methods can be used to enzymatically remove at least 10% of the original O-linked mannose present on the recombinant glycoprotein. In some embodiments, depending on the reporter protein and the reaction conditions, greater than 74% of the O-linked mannose is removed.

Alternatively, the methods can be used to enzymatically cleave at least 10% of the Man-α-O-Ser/Thr glycosidic bonds present on an O-glycosylated glycoprotein. In particular embodiments, greater than 74% of the Man-α-O-Ser/Thr glycosidic bonds are cleaved. In alternative aspects of the invention, the disclosed methods can be used to remove at least 50 %, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 97.5%, or at least about 99% of the O-linked mannose residues present on the glycoprotein. In yet other alternative aspects of the invention, the disclosed methods can be used to enzymatically cleave at least 50 %, 60%, 70%, 80%, 85%, 90%, 95%, 97.5%, or at least about 99% of the Man-α-O-Ser/Thr glycosidic bonds present on an O-glycosylated glycoprotein. In a particular aspect of the invention the disclosed methods can be used to completely remove all (e.g, 100% reduction) of the mannose residues and/or all of the Man-α-O-Ser/Thr glycosidic bonds present on an O-glycosylated protein of interest.

As disclosed herein, the methods of the invention can be practiced *in vitro* or *in vivo* using a recombinant lysosomal mannosidase (LMann) selected from the enzyme class consisting of E.C.3.2.1.24. Suitable lysosomal mannosidases can be isolated from a eukaryotic source. As shown herein the methods of the invention can be practiced using a LMann isolated from a source organism selected from *Arabidopsis thaliana, Dictyostelium discoideum, Glycine max, Cavia porcellus, Homo sapiens, Medicago truncatula, Mus musculus, Ricinus communis, Sulfolobus solfataricus, Trypanosoma cruzi, Solanum lycopersicum,* or *Vitis vinifera.*

As shown here, the methods of the invention can be practiced using LMann isolated from a plant or non-plant eukaryotic source. As shown herein the disclosed methods can be practiced using a lysosomal mannosidase selected from *Glycine max* (GmMann), *Medicago truncatula* (MtLMann) and *Vitis vinifera* (VvLMann) or a lysosomal mannosidase selected from *Arabidopsis thaliana* (AtLMann) and *Homosapiens* (HsLMann). Alternatively, the disclosed methods can be practiced using a lysosomal mannosidase obtained from another eukaryotic source. For example, a LMann obtained from a eukaryotic cell can be expressed, purified and screened using the methods disclosed in this invention. The methods and Examples demonstrate how a LMann can be recombinantly expressed in *P. pastoris*, isolated and then used in vitro to reduce O-mannosylation of a glycoprotein possessing O-linked mannose residues. Another approach is to co-secrete the LMann from the same cell as that expressing the glycoprotein of interest.

More specifically, the methods of the invention can be practiced using a LMann selected from, but not limited to homologues of the human lysosomal mannosidase (HsLMann) enzyme (AAC34130). For example, the methods of the invention can be practiced using a LMann selected from the mannosidases described using the designations provided in Table 1 to denote the LMann sequences having the amino acid sequences disclosed herein, including: AtLMann

(SEQ ID NO: 1), DdLMann (SEQ ID NO: 2), GmMann (SEQ ID NO: 3), GpLMann (SEQ ID NO: 4), HsLMann (SEQ ID NO: 5), MtLMann (SEQ ID NO: 6), MmLMann (SEQ ID NO: 7), RcLMann (SEQ ID NO: 8), SsLMann (SEQ ID NO: 9),TcLMann (SEQ ID NO: 10), ToLMann (SEQ ID NO: 11), or VvLMann (SEQ ID NO: 12).

As disclosed herein, the methods of the invention describe the identification and small-scale application of LMann to reduce O-mannosylation. It is foreseeable that reaction conditions, (including buffer composition, pH, time of incubation, temperature, substrate conentration, enzyme concentration, etc.) can be optimized to further enhance O-linked mannose removal. Likewise, it is foreseeable that a person having ordinary skill could readily use the reaction conditions described in the invention, or optimized conditions to scale the disclosed methods to faciliate O-mannose reduction of commercial scale preparations of therapetuic O-mannosylated glycoproteins. Thus providing a means to produce an intact commercial product with reduced and/or eliminated O-mannosylation using a yeast expression system.

Typically, lysosomal α-mannosidases (LManns) are large proteins, typically comprising a heterodimer of around 150kDa, which later self-dimerizes. The heterodimers are cleavage products of cleaved single chain precursor peptides. It is this latter single chain precursor that has been used to exemplify the utility of LMann to remove O-linked mannose. It is foreseeable that a cleaved, truncated or subdomain variant of the enzyme may be more efficient at removing O-linked mannose on particular proteins due to their differing structural conformations. Accordingly, the methods described herein can be practiced using a subdomain variant of a recombinant LMann. For example, in some embodiments artificial internal protease cleavage sites have been added to facilitate processing of the single chain precursor peptide into the heterodimeric form. In another example, in some embodiments the methods of the invention can be practiced using an N- or C-terminal variant of one of the recombinant lysosomal mannosidases disclosed herein.

As shown herein, the methods of the invention can be practiced *ex vivo* (e.g., *in vitro* using a glycoprotein that is secreted by a host cell), by the exogenous addition of a lysosomal mannosidase to a glycoprotein of interest following expression and secretion and/or optionally purification of the glycoprotein. When a method of the invention is practiced *in vitro,* the glycoprotein can remain in the culture media or can be purified. In other embodiments, the methods of the invention are practiced *in vivo* using yeast production strains that are engineered to co-secrete a lysosomal mannosidase in the presence of the glycoprotein of interest under conditions which promote the removal of O-linked mannose residues and/or the hydrolysis of the Man-α-*O*-Ser/Thr glycosidic bond during secretion or after secretion from the host cell.

Alternatively, the methods of the invention can be practiced *in vivo*. For example, one of the LMann expression vectors described herein, could be used to directly transform a yeast production strain that is already engineered to express the protein of interest. The co-secretion of the LMann along with the O-mannosylated protein of interest results in the production of a glycoprotein of interest with reduced O-mannosylation, which in turn avoids the need to perform an *in vitro* de-O-mannosylation step post-purification of the protein. It is forseeable that modification of the expression and/or media conditions could further enhance the ability to manipulate the extent of O-mannose removal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Illustrative representation of native and engineered O-linked glycosylations. (A) Comparison of mammalian mucin- and α-dystroglycan-type O-glycosylations to wild-type *P. pastoris* O-glycosylation. (B) A summary of *in vivo* engineering and *in vitro* manipulations to reduce O-mannosylation on proteins secreted from glycoengineered *P. pastoris*.

Figure 2. Exemplification of an expression vector used to produce recombinant lysosomal mannosidase. (A) The vector map for pGLY12387 represents the expression vector used to express the VvLMann under the control the AOX1 promoter. This vector integrates at the TRP2 loci of the *P. pastoris* genome and uses the Zeocin resistance marker for selection. Restriction enzyme sites used for subcloning or linearization (underlined) of the vector for transformation are highlighted. (B) The open reading frame encoding the VvLMann possesses an N-terminal α-mating factor secretion signal (SEQ ID NO: 13) fused in frame to a double FLAG tag, the sequence of which is in the upper box. C-terminal to this is fused the LMann fragment encoded by the amino acid residues described in Table 8 and a C-terminal 6xHIS tag possessing a GGGGS linker (SEQ ID NO: 14), as depicted in the lower box.

Figure 3. Preliminary screening to identify potential O-glycan cleavage ability of LMann secreted from *P. pastoris*. The supernatant from *P. pastoris* transformed with vectors encoding twelve homologues of lysosomal mannosidase (see Table 1) were assessed for their ability to reduce the O-glycan profile of recombinant SCI-CTP produced in a GSO1.0 glycoengineered *P. pastoris* strain. The SCI-CTP glycoprotein profile was analyzed by Q-TOF (A) and demonstrated to possess between six and twelve mannose residues per molecule. Incubation of supernatants from *P. pastoris* transformed with vectors encoding AtLMann (B), MtLMann (C) and VvLMann (D) showed no detectable, moderate and elevated ability to reduce the O-linked mannose content of SCI-CTP, respectively. The black arrow in panel D, highlights a peak corresponding to the

unglycosylated mass (8726 amu) of SCI-CTP, where all O-linked mannose has been removed. A scale has been added to the top of the figure to facilitate interpretation of the number of mannose residues present on the intact SCI-CTP. 0M, 1M, 2M etc. represents the addition of 0, 1, 2 etc. mannose residues respectively. Reactions were performed as described in the Materials and Methods section, with incubation time being 24h.

Figure 4. Preliminary characterization of VvLMann. Panels A and B represent untreated control SCI-CTP or that incubated for 24h with supernatant from *P. pastoris* transformed with the VvLMann expression vector. Continued incubation of the sample in B for a further 24h demonstrates that further reduction in the O-mannose profile can be obtained (C). Incubation of SCI-CTP with α-1,2-mannosidase from *Trichodema reesei* (TrMannI) in the presence or absence of VvLMann are respresented in (D) and (E) respectively. The black arrows highlight peaks corresponding to the unglycosylated mass (8726 amu) of SCI-CTP.

Figure 5. Purification of LMann. Recombinant VvLMann GmLMann and MtLMann were purified by nickel column chromatography from *P. pastoris* supernatants. Aliquots of the purified enzymes were run non-reduced (NR) or reduced (R) on SDS-PAGE gels and detected by Coomassie blue staining (A) or immunoblotted with anti-HIS tag antibody (B). Molecular weights markers are included for confirmation of molecular weight.

Figure 6. Characterization of the ability of purified LMann to reduce the O-glycan profile of SCI-CTP. Aliquots of VvLMann, GmLMann or MtLMann incubated with SCI-CTP for 24h produced the Q-TOF profiles represented in panels B, C, and D respectively. Untreated control SCI-CTP incubated for 24h in the absence of LMann is represented in panel A. The black arrows highlight peaks corresponding to the unglycosylated mass (8726 amu) of SCI-CTP.

Figure 7. Characterization of the ability of purified LMann mannosidases, which did not show activity when screened as supernatant, to reduce the O-glycan profile of SCI-CTP. Incubation of AtLMann and HsLMann with SCI-CTP for 24h produced the Q-TOF profiles represented in panel (B) and (C) respectively. Untreated control SCI-CTP incubated for 24h in the absence of LMann is represented in panel (A). The black arrows highlight peaks corresponding to the unglycosylated mass (8726 amu) of SCI-CTP.

Figure 8. Purification of AtLMann and HsLMann. Recombinant AtLMann and HsLMann were purified by nickel column chromatography from *P. pastoris* supernatants. Aliquots of the purified enzyme were run reduced on SDS-PAGE gels and detected by Coomassie blue staining (A) or immunoblotted with anti-HIS tag antibody (B). Molecular weights markers are included for confirmation of molecular weight.

Figure 9. Characterization of VvLMann enzymatic activity. Q-TOF analysis of SCI-CTP incubated for 24h in the absence of LMann is represented in panel A. Q-TOF analyses of parallel reactions following the addition of VvLMann to SCI-CTP in the presence of 1mM zinc (B), no additional zinc (C) or 1mM EDTA (D) are represented. The black arrows highlight peaks corresponding to the unglycosylated mass (8726 amu) of SCI-CTP.

Figure10. Characterization of the ability of purified VvLMann to reduce the O-glycan profile of human FcDM SEQ1. Human FcDM SEQ1 incubated overnight in the absence (A) or presence (B) of purified VvLMann was assessed by Q-TOF. The black arrows highlight peaks corresponding to the unglycosylated mass (50393+/-1 amu) of hFcDM SEQ1.

Figure 11. Characterization of the ability of purified VvLMann to reduce the O-glycan profile of SCI-OPEP. SCI-OPEP incubated overnight in the absence (A) or presence (B) of purified VvLMann was assessed by Q-TOF. The black arrow highlights the peak corresponding to the unglycosylated mass (7869 amu) of SCI-OPEP.

Figure 12. Characterization of the ability of purified VvLMann to reduce the O-glycan profile of commercial Leukine®. An aliquot of commercial Leukine® was PNGase treated to remove N-linked glycosylation. Subsequently this sample was incubated overnight in the absence (A) or presence (B) of VvLMann and assessed by Q-TOF. The black arrow highlights the peak corresponding to the unglycosylated mass (14431 amu) of Leukine®. Phosphomannose containing O-linked glycoforms have been labeled with a "P".

Figure 13. Production of recombinant LMann subdomains. Lysosomal mannosidase is typically synthesized as a precursor peptide which is processed into N- and C-terminal domains. An alignment (A) of the human LMann sequence with that of Vv-, GM- and Mt-LMann depicts where the human enzyme is cleaved (black arrow) into the two separate subdomains, based on the characterization by Berg et al. (2001) Mol. Gen. Met. 73, 18-29. Panel B depicts a variety of forms of secreted recombinant VvLMann. Form I represents the intact secreted precursor of the

VvLMann (regular hatched box) possessing an N-terminal double FLAG tag (black box) and a C-terminal HIS tag (white box). The black arrow depicts where the VvLMann would be proteolytically processed into two subdomains, based on homology to the HsLMann. Form II represents the introduction of a Kex2p cleavage (bolded hatched box) site into the VvLMann precursor sequence to facilitate N- and C-terminal processing. Form III represents Form II with the addition of a HIS tag N-terminal to the Kex2p site to facilitate purification. Form IV is similar to Form II except that a factor Xa site (FXa, dotted box) is introduced in place of the Kex2p site, thus allowing cleavage post-secretion. Form V represents the individual expression of the VvLMann N-terminal domain with an N-terminal double FLAG tag and a C-terminal HIS tag. Form VI represents individual expression of the VvLMann C-terminal domain with an N-terminal double FLAG tag and a C-terminal HIS tag.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that lysosomal α-mannosidases, can reduce the extent of O-mannosylation on intact glycoproteins and hydrolyze the Man-α-*O*-Ser/Thr glycosidic bond of glycoproteins, particularly those produced in wild type and engineered yeast expression systems. As shown herein the disclosed lysosomal α-mannosidases are efficient at O-mannose removal and because they are recombinant they provide purified mannosidase preparations that are devoid of the undesired proteolytic activity associated with crude cell extracts. Using the disclosed lysomsomal mannosidases in the methods disclosed and claimed herein provides a scaleable strategy for the removal of O-linked glycosylation from glycoproteins expressed in *P. pastoris*, or any other expression system characterized by O-mannosylation. Thus, the disclosed invention provides a means to produce biotherapeutic glycoproteins in non-mammalian expression systems that are more homologous to glycoproteins produced in mammalian expression systems.

Recent advances in glycoengineering of *P. pastoris* have successfully demonstrated the humanization of the N-glycosylation pathway and the subsequent ability to produce therapeutic glycoproteins with human-like N-linked glycans. *P. pastoris* like other yeast, add O-linked oligosaccharides to the hydroxyl groups of serine and threonine residues of secreted proteins. In *P. pastoris*, the most abundant O-linked glycan structures are typically polymers of between 1 and 4 α-1,2-linked mannose residues, with a subset of glycans being potentially capped by a ß-1,2-Man disaccharide or phosphomannose. Such O-mannosylation of recombinant proteins is considered a key factor in immunomodulation, with mannose-specific receptors binding and promoting enhanced immune responses. As a result of the glycoengineering of *P. pastoris*, the

recombinant proteins expressed in this system are devoid of phosphomannose and ß-mannose on O-linked glycans, resulting only in α-1,2-mannose polymers (Hopkins *et al.* 2011).

Examples of O-glycosylated recombinant proteins expressed in *P. pastoris* are well documented in the literature (Duman *et al.* 1998; Bewley *et al.* 1999; Letourneur *et al.* 2001; Boraston *et al.* 2003; Trimble *et al.* 2004; O'Leary *et al.* 2004). In humans and higher eukaryotes, extended sialylated O-linked glycosylation is typically of the mucin- or α-dystroglycan-type O-glycosylation (Hanisch 2001; Lommel and Strahl 2009). These are respectively composed of either an N-acetylgalactosamine (GalNAc) or a mannose (Man) residue attached to a serine or threonine residue, and subsequently extended by other monosaccharides, including N-acetylglucosamine (GlcNAc), galactose (Gal), sialic acid (Sia) and/or fucose (Fuc) (Figure 1A). In *P. pastoris*, O-glycosylation is often referred to as O-mannosylation since it is primarily composed of one, two, or three α-1,2-linked mannose (Man) residues, which are attached to an initiating α-linked mannose residue attached to either a serine or threonine on the protein backbone (Duman *et al.* 1998). Furthermore, the α-1,2-Man polymers can be additionally capped by a β-1,2-Man disaccharide or phosphomannose (Figure 1A) (Trimble *et al.* 2004).

The presence of non-native glycosylation on therapeutic proteins intended for human use can be of concern. For example, Hopkins *et al.* demonstrated that the occurrence of β-linked mannose on recombinant erythropoietin produced in glycoengineered *P. pastoris* had increased immunogenicity (Hopkins et al. 2011). Also of concern is that the presence of linear chains of α 1,2-linked mannose residues may produce off-target interactions with *in vivo* receptors, including Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (Cukan et al. 2012). There are also examples of proteins, such as human midkine and IGF1, which are not normally O-glycosylated in humans but which are when expressed in *P. pastoris* (Asami et al. 2000)(Gellissen 2000).

The reduction of O-linked glycans in yeast can be approached from many angles. One approach is to use a series of chromatographic purification steps to remove all of the mannosylated species from a glycoprotein composition. An alternative approach is to modulate the activity of the protein O-mannosyltransferase (PMT) family of genes, which are responsible for the addition of the initiating mannose to the serine or threonine residues of the protein backbone. However, the knockout of individual PMT genes can cause severe growth defects in yeast (Gentzsch and Tanner 1996; Gentzsch and Tanner 1997). As such, disrupting the entire repertoire of PMT genes is considered to be difficult, with any resultant strains being potentially non-viable (Hopkins *et al.* 2011). An alternative approach is to suppress Pmtp activity by adding chemical inhibitors (PMTi) to the fermentation media during cultivation ((Orchard et al. 2004).

However this strategy only reduces the occupancy of O-linked glycans, and does not completely eliminate it.

Enzymatic demannosylation provides yet another option for reducing the extent of O-mannosylation of glycoproteins produced in *Pichia* production strains. For example, Jack bean α-1,2/3/6-mannosidase has been shown to remove O-linked mannose from proteins expressed in wild type yeast. For example, Bergwerff demonstrated that Jack bean mannosidase could remove O-linked mannose fromrecombinant leech-derived tryptase inhibitor (rLDTI), following treatment will alkaline phosphatase to remove phosphomannose residues (Bergwerff et al. 1998). However, the reported ability of Jack bean α-mannosidase activity to remove O-linked mannose is primarily restricted to relatively small glycopeptides. To date, the only report indicating that Jack bean mannosidase can cleave the Man-O-Ser/Thr O-glycosidic linkage of an intact undenatured protein, is in the Bergwerff *et al.* report mentioned above , where the rLDTI is a 5 kDa protein.

The hydrolysis of the Man-O-Ser/Thr O-glycosidic linkage is generally considered not to occur on intact proteins, due to inaccessibility of the glycosidic linkage (Bretthauer 2007). This is consistent with the results of a study published by Ibatullin *et al.*, which describes the use of Jack bean mannosidase for the enzymatic demannosylation of glucoamylase from *Aspergillus awamori* (Ibatullin et al. 1993). The study indicated that the mannosidase removed only 24-26% of the total mannose from isolated glucoamylase, with only terminal mannose cleaved from the di- and trisaccharides. The study further reported that cleavage of the Man-O-Ser/Thr bond did not occur on the intact glucoamylase, and could not be achieved using either a higher concentration of mannosidase or a longer incubation time. Indeed, complete demannosylation (cleavage of O-linkages between mannose and serine/threonine) required proteolysis, which was achieved by treating native glucoamylase with trypsin, pronase or proteinase K. Therefore, the use of Jack bean mannosidase does not provide a universal option for the removal of O-mannosylation, because the enzymatic activity is believed to be limited by the conformation of the particular target protein and there is an inherent resistance of the Man-O-Ser/Thr bond to hydrolysis. Another potential limitation on the utility of Jack bean mannosidase as an option for reducing the extent of O-mannosylation on therapeutic glycoproteins, is that the only current source of Jack bean mannosidase is from crude cell extracts. As such, these possess numerous proteases which can degrade the therapeutic glycoprotein of interest during the deglycosylation incubation.

WO2005/033325 purports to describe a method of producing recombinant polypeptides in fungal host cells that are free of O-linked mannose residues using either a commercially available Jack bean α-mannosidase preparation or recombinant *Thermotoga maritima* α-

mannosidase (TMM) expressed in *E.coli*. Activities of the Jack bean mannosidase and the TMM were shown using two different relatively simple reporter proteins (tetra-glycosylated human GLP-1 and mono-glycosylated human insulin MG2). The results produced using Jack bean mannosidase agreed with what had been previously published by others, including Bergwerff *et al.* and Ibatullin *et al.*, which were that Jack bean mannosidase was not efficient at removing O-linked mannose from intact glycoproteins. Specifically, Jack bean mannosidase was only able to demannosylate 24% and 11% of the O-linked mannose on the GLP1 and human insulin glycoproteins respectively (See Tables 1 and 2 in WO2005/033325). By contrast, the results provided in Tables 3 and 4 of WO2005/033325 showed that the recombinant TMM was more efficient at demannosylating both of these reporter proteins. Specifically, up to 99% of the GLP1 and approximately 50% of the human insulin samples were able to be demannosylated. While the demannosylation by TMM of the GLP1 was efficient at 40°C, elevated temperatures (70°C) were required for the human insulin demannosylation. Such elevated temperatures are not desirable when handling therapeutic glycoproteins on a commercial scale since it may lead to increased aggregation, proteolysis and/or denaturation of the therapeutic protein. Furthermore, the methods provided for the TMM, indicate that the incubation conditions are protein specific and require optimization for each protein demannosylated, specifically optimization of the incubation temperature.

Sequence analysis of *T. maritima* and the lysosomal mannosidases disclosed herein, using the DNASTAR Lasergene Megalign software, reveal that although the eukaryotic lysosomal mannosidases disclosed herein exhibit a high homology to each other (>30% identity), the α-mannosidase from *T. maritima* showed less than 11% identity to the lysosomal mannosidases, with the exception of archaean mannosidase assessed (SsLMann), to which it had 30% identity. Indeed, the homology of the *T. maritima* mannosidase was so low towards the human LMann used in the Blastp search described below, that it was not returned as a homologue in the BLAST search that identified the eleven homologues used to exemplify the current invention. As such, based on sequence homology, both the *T. maritima* mannosidase and the SsLMann are considered to be significantly different to the eurkaryotic lysosomal mannosidases, which are the primary focus of the current invention, and represent a different family of enzymes. For the purposes of this invention, the Sulfolobus solfataricus protein was named SsLMann and characterized in parallel to the eukaryotic LManns, though this former enzyme is clearly not a lysosomal mannosidase.

Furthermore, the sequence of Jack bean mannosidase has not been reported to date in public databases, and commercial preparations are prepared from crude cellular extracts, and not a recombinant enzyme. As such, it is possible that non-desirable cellular components may be

present, including proteases which may hydrolyze the glycoprotein of interest. Accordingly, the use of Jack bean mannosidase in a production scale process is not desirable from the perspective of sourcing, with the possibility of contaminating protease activities, reagent quality, and cost.

Definitions

As used herein the term "lysosomal mannosidase" (LMann) refers to a eukaryotic mannosidase that localizes to the lysosome of a eukaryotic cell and possesses α-mannosidase activity. Lysosomal mannosidases are members of the glycoside hydrolase family 38 (GH38, Class II). Lysosomal mannosidases catalyze the hydrolysis of α-1,2-, α-1,3- and α-1,6-glycosidic bonds with retention of configuration of the anomeric carbon of the released mannose residue. Other synonyms for lysosomal mannosidase are," lysosomal alpha-mannosidase", "mannosidase alpha-B", "mannosidase (alpha B) lysosomal" or "lysosomal acid alpha-mannosidase"

As used herein, the term "reduce" as it relates to the decrease in the percentage amount of a defined or a group of glycoform structures, relative to the percentage amount started with.

As used herein, the term "demannosylated" as it relates to the decrease in the percentage amount of a defined or a group of mannose structures, relative to the percentage amount started with.

As used herein, the term "O-mannosylation" as it relates to the covalent conjugation of one or more mannose residues to serine or threonine residues of a protein sequence.

As used herein the term "O-glycoprofile" as it refers to the O-linked glycosylation profile of a released O-linked glycan population or the profile of the said O-linked glycan population while conjugated to a peptide sequence.

As used herein, the term "Man-α-O-Ser/Thr glycosidic bond" refers to the glycosidic bond connecting a mannose residue to a serine or threonine residue in a peptide sequence, where the mannose residue is in the α-configuration. The term "Man-α-O-Ser/Thr glycosidic bond" is also referred to as "Man-O-Ser/Thr glycosidic bond" or "Man-O-Ser/Thr O-glycosidic bond", all meaning the same defined glycosidic bond structure.

As used herein, the term "intact" refers to a glycoprotein that has not been manipulated *in vitro* to alter the conformation or structure of the protein. Examples of manipulation include, but are not limited to, denaturization of the protein by physical or chemical means and/or degradation of the protein by physical, chemical or enzymatic means. Typical manipulations used in the art are heat denaturization through increased temperature, proteolytic degradation using proteolytic enzymes and chemical denaturization using detergents or reducing agents.

Lysosomal Mannosidases

α-Mannosidases are ubiquitous in nature and have been purified and characterized from various plant, microbial and animal sources. α-Mannosidases have been classified into two independently derived groups, class I and class II, based on biochemical properties, substrate specificity, inhibitor profiles and sequence alignments. Generally speaking, Class I contains mannosidases found in the endoplasmic reticulum (ER) and Golgi. In eukaryotes, class I α-mannosidases are involved in early N-glycan processing reactions and in N-glycan dependent quality control in the ER. Class I α-mannosidases are conserved throughout eukaryotic evolution and do not share sequence homology with class II α-mannosidases.

The second group (class II) is more heterogeneous and contains the lysosomal mannosidases and other distantly related enzymes. A class of lysosomal mannosidases (LManns) with broad substrate specificity and the ability to catalyze the hydrolysis of α-1,2, α-1,3 and α-1,6-glycosidic bonds reside in the lysosome of eukaryotic cells and function to degrade mannose containing glycans. LManns (EC 3.2.1.24) (designated lysosomal α-1,2/3/6-mannosidases), are members of the glycoside hydrolase family 38 (GH38, Class II). They are involved in the catabolism of Asn-linked glycans and play a vital role in maintaining cellular homeostasis.

The lack of lysosomal mannosidases in humans results in the lysosomal storage disease α-mannosidosis (Malm and Nilssen 2008). Patients having this disease display a wide range of neurological, immunological and skeletal symptoms caused by a multisystemic accumulation of mannose containing oligosaccharides (Malm and Nilssen 2008). As such, much work has been done to characterize and understand this enzyme (Berg et al. 2001). Much of this work has focused on characterizing the enzyme in terms of its activity towards released free N-linked glycoforms (Aronson, Jr. and Kuranda 1989). As a consequence, lysosomal mannosidase has been shown to have a similar activity towards free N-linked glycans as Jack bean α-1,2/3/6-mannosidase.

Based on the hypothesis that lysosomal mannosidases would be able to reduce O-linked mannose chain length and cleave the Man-O-Ser/Thr glycosidic bond, homologues of the human lysosomal mannosidase (HsLMann) enzyme were identified. The entire human lysosomal mannosidase sequence (AAC34130) was used to BLAST search NCBI databases for homologues. Either the BLAST or the BLAST 2.0 algorithms, described in Altschul *et al.* (1977) Nucl. Acids Res. 25:3389 and Altschul *et al.* (1990) J. Mol. Biol. 215:403, respectively could be used to search for homologues. Specifically the NCBI Standard Protein BLAST website: (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome)

was used selecting the "non-redundant protein sequences (nr)" database and the "Blastp" algorithm. The "Blastp" algorithm was set to collect a maximum of 1000 sequences using the BLOSUM62 matrix with Gap costs of (Existence: 11, extension: 1). From the Blastp results returned, eleven homologues (Table 1) from various organisms were selected to represent a broad representation of the LMann family. The "homologue" identified from *S.* solfataricus (SsLMann), showed relatively low homology (approxiamtely 11% homology), and was the only non-eukaryotic protein assessed in the current invention. For the purposes of this invention, the Sulfolobus solfataricus protein was named SsLMann and characterized in parallel to the eukaryotic LManns, though this former enzyme is clearly not a true lysosomal mannosidase. It is foreseeable that other homologues identified in the described Blastp search, or from an expanded search, can be selected and screened using the methods disclosed for their ability to demannosylate O-linked mannose glycans.

Having identified twelve LMann homologues the methods described in the current invention were used to express and screen for O-mannose cleavage activity in supernatants isolated from *P. pastoris* into which expression vectors for each of the homologues had been transformed. Preliminary screening identified that detectable O-mannose hydrolysis was observed in the supernatants isolated from GmLann, MtLMann and VvLMann (Table 2). The screen described is dependent on sufficient expression of the homologues in P. pastoris, to detect activity in the supernatants isolated. It is foreseeable that O-mannose hydrolysis activity is present at very low levels in the supernatants from the other nine homologues, but the level of which is too low to be detected. As such, either by enriching the LMann from these other supernatants (e.g. by nickel or anti-FLAG chromatography, using the engineered tags on the proteins) or by expressing these homologues in an alternative expression system which gives enhanced expression of the LMann homologues, it could be possible to also detect O-mannose hydrolysis activity. Likewise, it is foreseeable that by expressing the LMann homologues in a glycoengineered *P. pastoris* strains, capable of producing alternative glycoforms on theexpressed LMann, could also alter the activity of the expressed LMann homologue. For example, expression of the LMann homologues in a strains engineered to produce N-linked glycans possessing Man5GlcNAc2 glycoforms, designated GS2.0 glycans, could be more active due to reduced hyperglycosylation of the N-linked glycans. Likewise, producing the LMann with complex-type N-linked glycans may be more beneficial.

Having identified three LMann homologues that demonstrate significant O-mannose hydrolysis activity, it is foreseeable that these homologues could be used to repreform the Blastp search to identify an expanded set of homologues with O-mannose hydrolytic activity.

Assays

In some embodiment the treated glycoprotein is optionally isolated/purified and its O-glycoprofile is characterized using an appropriate analytical protocol (e.g.,, high-performance anion exchange chromatorgraphy with pulsed amperometric detection (HPAEC-PAD or quadrople time-of-flight (Q-TOF) mass spectrometry of the glycopeptide or a parallel mass spectrometry based technology to analyze either the O-glycan composition or O-glycoprofile of a peptide/protein; gel-shift assays can be used to monitor overall peptide size and changes in mass following deglycosylation using the methods disclosed; likewise capillary electrophoresis may be used to monitor changes in the mass of a glycopeptide or glycan structure, for instance using a microchip such as that used in the LabChip technology; nuclear magnetic resonance (NMR), or a similar structural determination technique, can be used to monitor the structure of released O-glycans or glycopeptides; a functional assay can be used to evaluate the effect of the reduced level of O-mannosylation on the activity profile of the glycoprotein to be determined).

In some embodiment the treated glycoprotein is a glycoprotein produced in a wild-type or engineered yeast or fungal cell selected from SCI-CTP, SCI-OPEP, human Fc, GM-CSF or another protein which has been previously demonstrated to be O-glycosylated. Examples of such O-glycosylated therapeutic proteins are reviewed by Higgins (Higgins 2010). Other potential proteins that can benefit from the methods disclosed, are those which are traditionally not O-glycosylated in their native state but which are when expressed in a heterologous system, such as in yeast or fungal systems. Furthermore, the methods described herein can be used to remove O-linked mannose on proteins from mammalian or higher eukaryotic sources. For example, IgG2 produced in CHO and COS cell lines has been reported to possess a single O-linked mannose on the light chain (Martinez *et al.* 2007). Likewise, the methods disclosed herein can be used in combination with other glycosidic enzymes to remove α-dystroglycan-type glycans on proteins expressed in higher eukaryotes. In such instances, this can not only be used in the production of recombinat therapeutic proteins, but also be used to improve the analytical characterization of these proteins, which may be restricted by the presence of O-linked glycan structures.

Host Cells

The present invention encompasses any isolated *Pichia* sp. host cell (*e.g.*, such as *Pichia pastoris*) including wild-type and engineered host cells comprising various modified constructs, including host cells that comprise a promoter *e.g.*, operably linked to a polynucleotide encoding a heterologous polypeptide (*e.g.*, a reporter or immunoglobulin heavy and/or light chain) as well as methods of use thereof, *e.g.*, methods for expressing the heterologous polypeptide in the host

cell. Host cells of the present invention, may be also genetically engineered so as to express particular glycosylation patterns on polypeptides that are expressed in such cells. Host cells of the present invention are discussed in detail herein. Any engineered *Pichia* host cell cultured under any of the described conditions forms part of the present invention. In an embodiment of the invention, the host cell is selected from the group consisting of any *Pichia* cell, such as *Pichia pastoris*, *Pichia angusta (Hansenula polymorpha)*, *Pichia finlandica*, *Pichia trehalophila*, *Pichia koclamae*, *Pichia membranaefaciens*, *Pichia minuta* (*Ogataea minuta*, *Pichia lindneri*), *Pichia opuntiae*, *Pichia thermotolerans*, *Pichia salictaria*, *Pichia guercuum*, *Pichia pijperi*, *Pichia stiptis*, *Pichia methanolica*, *Pichia*.

In some aspects of the invention, *Pichia* host cells (*e.g.*, *Pichia pastoris*) can be genetically engineered to eliminate glycoproteins having α-mannosidase-resistant N-glycans by deleting or disrupting one or more of the beta-mannosyltransferase genes (*e.g.*, *BMTl*, *BMT2*, *BMT3*, and *BMT4*) (See, U.S. Patent No. 7,465,577) or abrogating translation of RNAs encoding one or more of the beta-mannosyltransferases using interfering RNA, antisense RNA, or the like. The scope of the present invention includes the use of such engineered *Pichia* host cells (*e.g.*, *Pichia pastoris*) comprising an expression cassette (*e.g.*, a promoter operably linked to a heterologous polynucleotide encoding a heterologous polypeptide).

Engineered host cells (*e.g.*, *Pichia pastoris*) cultured under conditions of the present invention also include those that are genetically engineered to eliminate glycoproteins having phosphomannose residues, *e.g.*, by deleting or disrupting one or both of the phosphomannosyl transferase genes *PNO1* and *MNN4B* (See for example, U.S. Patent Nos. 7,198,921 and 7,259,007), which can include deleting or disrupting the *MNN4A* gene or abrogating translation of RNAs encoding one or more of the phosphomannosyltransferases using interfering RNA, antisense RNA, or the like. In an embodiment of the invention, an engineered *Pichia* host cell has been genetically modified to produce glycoproteins that have predominantly an N-glycan selected from the group consisting of complex N-glycans, hybrid N-glycans, and high mannose N-glycans wherein complex N-glycans are, in an embodiment of the invention, selected from the group consisting of $Man_3GlcNAc_2$, $GlcNAc_{(1-4)}Man_3GlcNAc_2$, $Gal_{(1-4)}GlcNAc_{(1-4)}Man_3GlcNAc_2$, and $NANA_{(1-4)}Gal_{(1-4)}$ $GlcNAc_{(1-4)}Man_3GlcNAc_2$; hybrid N-glycans are, in an embodiment of the invention, selected from the group consisting of $GlcNAcMan_{(3-5)}GlcNAc_2$, $GalGlcNAcMan_{(3-5)}GlcNAc_2$, and $NANAGalGlcNAcMan_{(3-5)}GlcNAc_2$; and high mannose N-glycans are, in an embodiment of the invention, selected from the group consisting of $Man_5GlcNAc_2$, $Man_6GlcNAc_2$, $Man_7GlcNAc_2$, $ManG1cNAc_2$, and $Man_9GlcNAc_2$. In an embodiment of the invention, an engineered *Pichia* host cell has been genetically modified to produce glycoproteins that have predominantly an O-glycan selected from the group consisting of wild-type or

engineered O-glycans wherein wild-type glycans are inititated by an α-linked mannose residue to serine or threonine of a peptide sequence, which may subsequently be extended by 1-5 mannose residues in an α- or β-linked configuration and can possess phosphate linked to particular mannose residues. Engineered O-glycans are composed of those where the extent of α-, β- or phospho-mannose have been reduced and can further result in the production of O-linked glycans where single mannose residues are conjugated to serine or threonine residues of the peptide backbone.

The scope of the present invention includes such engineered *Pichia* host cells (*e.g.*, *Pichia pastoris*) comprising a modified, truncated, or deleted form of the *XRN1* gene. Additional embodiments of the present invention include engineered *Pichia* host cells (*e.g.*, *Pichia pastoris*) cultured under conditions of the present invention that are genetically engineered to include a nucleic acid that encodes the *Leishmania sp.* single-subunit oligosaccharyltransferase *STT3A* protein, *STT3B* protein, *STT3C* protein, *STT3D* protein, or combinations thereof such as those described in WO2011/06389. Additionally, engineered host cells (*e.g.*, *Pichia pastoris*) cultured under conditions of the present invention also include those that are genetically engineered to eliminate nucleic acids encoding Dolichol-P-Man dependent alpha(1-3) mannosyltransferase, *i.e.*., Alg3, such as described in US Patent Publication No. US2005/0170452.

Additional embodiments include the use of other lower eukaryotic organisms including yeast and fungal cells for the production of therapeutic proteins, and using methods similar to those described herein to removal O-linked mannose residues. In further aspects, the yeast or filamentous fungus host cell is selected from the group consisting of *Saccharomyces cerevisiae*, *Saccharomyces* sp., *Hansenula polymorpha*, *Kluyveromyces* sp., *Kluyveromyces lactis*, *Candida albicans*, *Aspergillus nidulans*, *Aspergillus niger, Aspergillus oryzae, Trichoderma reesei, Chrysosporium lucknowense*, *Fusarium* sp., *Fusarium gramineum, Fusarium venenatum*, *Physcomitrella patens* and *Neurospora crassa.*

Additional embodiments include the use of other eukaryotic organisms including higher eukaryotics organism, such as insect or mammalian cells for the production of therapeutic proteins, and using methods similar to those described herein to removal O-linked mannose residues. In such examples, the O-linked mannose structures may be α-dystroglycan-type in nature, which have been reduced to single O-linked mannose residues through genetic engineering of the host cell or through the prior/combined digestion of the α-dystroglycan-type glycans with other glycosidic enzymes, including but not limited to sialidase, galactosidase and hexosaminidase.

Molecular Biology

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Unless otherwise defined herein, scientific and technical terms used in connection with the present invention shall have the meanings that are commonly understood by those of ordinary skill in the art. Further, unless otherwise required by context, singular terms shall include the plural and plural terms shall include the singular. Generally, nomenclatures used in connection with, and techniques of biochemistry, enzymology, molecular and cellular biology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well known and commonly used in the art.

The methods and techniques of the present invention are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. *See*, e.g., James M. Cregg (Editor), *Pichia* Protocols (Methods in Molecular Biology), Humana Press (2010), Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989); Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates (1992, and Supplements to 2002); Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1990); Taylor and Drickamer, Introduction to Glycobiology, Oxford Univ. Press (2003); Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, N.J.; Handbook of Biochemistry: Section A Proteins, Vol I, CRC Press (1976); Handbook of Biochemistry: Section A Proteins, Vol II, CRC Press (1976); Essentials of Glycobiology, Cold Spring Harbor Laboratory Press (1999), Animal Cell Culture (R.I. Freshney, ed. (1986)); Immobilized Cells And Enzymes (IRL Press, (1986)); B. Perbal, A Practical Guide To Molecular Cloning (1984);

A “polynucleotide”, “nucleic acid” includes DNA and RNA in single stranded form, double-stranded form or otherwise.

A "polynucleotide sequence" or "nucleotide sequence" is a series of nucleotide bases (also called “nucleotides”) in a nucleic acid, such as DNA or RNA, and means a series of two or more nucleotides. Any polynucleotide comprising a nucleotide sequence set forth herein (*e.g.*, promoters of the present invention) forms part of the present invention.

A "coding sequence" or a sequence "encoding" an expression product, such as an RNA or polypeptide is a nucleotide sequence (*e.g.*, heterologous polynucleotide) that, when expressed,

results in production of the product (*e.g.*, a heterologous polypeptide such as an immunoglobulin heavy chain and/or light chain).

As used herein, the term "oligonucleotide" refers to a nucleic acid, generally of no more than about 100 nucleotides (*e.g.*, 30, 40, 50, 60, 70, 80, or 90), that may be hybridizable to a polynucleotide molecule. Oligonucleotides can be labeled, *e.g.*, by incorporation of ^{32}P-nucleotides, ^{3}H-nucleotides, ^{14}C-nucleotides, ^{35}S-nucleotides or nucleotides to which a label, such as biotin, has been covalently conjugated.

A "protein", "peptide" or "polypeptide" (*e.g.*, a heterologous polypeptide such as an immunoglobulin heavy chain and/or light chain) includes a contiguous string of two or more amino acids.

A "protein sequence", "peptide sequence" or "polypeptide sequence" or "amino acid sequence" refers to a series of two or more amino acids in a protein, peptide or polypeptide.

The term "isolated polynucleotide" or "isolated polypeptide" includes a polynucleotide or polypeptide, respectively, which is partially or fully separated from other components that are normally found in cells or in recombinant DNA expression systems or any other contaminant. These components include, but are not limited to, cell membranes, cell walls, ribosomes, polymerases, serum components and extraneous genomic sequences. The scope of the present invention includes the isolated polynucleotides set forth herein, *e.g.*, the promoters set forth herein; and methods related thereto, *e.g.*, as discussed herein.

An isolated polynucleotide or polypeptide will, preferably, be an essentially homogeneous composition of molecules but may contain some heterogeneity.

"Amplification" of DNA as used includes the use of polymerase chain reaction (PCR) to increase the concentration of a particular DNA sequence within a mixture of DNA sequences. For a description of PCR see Saiki, *et al.*, Science (1988) 239:487.

In general, a "promoter" or "promoter sequence" is a DNA regulatory region capable of binding an RNA polymerase in a cell (*e.g.*, directly or through other promoter-bound proteins or substances) and initiating transcription of a coding sequence to which it operably links.

A coding sequence (*e.g.*, of a heterologous polynucleotide, *e.g.*, reporter gene or immunoglobulin heavy and/or light chain) is "operably linked to", "under the control of", "functionally associated with" or "operably associated with" a transcriptional and translational control sequence (*e.g.*, a promoter of the present invention) when the sequence directs RNA polymerase mediated transcription of the coding sequence into RNA, preferably mRNA, which then may be RNA spliced (if it contains introns) and, optionally, translated into a protein encoded by the coding sequence.

The present invention includes vectors or cassettes which comprise various modified constructs, including promoters optionally operably linked to a heterologous polynucleotide. The term "vector" includes a vehicle (*e.g.*, a plasmid) by which a DNA or RNA sequence can be introduced into a host cell, so as to transform the host and, optionally, promote expression and/or replication of the introduced sequence. Suitable vectors for use herein include plasmids, integratable DNA fragments, and other vehicles that may facilitate introduction of the nucleic acids into the genome of a host cell (*e.g.*, *Pichia pastoris*). Plasmids are the most commonly used form of vector but all other forms of vectors which serve a similar function and which are, or become, known in the art are suitable for use herein. See, *e.g.*, Pouwels, *et al.*, Cloning Vectors: A Laboratory Manual, 1985 and Supplements, Elsevier, N.Y., and Rodriguez *et al.* (eds.), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, 1988, Buttersworth, Boston, MA.

A polynucleotide (*e.g.*, a heterologous polynucleotide, *e.g.*, encoding an immunoglobulin heavy chain and/or light chain), operably linked to a promoter, may be expressed in an expression system. The term "expression system" means a host cell and compatible vector which, under suitable conditions, can express a protein or nucleic acid which is carried by the vector and introduced to the host cell. Common expression systems include fungal host cells (*e.g.*, *Pichia pastoris*) and plasmid vectors, insect host cells and Baculovirus vectors, and mammalian host cells and vectors.

The following references regarding the BLAST algorithm are herein incorporated by reference: BLAST ALGORITHMS: Altschul, S.F., *et al.*, J. Mol. Biol. (1990) 215:403-410; Gish, W., *et al.*, Nature Genet. (1993) 3:266-272; Madden, T.L., *et al.*, Meth. Enzymol. (1996) 266:131-141; Altschul, S.F., *et al.*, Nucleic Acids Res. (1997) 25:3389-3402; Zhang, J., *et al.*, Genome Res. (1997) 7:649-656; Wootton, J.C., *et al.*, Comput. Chem. (1993) 17:149-163; Hancock, J.M., *et al.*, Comput. Appl. Biosci. (1994) 10:67-70; ALIGNMENT SCORING SYSTEMS: Dayhoff, M.O., *et al.*, "A model of evolutionary change in proteins." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3. M.O. Dayhoff (ed.), pp. 345-352, Natl. Biomed. Res. Found., Washington, DC; Schwartz, R.M., *et al.*, "Matrices for detecting distant relationships." in Atlas of Protein Sequence and Structure, (1978) vol. 5, suppl. 3." M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC; Altschul, S.F., J. Mol. Biol. (1991) 219:555-565; States, D.J., *et al.*, Methods (1991) 3:66-70; Henikoff, S., *et al.*, Proc. Natl. Acad. Sci. USA (1992)89:10915-10919; Altschul, S.F., *et al.*, J. Mol. Evol. (1993) 36:290-300; ALIGNMENT STATISTICS: Karlin, S., *et al.*, Proc. Natl. Acad. Sci. USA (1990) 87:2264-2268; Karlin, S., *et al.*, Proc. Natl. Acad. Sci. USA (1993) 90:5873-5877; Dembo, A., *et al.*, Ann. Prob. (1994) 22:2022-2039; and Altschul, S.F. "Evaluating the statistical significance of

multiple distinct local alignments." in Theoretical and Computational Methods in Genome Research (S. Suhai, ed.), (1997) pp. 1-14, Plenum, New York.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

The present invention is not to be limited in scope by the specific embodiments described herein; the embodiments specifically set forth herein are not necessarily intended to be exhaustive. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Examples

The following examples are intended to exemplify the present invention and not to be a limitation thereof. The methods and compositions disclosed below fall within the scope of the present invention.

Experimental Methods

Reagents

The α-1,2-mannosidase was cloned from *Trichoderma reesei* (TrMannI), a C-terminal 6xHIS tag was added and it was expressed in-house in glycoengineered *P. pastoris* under the control of the inducible AOX1 promoter.

Reporter Proteins

The reporter proteins, single chain insulin fused to the CTP peptide (SCI-CTP) and single chain insulin fused to the highly O-glycosylated peptide of the human TNFR2 ectodomain (SCI-OPEP) were expressed in a GFI1.0 glycoengineered *P. pastoris* strain (Li et al. 2006), which produced extended α-1,2-mannose containing O-linked chains. The reporter protein human Fc double mutein sequence 1 was expressed in a GFI2.0 glycoengineered P. pastoris strain (Li *et al.* 2006), which produced O-linked glycans composed primarily of single O-linked mannose residues. Both the GFI1.0 and GFI2.0 glycoengineered strains secreted recombinant protein devoid of β-mannose and phosphomannose additions. Commercial Leukine® was purchased from a Pharmacy. This protein was produced in a *Saccharomyces cerevisiae* yeast strain.

Both of the SCI fusion proteins were chosen due to the non-insulin fusion partners being heavily O-glycosylated. A GFI1.0 glycoengineeered background was chosen for these reporter proteins to allow screening for both reduction of extended α-1,2-mannose polymers and the cleavage of the Man-O-Ser/Thr glycosidic bond. The hFcDM reporter protein was chosen as an example of a different protein class. A GFI2.0 glycoengineered background was chosen to show specific cleavage of the Man-O-Ser/Thr glycosidic bond. Commercial Leukine® (a *S. cerevisiae* produced GM-CSF) was chosen as it exemplifies how the LMann can faciliate O-mannose reduction on a non-*Pichia pastoris* produce glycotherapeutic.

Generation of O-glycosylated reporter proteins

Synthetic single chain insulin fusion proteins were generated by fusing either the O-glycosylated carboxyl-terminal peptide (amino acids 136 to 163 of the precursor) of the human chorionic gonadotropin beta-subunit or an O-glycosylated ectodomain fragment (amino acids 221 to 240 of the precursor) of human tumor necrosis factor receptor 2 peptide, and were

designated SCI-CTP and CTP-OPEP respectively. Both SCI-CTP and SCI-OPEP were expressed in a GFI1.0 glycoengineered *P. pastoris* strain (Li et al. 2006), which produced extended α-1,2-mannose containing O-linked chains. Human Fc double mutein (hFcDM) sequence1 was expressed in a GFI2.0 glycoengineered *P. pastoris* strain (Li et al. 2006), which produced O-linked glycans composed of a single O-linked mannose residue. Both the GFI1.0 and GFI2.0 glycoengineered strains secreted recombinant protein devoid of β-mannose and phosphomannose additions.

Generation and transformation of LMann expression vectors

Twelve *P. pastoris* expression vectors were generated to secrete twelve lysosomal mannosidase homologues into the culture supernatant to facilitate assessment of the enzymes abilities to reduce O-linked mannose. Table 1 overviews the homologues assessed, the respective fragments of each ORF expressed and the resultant vector generated for each. Briefly, each vector was based upon the previously described vector pGLY2088 (Nett *et al.* 2012). This latter vector is a roll-in integration vector that targets integration of the vector to the *TRP2* locus and contains the bleomycin (ble) resistance gene to confer selection on Zeocin™. The vector pGLY2088 also possesses an *AOX1* inducible expression cassette, which contains functional fragments of *P. pastoris AOX1* promoter and the *S. cerevisiae CYC1* terminator. Located between these regions are the restriction sites *Fse*I, *Eco*RI and *Swa*I, which have been used to facilitate generation of the recombinant LMann expression cassettes. PCR was used to amplify the DNA encoding amino acids 1-89 of the α-mating factor prepro (αMFpp) sequence from *P. pastoris* genomic DNA. Subsequently this was used as template to generate a 350bp DNA fragment possessing from 5' to 3' termini, an *Fse*I restriction site, a kozak sequence, DNA encoding amino acids 1-89 of the αMFpp sequence, a double FLAG tag (encoded by the amino acid sequence "DYKDDDDK" in duplicate) and an *Eco*RI restriction site. This fragment was subsequently subcloned into the *TRP2* integration vector between the *Fse*I/*Eco*RI restriction sites of pGLY2088. This vector was designated pGLY2088-αMFpp-2xFLAG.

Partial synthetic open reading frames encoding fragments of the twelve LMann ORFs were generated by Life Technologies/ GeneArt® (Regensburg, Germany) using the GeneOptimizer® software and codon-optimized for *P. pastoris* expression. Briefly, fragments possessing from 5' to 3' an *Eco*RI restriction site, DNA encoding codon-optimized amino acid fragments of each LMann as highlighted in Table 1 and SEQ ID NO.1 to 12 , DNA encoding an amino acid "GGGGS" linker, DNA encoding a six histidine tag (6xHIS), a stop codon and a *Swa*I restriction site were generated for each LMann. Subsequently, these fragments were

subcloned in-frame into the *Eco*RI/*Swa*I restriction sites of pGLY2088-αMFpp-2xFLAG, so generating the expression vectors pGLY12376 to pGLY12387 as summarized in Table 1. Figure 2A shows a representative final vector, pGLY12387, and Figure 2B summarizes the domain structures of the synthetic ORF generated for each of the recombinant LMann. The upper and lower panels, containing amino acid sequences, represent the amino acids added to the N- and C-termini of the amino acid fragments of each of the LMann fragments highlighted in Table 1.

For transformation of the vectors into *P. pastoris*, the LMann expression vectors pGLY12376 to pGLY12387 were digested with the restriction enzyme *Spe*I to linearize, transformed into the wild-type *P. pastoris* strain NRRL-Y11430 (ATCC, Manassas, VA) by electroporation (Choi et al. 2003) and plated on YSD plates containing 100μg/ml Zeocin™ (Life Technologies, Grand Island, NY). Several representative clones potentially expressing each LMann were either screened directly for their ability to hydrolyze O-linked mannose using SCI-CTP.

Small scale expression of LMann in *P. pastoris*

Representative clones of each LMann homologue transformed into NRRL-Y11430 were grown up in shake flasks containing 50ml BSGY (40 g/L glycerol, 20 g/L soytone, 10 g/L yeast extract, 11.9 g/L KH2PO4, 2.3 g/L K2HPO4, 18.2 g/L sorbitol, 13.4 g/L YNB with ammonium sulfate without amino acids, 8 mg/L biotin) for 72h at 26°C. Following which the cells were pelleted and washed once in 10ml BSMY (the composition of which was identical to BSGY, except that it contained 1% methanol (containing 0.88 mg/L of PMTi4) in place of the glycerol) prior to resuspension in 5ml BSMY and incubation at 26°C. After 24h an additional charge of methanol to a final concentration of 1% (v/v) was added. The culture supernatants were harvested after a further 24h incubation at 26°C. A control sample was prepared where untransformed NRRL-Y11430 was grown and mock-induced in parallel, to provide supernatant devoid of expressed LMann.

Screening *P. pastoris* supernatant expressing LMann for O-linked mannose hydrolysis activity

10μl of harvested culture supernatant was added to a vial containing 10μl of 5x reaction buffer (100mM sodium acetate, 2mM Zn2+, pH 5.0), 1.5μl SCI-CTP (3.3mg/ml), 0.5μl 100x protease inhibitor cocktail (330mg/L Pepstatin A and 220mg/L Chymostatin in methanol) and the volume brought up to 50μl with distilled water. Reactions were incubated for 16-24h at 37°C prior to submitting samples for Q-TOF analysis.

To reduce the extent of α-1,2-mannose extensions on the O-linked glycans of SCI-CTP reactions were performed in the presence of both LMann supernatants and recombinant TrMannI. For these 10µl of harvested culture supernatant was added to a vial containing 1µl of purified TrMannI (0.35 mg/ml), 5µl of 10x reaction buffer (0.2M ammonium acetate, pH6.0 containing 10mM calcium chloride), 1.5µl SCI-CTP (3.3 mg/ml), 0.5µl 100x protease inhibitor cocktail (330 mg/L Pepstatin A and 220mg/L Chymostatin in methanol) and the volume brought up to 50µl with distilled water. A control lacking TrMannI was run in parallel, with the volume of distilled water being adjusted accordingly. Reactions were incubated for 16-24h at 37oC prior to submitting samples for Q-TOF analysis.

Large-scale generation of LMann

The growth of strains was performed in bioreactors using inoculum seed flasks as described below. The inoculum seed flasks were inoculated from yeast patches (isolated from a single colony) on agar plates into 0.1 L of 4% BSGY in a 0.5-L baffled flask. Seed flasks were grown at 180 rpm and 24°C (Innova 44, New Brunswick Scientific) for 48 hours. Cultivations were done in 1 L (fedbatch-pro, DASGIP BioTools) bioreactors. Vessels were charged with 0.54 L of 0.22 µm filtered 4% BSGY media (with 4 drops/L Sigma 204 antifoam and 50 g/L Maltitol) and autoclaved at 121°C for 60 min.

After sterilization and cooling; the aeration, agitation and temperatures were set to 0.7 vvm, 620 rpm and 24°C respectively. The pH was adjusted to and controlled at 6.5 using 30% ammonium hydroxide. Inoculation of a prepared bioreactor occurred aseptically with 60 mL from a seed flask. Agitation was ramped to maintain 20% dissolved oxygen (DO) saturation. After the initial glycerol charge was consumed, denoted by a sharp increase in the dissolved oxygen, a 50% w/w glycerol solution containing 5 mg/L biotin and 32.3 mg/L PMTi4 was triggered to feed at 3.68 mL/hr for 8 hours. During the glycerol fed-batch phase 0.375 mL of PTM2 salts were injected manually. Completion of the glycerol fed-batch was followed by a 0.5 hour starvation period and initiation of the induction phase. A continuous feed of a 50% v/v methanol solution containing 2.5 mg/L biotin and 6.25 mL/L PTM2 salts was started at a flat rate of 2.16 mL/hr. Injections of 0.5 mL of protease inhibitor solution containing 3.6 mg/mL Pepstatin A and 2.2 mg/mL Chymostatin (in methanol) were added at the start of induction and after each 24 hours of induction time. Additionally, injections of 0.25 mL of 1.9 mg/ml PMTi4 (in methanol) were added each 24 hours of induction. Individual fermentations were harvested after 60 hours of induction. The culture broth was clarified by centrifugation (Sorvall Evolution RC, Thermo Scientific) at 8500 rpm for 40 min and the resulting supernatant was submitted for purification.

Purification of active LMann

Immobilized metal ion affinity chromatography (IMAC) was used for the purification of 6×His-tagged recombinant LMann enzyme produced in *Pichia pastoris*. The cell free supernatant sample was transferred to a 10 ml STREAMLINE™ Chelating column (GE healthcare, Cat:17-1280-01) preloaded with nickel ions, at 2.0 ml/min and equilibrated with 20mM TRIS-HCl pH7.0, 150mM NaCl containing running buffer. After loading, the column was washed with three column volume of the running buffer to remove unbound proteins. The bound LMann enzyme was eluted with a linear gradient of twelve column volume from 0–500 mM Imidazole in 20mM TRIS-HCl pH7.0, 150mM NaCl. Fractions containing the purified LMann enzyme were assessed by Coomassie blue staining, pooled, and dialyzed against 4L of Phosphate Buffered Saline pH7.4 over a 16h period at 4°C. After dialysis the purified LMann enzyme was stored at -20°C until analysis.

Exoglycosidase enzyme treatments

Intact glycoprotein (SCI-CTP, hFcDM SEQ1 or SCI-OPEP) 50μg were dissolved in reaction buffer containing 20 mM sodium acetate, 0.4 mM Zn2+ pH 5.0 and incubated with 3.8μg of LMann enzyme for 16h at 37°C prior to analysis. For α-1,2-mannosidase (TrMannI) treatment, 50μg of glycoprotein was dissolved in 20mM ammonium acetate pH6.0 containing 1mM CaCl2 and incubated with 2μg of enzyme for 16h at 37°C prior to analysis. Commercial Leukine® (sargramostim) yeast expressed recombinant GM-CSF was subjected to similar enzymatic digest as mentioned above.

Quadrupole time-of-flight mass spectrometry (Q-TOF) analysis

Mass spectrometric analysis was done in positive ion mode on Accurate-Mass Q-TOF LC/MS 6520 (Agilent technologies, Santa Clara, CA). The protocol used was as previously described (Choi et al. 2012), except that only intact glycoprotein analysis was performed.

O-glycan composition analysis

For O-glycan analysis, the O-glycans were released from the reporter protein by β-elimination under alkaline conditions, processed and analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described previously (Stadheim et al. 2008).

SDS-PAGE gels and immunodetection

Purified LMann (2.5μg each) were separated on the 4-20% gradient SDS-PAGE TRIS-HCl gels (BioRad Laboratories, Hercules, CA) and protein bands were visualized with Coomassie blue stain. For immunoblotting, proteins were transferred onto a nitrocellulose membrane, and blocked overnight in 1% blocking solution (Western blocking reagent, Roche Diagnostics, Indianapolis, IN). The membrane was then incubated with 1:100 dilution of His-Probe (H-3) HRP (horseradish peroxidase) mouse monoclonal IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA) in 0.5% blocking solution for 1 hour at room temperature. After incubation, the nitrocellulose membrane was washed three times with TBST wash buffer (TRIS-saline buffer containing 0.1% Tween 20). After washing, protein bands on the membrane were detected by colorimetric method using Metal Enhanced DAB substrate Kit (Pierce Biotechnology, Rockford, IL).

Examples

Example 1: Recombinant Expression and Secretion of Lysosomal Mannosidases

Purpose: To produce recombinant LMann to screen for O-linked mannose hydrolysis ability. Twelve lysosomal mannosidases (See Table 1), with broad sequence homology to human lysosomal mannosidase (HsLMann), were identified and selected for expression and characterization.

To produce recombinant enzyme the LMann sequences were cloned into *P. pastoris* expression vectors which would facilitate expression and secretion recombinant versions of each from this host organism. The yeast expression vector used integrated at the *TRP2* loci and used the bleomycin resistance marker to confer resistance to Zeocin™ (Figure 2A). The lysosomal mannosidase was under the transcriptional control of the methanol inducible *AOX1* promoter. The ORF encoding the LMann sequence possessed and N-terminal alpha-mating factor prepro secretion signal, a double FLAG tag, the LMann seqeunce and a C-terminal HIS tag (Figure 2B). During secretion the alpha-mating factor prepro sequence is cleaved to produce a secreted N-terminally FLAG tagged and C-terminally HIS tagged protein. Each of these tags can be used for purification or detection purposes. The LMann encoded amino acids of each vector is highlighted in Table 1 and SEQ ID NOS: 1 through 12.The N- and C-terminal amino acid sequence added to each LMann is depicted in Figure 2B.

Table 1: List of LMann homologues used in the current study

Source organism	NCBI ID	Enzyme abbreviation	Amino acid residues expressed	Expression vector
Arabidopsis thaliana	NP_189306	AtLMann	23-1019	pGLY12376
Dictyostelium discoideum	AAA33224	DdLMann	33-1005	pGLY12377
Glycine max	XP_003548841	GmMann	26-1012	pGLY12378
Cavia porcellus	MA2B1_CAVPO	GpLMann	48-1007	pGLY12379
Homo sapiens	AAC34130	HsLMann	50-1011	pGLY12380
Medicago truncatula	XP_003629280	MtLMann	30-1018	pGLY12381
Mus musculus	AAC53369	MmLMann	28-991	pGLY12382
Ricinus communis	XP_002530831	RcLMann	29-891	pGLY12383
Sulfolobus solfataricus	NP_344318	SsLMann	1-968	pGLY12384
Trypanosoma cruzi	XP_819105	TcLMann	26-977	pGLY12385
Solanum lycopersicum	NP_001234851	ToLMann	26-1028	pGLY12386
Vitis vinifera	XP_002265360	VvLMann	25-1006	pGLY12387

Results: Transformation of the twelve LMann expression vectors, and immunoblot detection indicated that recombinant protein had been expressed at varying levels.

One known caveat to removing O-linked mannose from intact proteins is that in the lysosome glycoproteins are rapidly processed by proteases into simple peptides, thus removing any restriction caused by steric hindrance of an intact protein. As such it was not apparent that lysosomal mannosidase would hydrolyze O-linked glycans on intact glycoproteins. To this end, we designed a screen to assess the potential of lysosomal mannosidases that could be used *in vitro* to not only reduce the extent of O-mannosylation on intact glycoproteins produced in yeast, but also for their ability to cleave the Man-O-Ser/Thr glycosidic linkage. In doing so, we have preliminarily identified three lysosomal mannosidases that possess the desired activities. Herein we provide a more in-depth characterization of the VvLMann activity and demonstrate its utility to reduce and even eliminate O-linked mannose structures on several intact glycoproteins.

The data provided herein establishes the activity of lysosomal α-1,2/3/6-mannosidases towards O-linked mannose. Furthermore, this strategy demonstrates a practical approach for using recombinant lysosomal mannosidases to remove O-linked mannose glycoforms from intact glycoproteins produced in yeast expression systems.

Example 2: Lysosomal Mannosidase Screening Assay for O-mannose Removal

Purpose: To identify LMann homologues capable of reducing O-linked mannose.

As described previously, the O-mannose structures on the recombinant proteins expressed in glycoengineered *P. pastoris* consist primarily of α-linked mannose. Therefore, a panel of lysosomal mannosidase enzymes were recombinantly expressed and screened for their

ability to reduce extended O-linked mannose polymers, in addition to specifically cleaving the Man-O-Ser/Thr glycosidic bond.

However, one known caveat to this concept was that in the lysosome glycoproteins are rapidly processed by proteases into simple peptides, thus removing any restriction caused by steric hindrance of an intact protein. As such it was not apparent that lysosomal mannosidase would hydrolyze O-linked glycans on intact glycoproteins. To this end, we designed a screen to assess the potential of lysosomal mannosidases that could be used *in vitro* to not only reduce the extent of O-mannosylation on intact glycoproteins produced in yeast, but also for their ability to cleave the Man-O-Ser/Thr glycosidic linkage.

Table 2 summarizes the preliminary data obtained by screening the supernatants harvested from cultures of *P. pastoris* which had been transformed with each of the LMann expression vectors. Those homologues reported as having no detectable O-mannosidase activity gave Q-TOF profiles similar to Figure 3B, which in turn is similar to the Q-TOF profile of the SCI-CTP substrate used (Figure 3A). The GmLMann supernatant gave a Q-TOF profile similar to that for the MtLMann, represented in Figure 3C. Thus, indicating that both of these enzymes could reduce the extent of O-mannosylation on SCI-CTP but not as of yet confirming that they could specifically cleave the Man-O-Ser/Thr glycosidic both, due to the absence of the mass for the intact unglycosylated SCI-CTP. This latter ability was specifically demonstrated by the VvLMann, thus confirming that it could not only reduce extended O-linked mannose structures but that it could also specifically cleave the Man-O-Ser/Thr glycosidic bond, due to the appearance of a mass which coincides with the mass of unglycosylated intact SCI-CTP (Visualized by the black arrow in Figure 3D).

Table 2 Mannosidase Activity

Enzyme abbreviation	Preliminary Activity detected
AtLMann	No
DdLMann	No
GmMann	Yes
GpLMann	No
HsLMann	No
MtLMann	Yes
MmLMann	No
RcLMann	No
SsLMann	No
TcLMann	No
ToLMann	No
VvLMann	Yes

Results: As summarized in Table 2 the lysosomal mannosidases from *Glycine max* (GmMann), *Medicago truncatula* (MtLMann) and *Vitis vinifera* (VvLMann) are all capable of reducing the degree O-linked mannose on SCI-CTP. This data for the first time confirms the activity of lysosomal α-1,2/3/6-mannosidases towards O-linked mannose. Furthermore, this strategy demonstrates a practical approach for using recombinant lysosomal mannosidases to remove O-linked mannose glycoforms from intact glycoproteins produced in yeast expression systems.

Example 3: Enhancement of VvLMann ability to specifically cleave the Man-O-Ser/Thr glycosidic bond.

Purpose: To assess the ability of VvLMann (in supernatant) to further reduce the extent of O-mannosylation. To achieve this the reaction time was extended or the extent of extended α-1,2-mannose polymers was reduced using *Trichoderma reesei* α-1,2-mannosidase (TrMannI) (Figure 4).

Results: An improvement in the reduction of O-mannosylation was observed when the reaction time was extended from 24 hours to 48 hours. By comparing Figure 4B and 4C, it is apparent that the overall state of mannosylation is reduced by increasing the incubation time for which the VvLMann is in contact with the SCI-CTP substrate. It is also apparent that a higher proportion of the Man-O-Ser/Thr glycosidic bonds have been cleaved by the increase in the signal of the unglycosylated SCI-CTP intact mass, as indicated by the black arrow.

As mentioned above, the SCI-CTP was produced in a GFI1.0 glycoengineered background, which produces O-linked glycans with extended α-1,2-mannose chains. To further clarify that the VvLMann was cleaving the Man-O-Ser/Thr, the extent of extended α-1,2-mannose chains were reduced using TrMannI. As can be seen comparing Figures 4C and 4D, which were run in parallel but with the latter also including TrMannI, the relative proportion of unglycosylated SCI-CTP increased, thus showing that the VvLMann could more efficiently cleave the Man-O-Ser/Thr glycosidic bond when extended α-1,2-mannose chains were reduced. As shown in Figure 4E, incubation of SCI-CTP with only TrMannI on resulted in a reduction of O-mannosylation, but did not produce any unglycosylated SCI-CTP. Thus indicating that the TrMannI could only reduce the extent of α-1,2-mannose polymerization but not cleave the Man-O-Ser/Thr glycosidic bond.

Example 4: Assessing the O-mannose reduction potential of purified LMann.

Purpose: Having detected O-mannose reduction activity in supernatants of LMann homologues from *Glycine max* (GmMann), *Medicago truncatula* (MtLMann) and *Vitis vinifera* (VvLMann), we further clarified the activity of the purified forms of each. As such it was expected that purified forms would be more efficient in O-mannose reduction.

Results: Clones expressing each of the Vv-, Gm- and Mt-Lmann were growth in DASGIP fermentors as described above in the Methods section. Following the growth and induction of these clones, the supernatant was harvested and the recombinant mannosidases purified using nickel column purification as described in the Methods section.

As can be seen in Figure 5, a major product was isolated from each supernatant which migrated at approximately 130kDa when analyzed by SDS-PAGE and Coomassie stained (Figure 5A). This mass corresponded with that expected for intact precursor forms of each of the LMann homologues. Anti-HIS tag western blot confirmed that each of these major products visualized by Coomassie staining, did possess a HIS tag, though the extent of staining did vary (Figure 5B). Aliquots of each of these purified LMann samples were then subjected to *in vitro* analysis as described in the Methods section. As can be seen in Figure 6B, C & D, no significant O-mannosylation is detected on SCI-CTP when purified Vv-, Gm- or Mt-LMann are used in the standard *in vitro* assay, when compared to the substrate only reaction (Figure 6A). Thus confirming that the purified enzymes could efficiently remove O-linked mannose residues.

Table 3

Molecular form	Peak mass	% of total			
		Control	VvLMann	GmLMann	MtLMann
SCI-CTP	8727	0	96.9	97.3	96.7
+1M	8889	0	3.1	2.7	3.3
>1M	9051	100	0	0	0

To estimate the efficiency of O-mannose removal, the peak area of defined SCI-CTP molecular forms were assessed using the Agilent Chameleon software to calculate peak area. The data provided in Table 3 provides an estimation of the relative degree of O-mannosylation of individual or collated species based on the peak area(s) of the Q-TOF profile (Note: the 9051 peak mass value corresponds to the lowest peak mass in range, additional peak masses can be obtained by adding 162 amu).

The peak area percentage values summarized in Table 3 indicates that prior to treatment 100% of the SCI-CTP possessed more than one O-linked mannose residue. However, following incubation with each of the three LMann greater than 96% of the molecular forms of SCI-CTP

were unglycosylated, with the remaining forms possessing a single O-linked mannose residue. This data indicates that the Vv-, Gm- and Mt-LMann can efficiently reduce not only the extended O-linked mannose polymers but that they can all specifically cleave the Man-O-Ser/Thr glycosidic bond efficiently.

These observations raised the possibility that the lack of activity observed when the other nine homologues were tested may have been a result of their low expression level. To address this concern, the strains expressing AtLMann and HsLMann were scaled up (using 1 L bioreactors), the recombinant LManns purified and their activities characterized using SCI-CTP as a substrate. On comparing undigested SCI-CTP by Q-TOF (Figure 7A) with that incubated with purified AtLMann (Figure 7B), it was evident that this enzyme could remove a proportion of the mannose residues. However, even though the same amount of purified AtLMann was added to the *in vitro* reaction as VvLMann previously (see Figure 6B), it was much less efficient at removing O-linked mannose. By contrast, when the purified HsLMann was incubated at the same protein concentration as VvLMann in earlier experiments, it showed comparable O-linked mannose removal activity when analyzed by Q-TOF (compare Figure 7C to Figure 6B). Quantitation of HsLMann activity using the Agilent Chameleon software indicated that this enzyme was able to remove 96.4 % of the O-linked mannose on SCI-CTP, comparable to the 96.9 % efficiency of VvLMann (see Table 3). To help understand the differences observed in enzyme activity in the initial screening of supernatants, both the expression levels and the quality of the recombinant enzymes were further analyzed. To determine relative expression levels the amount of protein eluted from the nickel columns was quantitated and back-calculated to a titer in the culture supernatant. For the AtLMann and HsLMann preparations 4.3 mg/L and 0.3 mg/L of nickel-column binding protein was obtained in the supernatant as compared to 24.9 mg/L for the VvLMann. Comparing the quality of the recombinant enzymes, it was observed that both the AtLMann and the HsLMann preparations possessed a number of bands when analyzed by SDS-PAGE (Figure 8A). Western blot analysis confirmed that both the AtLMann and HsLMann had undergone extensive proteolysis, in-so-far that no intact HsLMann could be detected (Fig. 8B). These compared to the VvLMann, which we had previously shown (Figure 5) to be primarily intact. As such, the lower degree of expression and the increased proteolysis of particular LManns are both feasible reasons for why no significant mannosidase activity was observed for a number of the LManns during the early screening process. These results confirm that the ability of lysosomal mannosidases to remove O-linked mannose, including cleavage of the Man-O-Ser/Thr glycosidic bond, is not restricted to plant derived lysosomal mannosidases and can include lysosomal mannosidases from other sources, including mammalian, as demonstrated by the human homologue. In particular cases the activities of the other homologues may be

enhanced through further purification, or the use of protease inhibitors or expression conditions to improve the quality (and hence activity) of the LMann protein.

Having demonstrated that Vv-, Gm-, Mt-LMann and HsLMann have similar O-glycan reduction activity, subsequent examples will expand only on characterizing the VvLMann, with the assumption that the other enzymes behave in a similar fashion.

Example 5: Assessing the co-ion dependency of O-mannose reduction by VvLMann

Purpose: To determine the co-ion dependency of LMann during cleavage of O-linked mannose glycans.

Results: Figure 9 depicts the digestion of O-linked glycans under varying co-ion conditions. The Q-TOF profile of the starting SCI-CTP can be seen in Figure 9A, and indicates that the molecule is heavily O-glycosylated, and that the mass for unglycosylated intact SCI-CTP is not detected. Q-TOF analysis of SCI-CTP in the presence or absence of adding additional zinc ions (Figure 9B & 9C respectively), shows that the majority of the O-linked glycans have been removed, as indicated by the prominent species detected being unglycosylated SCI-CTP (black arrow). The requirement for zinc ion dependency was chosen due to the presence of zinc in the commercial buffer provided with Jack bean α-1,2/3/6-mannosidase, which has a similar activity to LMann on N-linked glycans. To confirm that LMann did not require another co-ion for reduction of O-linked glycans, 1mM EDTA was added. Under these conditions near complete removal of O-linked mannose from SCI-CTP was observed (Figure 9D).

Table 4

Molecular form	Peak mass	% of total			
		Control	1mM Zinc	No Zinc	1mM EDTA
SCI-CTP	8727	0	97.5	97.6	96.7
+1M	8889	0	2.5	2.4	3.3
>1M	9051	100	0	0	0

Using the Agilent Chameleon software to calculate peak area, the Table inset in Figure 9 was compiled. The data in Table 4 provides an estimation of the relative degree of O-mannosylation of individual or collated species based on the peak area(s) of the Q-TOF profile (Note: the 9051 peak mass value corresponds to the lowest peak mass in range, additional peak masses can be obtained by adding 162 amu). The peak area percentage values summarized in Table 4 indicates that prior to treatment 100% of the SCI-CTP possessed more than one O-linked mannose residue. However, incubation of SCI-CTP with VvLMann under any of the three reactions conditions represented in Figure 9B, 9C or 9D resulted in greater than 96% of the molecular

forms of SCI-CTP being unglycosylated, with the remaining forms possessing a single O-linked mannose residue. This data indicates that the VvLMann can efficiently reduce O-linked mannose polymers including the Man-O-Ser/Thr glycosidic bond efficiently independent of co-ion requirement. A similar result was observed when human LMann was assessed for co-ion dependency *in vitro* using *p*-nitrophenyl-α-D-mannopyranoside as the substrate (Venkatesan et al. 2009).

Example 6: Assessment of LMann to removal O-linked mannose from alternative proteins.

Purpose: To confirm that LMann could efficiently remove O-linked mannose from a broad range of intact O-mannosylated protein substrates.

Results: Human Fc double mutein (hFcDM) sequence 1 was expressed in a GFI2.0 glycoengineered *P. pastoris* strain background. This resulted in the presence of intact protein containing a single O-linked mannose residue on each monomer, which is equivalent to an intact Fc dimer with two O-linked mannoses (as observed by Q-TOF in Figure 10A). Incubation of VvLMann with this substrate, under the conditions highlighted in the Methods section, resulted in the majority of the O-linked glycans being removed (Figure 10B).

Table 5

Molecular form	Peak mass	% of total	
		Control	VvLMann
hFcDM	50393	69.6	92.4
+1M	50554	6.4	5.8
+2M	50717	20.5	1.8
+3M	50878	2.1	0
+4M	51041	1.4	0

The data in Table 5 was compiled using the Agilent Chameleon software to calculate peak area. The data provides an estimation of the relative degree of O-mannosylation of individual molecular species based on the peak areas of the Q-TOF profile. The peak area percentage values summarized in Table 5 indicates that prior to treatment >30% of the hFcDM possessed more than one O-linked mannose residue. However, following incubation of hFcDM with VvLMann greater than 92% of the molecular forms of hFcDM were unglycosylated, with the remaining forms possessing primarily a single O-linked mannose residue. This data indicates that the VvLMann can efficiently reduce O-linked mannose polymers on hFcDM.

The activity of LMann was assessed with a third O-glycosylated reporter protein SCI-OPEP. This reporter protein was similar to the SCI-CTP reporter protein except that the CTP peptide was replaced with a highly O-glycosylated peptide from the human tumor necrosis factor receptor 2 (TNFR2) ectodomain. Expression of this fusion protein in a GFI1.0 glycoengineered *P. pastoris* strain resulted in the protein being extensively O-glycosylated with the majority of the molecular forms possessing five or more O-linked mannose residues, with the addition of up to twenty eight mannose residues being detected (Figure 11A). Incubation of VvLMann with this substrate, under the conditions highlighted in the Methodss section, resulted in the majority of the O-linked glycans being removed (Figure 11B, black arrow).

Table 6

Molecular form	Peak mass	% of total	
		Control	VvLMann
SCI-OPEP	7869	0	77.8
+1M	8031	0	15.4
+2M	8193	0	5.0
+3M	8355	0	1.8
M+>M3	8517	100	0

Using the Agilent Chameleon software to calculate peak area, the data in Table 6 was compiled (Note the 8517 peak mass value corresponds to the lowest peak mass in range, additional peak masses can be obtained by adding 162 amu). The data provided in Table 6 represents an estimation of the relative degree of O-mannosylation of individual or collated molecular species based on the peak areas of the Q-TOF profile. The peak area percentage values summarized in Table 6 indicates that prior to treatment 100% of the SCI-OPEP possessed more than three O-linked mannose residues. However, following incubation of SCI-OPEP with VvLMann greater than 77% of the molecular forms of SCI-OPEP were unglycosylated, with the remaining forms possessing primarily a single O-linked mannose residue. This data indicates that the VvLMann can efficiently reduce O-linked mannose polymers on SCI-OPEP, a third example of an O-mannosylated protein.

Example 7: Assessment of LMann to remove O-linked mannose from a non-*P. pastoris* produced O-glycosylated protein.

Purpose: To demonstrate that the utility of LMann to remove O-linked mannose glycans goes beyond O-mannosylated proteins produced in *P. pastoris*.

Results: Leukine® is a commercially available recombinant form of granulocyte macrophage colony-stimulating factor (GM-CSF) produced in the yeast *Saccharomyces cerevisiae*. Q-TOF analysis of PNGase treated Leukine® indicated that up to nine O-linked mannose residues could be detected on the molecule (Figure 12A), including two phosphomannose species (indicated by the "P"s). The extent of phosphomannose present was detectable but not significant, and as such was not quantitated. Incubation of VvLMann with this substrate, under the conditions highlighted in the Methods section, resulted in the majority of the O-linked glycans being removed (Figure 12B, black arrow).

Table 7

Molecular form	Peak mass	% of total	
		Control	VvLMann
Leukine	14431	47.3	95.6
+1M	14593	15.8	4.4
>1M	14755	36.9	0.0

The data in Table 7 was compiled using the Agilent Chameleon software to calculate peak area (Note: the 14755 peak mass value corresponds to the lowest peak mass in range, additional peak masses can be obtained by adding 162 amu). The table provides an estimation of the relative degree of O-mannosylation of individual or collated molecular species based on the peak areas of the Q-TOF profile. The peak area percentage values summarized in Table 7 indicates that prior to treatment greater than 52% of the Leukine® possessed more than one O-linked mannose residue. However, following incubation of Leukine® with VvLMann greater than 95% of the molecular forms of Leukine® were unglycosylated, with the remaining forms possessing primarily a single O-linked mannose residue. Also noted was that the phosphomannose structures collapsed into a smaller single phosphomannose form, indicating that the original heterogeneity in the phosphomannose structures was eliminated. This data indicates that the VvLMann can efficiently reduce O-linked mannose polymers on commercial Leukine®, a fourth example of an intact O-mannosylated protein and one which is not produced in *P. pastoris*.

Example 8: Production of subdomain variants of recombinant LMann.

Purpose: LMann is a large protein, typically a heterodimer around 150kDa, which later self-dimerizes. The heterodimer is a cleavage product of the LMann precursor peptide. It is this latter single peptide precursor that has been used to exemplify the utility of LMann to remove O-linked

mannose. It is foreseeable that a processed dimeric form of the enzyme may be more efficient at removing O-linked mannose on particular proteins due to their differing structural conformations. Here we have created several N- and C-terminal variants of the LMann.

Results: The internal proteolytic cleavage site for human LMann has been determined, which produces the N- and C-terminal domains of HsLMann from its precursor peptide (Berg et al. 2001). Alignment of Vv-, Gm and Mt-LMann with HsLmann indicated the potential proteolytic cleavage sites (black arrow) for each of these proteins (Figure 13A). This cleavage site is also represented on the intact VvLMann precursor enzyme recombinantly secreted from *P. pastoris* (Figure 13B, Form I, black arrow). A number of recombinant forms of the enzyme were generated based on this N- and C-terminal subdomain structure. As described in the Methods section, introduction of a Kex2p protease recognition site between the amino acid sequence highlighted in Figure 13B Form I, produces Form II following secretion from *P. pastoris*. In an alternative form (Form III), a second HIS-tag sequence is added N-terminal to the Kex2p recognition site, so that both N- and C-terminal subdomains possess a HIS-tag to facilitate downstream purification of the recombinant enzyme. In an alternative form (Form IV), a factor Xa (FXa) site has been added to facilitate cleavage into the two subdomains following the initial purification of the precursor peptide. Finally, Forms V and VI represent recombinants forms of the enzymes were the N- or C-terminal subdomains have been expressed independently, each with a C-terminal HIS-tag to facilitated purification. Forms V and VI may be used independently of combined for O-mannose removal activity. To date Form I has been able to efficiently remove O-linked mannose from a number of O-mannosylated proteins but it is anticipated that particular O-mannosylated proteins may be more efficiently processed with a subset of Forms II to VI.

Example 9: Reduction of O-linked mannose by co-secretion of LMann and the O-glycosylated glycoprotein of interest.

Purpose: Co-secretion of LMann in parallel to the secreted O-mannosylated glycoprotein of interest can be used to reduce the extent of O-linked mannose on the glycoprotein, prior to its purification from the culture broth.

For example, the LMann expression vectors described previously can be used directly to transform into a strain already expressing the protein of interest. Using the current vectors described previously the LMann integrates into the *TRP2* loci using Zeocin for selection. It is also possible to use the current vectors to integrate into the *AOX1* promoter loci, using the restriction enzyme *Pme*I to linearize the DNA prior to transformation into the yeast strain. The current vectors can be modified to increase their utility by replacing the Bleomycin resistance cassette with another selectable marker to facilitate selection of a host strain that already

possesses Zeocin resistance. Such examples of other selectable markers are the *URA5* blaster counter-selectable marker or any other auxotrophic marker, wherein the host strain is deficient in the activity of that essential enzyme. Alternatively another dominant marker can be used which confers resistance to a normally toxic compound. Such markers could confer resistance to hygromycin, kanamycin, G418, nouresothricin, arsenite etc.

The introduction of LMann outlined above can result in multiple integrations of the LMann expression cassette, and is referred to as roll-in integration. An alternative approach is to introduce the LMann expression cassette using a knock-in vector strategy were only one copy of the LMann expression cassette is integrated into the yeast genome. Examples of the structures of knock-in vectors are common in the literature and can be seen exemplified in (Nett et al. 2005). An alternative approach to introducing the LMann after the strain has been modified to produce the O-mannosylated protein of interest, is to either introduce the LMann prior to or while introducing the construct for expression of the glycoprotein of interest. Using the strategy outlined in the previous paragraph the LMann is under the control of the methanol inducible AOX1 promoter, similar to the putative glycoprotein of interest. By changing the promoter on either or both, it is possible to regulate the relative expressional timing of each. Alternatively constitutive expression of each can be desirable, where each is under the expressional control of a constitutive promoter such as the GAPDH promoter.

The co-secretion of the LMann along with the O-mannosylated protein of interest results in the production of a glycoprotein of interest with reduced O-mannosylation, which in turn avoids the need to perform an *in vitro* de-O-mannosylation step post-purification of the protein. By modifying the expression and media conditions the extent of O-mannose removal can be manipulated. For instance, varying the pH of the media can be used to enhance or reduce the activity of the LMann.

Patents, patent applications, publications, product descriptions, and protocols are cited throughout this application, the disclosures of which are incorporated herein by reference in their entireties for all purposes. All references cited herein are incorporated by reference to the same extent as if each individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, was specifically and individually indicated to be incorporated by reference. This statement of incorporation by reference is intended by Applicants, pursuant to 37 C.F.R. §1.57(b)(1), to relate to each and every individual publication, database entry (e.g. Genbank sequences or GeneID entries), patent application, or patent, each of which is clearly identified in compliance with 37 C.F.R. §1.57(b)(2), even if such citation is not immediately adjacent to a dedicated statement of incorporation by reference. The inclusion of dedicated statements of incorporation by reference, if any, within the specification does not in

any way weaken this general statement of incorporation by reference. Citation of the references herein is not intended as an admission that the reference is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

TABLE 8 LMann Amino Acid Sequences

AtLMann (SEQ ID NO: 1)
E Y I E Y N T K P R I V P E K I N V H L V P H S H D D V G W L K T V
D Q Y Y V G S N N S I R G A C V Q N V L D S V I A S L L D D E N R
K F I Y V E M A F F Q R W W R Q Q S N A K K V K V K K L V D S G Q
L E F I N G G M C M H D E A T P H Y I D M I D Q T T L G H Q F I K T
E F G Q V P R V G W Q I D P F G H S A V Q A Y L L G A E F G F D S L
F F A R I D Y Q D R A K R L R E K T L E V I W Q G S K S L G S S S Q
I F T G V F P R H Y D P P E G F T F E I N D V S A P I Q D D P L L F D
Y N V Q E R V N D F V A A A L A Q V N V T R T N H I M W L M G T
D F R Y Q Y A Y S W F R Q I D K F I H Y V N K D G R L N V L Y S T
P S I Y T D A K Y A A N E S W P L K T D D F F P Y A D K P N A Y W
T G Y F T S R P A F K K Y V R D L S G Y Y L A A R Q L E F L R G R
D S S G P T T D M L A D A L A I A Q H H D A V S G T Q R Q H V A A
D Y A L R L S M G Y L Q A E K L V A S S L S F L S A A K S S T E K K
N P G T K F Q Q C P L L N I S Y C P A S E A R L L S G K S L V V V V
Y N S L G W K R E E V V R V P V S S E N V I V K D A S G K E V V F
Q L L P L S E I A L R I R N E Y V K A Y L G R S P R D T A K H V L A
F T A S V P P L G F S S Y V I S D T G R T A R G L S A S Y V T S G S
M N Q N V E V G Q G N L K L R Y S E E G V K I T R H L S T K N Q V
T A E Q S Y A Y Y I G S N G T D K D P Q A S G A Y V F R P D G V L
P I K S K E E A Q L T I V Q G P L F D E V H Q E L N S W I S Q I T R V
Y K G K N H A E I E F T I G P I P A D D G I S K E I I T K L T T T M K
T N G T F Y T D S N G R D F I K R I R D F R T D W D L Q V Y Q P V A
G N Y Y P L N L G I Y M Q D K T S E L S V L V D R A V G G S S L E
N G Q I E L M L H R R M Q H D D I R G V G E I L N E T V C L P E G C
K G L T I Q G K F Y V Q I D K P G D G A K W R R T F G Q E I Y S P L
L I A F T E Q E G D S W I N S H K T T F S A F E P S Y S L P K N V A
L L T L Q E L E N G E V L L R L A H L F E V G E D S E Y S V M A K V
E L K K L F H N K K I R E V K E T S L S G N Q E K A E M E K R R L I
W K V E G S A G E E V K R G E A V D A E K L V V E L V P M E I R T
L L I K F D D Q I E M V G D K E Q Q H R L

DdLMann: (SEQ ID NO: 2)
S K P K L S S T L L N V H I V A H T H D D V G W L K T V D E Y Y Y
G S N M S I A F A G V Q Y T L D T A I T C L L A N P E R K F I Y V E
I A F F Q R W W D E Q S T T M Q N I V K G L V G K W S I E F I N G G
Y C M N D E A T T Y Y D D T I D Q M T L G H Q F L W E N F G V M P
K I G W H I D P F G H S A T Q A R I F G Q L G F D A F I I G R M D Y
Q D I E A R L E N K Q M E F M W R S T Q S T P E N Q V F T S V L R
A M Y C T P D G F N F E Q G D D P I Q D D P N L F D N N V D S R A
E Q F T Q V A L E Y A T H Y R T N N V L I P F G C D F A Y L N A Q
M Y Y K N I D K L I A H I N S N P D K Y G L N L L Y S T P S I Y I D
A V N D A N L V W E V K T D D L F P Y A D N E F S Y W T G Y F V S
R P A L K G Y V R Q N N A L L H V V E Q M L V T S S N L M P S S R
S E Q L V D D I V I M R E V M G I A Q H H D A V S G T E Q Q H V A

D D Y A E R L S I G N C A S L E T I N T V V G T L L T A N G N S K S
A A A T P T I S F C P L L N Q S I C P A T D P L S S G T S V P V L I Y
N S L S W T R N E P V R T L I P I A N V T V T S S S N G S I T S Q V N
Q I N G T F I L E F L A T I P P L G Y S T Y I I T S T A S D F V E P N S
I P A I I I Q D E I I V S G G G K I N E K V S Y N D P I I L E N D Y I N
V Q F S S Q D G S I L S I T N K T S G V T S S I T Q E Y I W Y N P S V
G N D D S A Q C S G A Y I F R P V E D F A Y P Y N N A T P S V S I I
R G E I S S S I R R F W S N E M V Q T F R L Y S N A D H L E V E E I I
G P I D I S D G I G K E I V S R Y T T T L V T D Q T W Y S D S Q G M
E M Q K R I T N Y R P S W N L T V V Q P T S G N Y V P V N A I A Y I
Q D P N Q S L Q F T I V T D R S R G C A S L R D G Q L D M M M H R
R T L K D D G R G V G Q P M N E S T Q I V T T S K L I F H D I S S Y
A Q S H Y R P A A L S L S H P L L P M F T T T Q Q S S N D W N S Q Y
Q G V Y S P L T S A S P L P N G L K I Q T L Q W L D N Q D N T I L L
R I E N I Y Q I D G Q D S Q D P Q T I T L D L S T I F S T I T I T S A T
E M N L T G V Q K L S N L S R L K W K T V D G K N Y Y H K S S S S
T K E D S S N G F V F T F S P I K S E L L

GmLMann: (SEQ ID NO: 3)

K Y M K Y N T G A S I V P G K L N V H L V P H S H D D V G W L K T
I D Q Y Y V G S N N S I Q G A C V E N V L D S V V V S L Q K D P N
R K F V F A E M A F F H R W W V E Q S P E T Q E Q V R K L V D A G
Q L E F I N G G W C M H D E A A T H Y I D M I D Q T T L G H R F I K
D Q F N K T P T V G W Q I D P F G H S A V Q A Y L L G A E L G F D
S I H F A R I D Y Q D R A K R K A D K S L E V V W R G S K T F G S S
A Q I F A N T F P V H Y S A P N G F N F E V N N P D V D V G P V Q D
D P L I F D Y N V K Q R V K E F I D A A T T Q A N V T R T N H I M W
T M G D D F Q Y Q Y A E S W F K Q M D K L I H Y V N K D G R V N
A L Y S T P S I Y T N A K N A A N Q L W P L K T D D Y F P Y A D S P
N A Y W T G Y F T S R P A L K R Y V R M L S G Y Y L A A R Q L E F
L V G K Q S T K Y N T Y D L G D A L G I A Q H H D A V S G T A K Q
H T T N D Y A K R L A I G A Y E A E A V V S S S L A C L T R K Q S G
D K C S T P A S A F A Q C Q L L N I S Y C P P A E D N I P E A K S L
V V V V Y N P L G W N R T D I V K I P V N D A N L V V K D S S G N
K L E V Q Y V D V D D V T T N L R K F Y V K A Y V G V S P K Q S P
K Y W L L F Q V S V P P L G W S T Y F I S K A T R K G T R R K D L S
H T N S Q K G D T I N I G S G N L K M S F S S T S G Q L K R M Y N S
R T G V D I P I Q Q S Y L W Y G S S E G D S D P Q A S G A Y I F R P
N G S P P N I V S R S V P T K V I R G P L V D E V H Q K F S S W I Y
Q V T R L Y K D K E H A E I E F T I G P I P T D D G V G K E V I T R
M T A N M A T N K E F Y A D S N G R D F L K R V R D H R E D W P L
Q V T Q P V A G N Y Y P L N L G I Y T K D E K S E F S V L V D R A T
G G A S I K D G E V E L M L H R R I L H D D S R G V G E P L D E Q V
C V N N N N T C E G L T V R G N Y Y I S I H K L G V G S R W R R T
T G Q E I Y S P F L V A F T H E I S E N W K S S H L T K G T I M D P
N Y S L P P N I A L I T L E E L D G G I V L L R L A H L Y E R S E D A
E Y S T L T K V E L K K L F A M K T I R E L K E V S L S S N Q E K S
E M K R M T W K V E G D K G Q E P Q A V R G G P V S Y H N L V V
E L G P M E I R T F L L K F

GpLMann: (SEQ ID NO: 4)

A G Y E T C P M V Q P G M L N V H L V A H T H D D V G W L K T V
D Q Y Y W G I H N D L Q Q A G V Q Y I L D S V I S A L L A E P T R R
F V Y V E M A F F S R W W H Q Q T N E T Q E V V R R L V R Q G R L
E F A N G G W V M N D E A A T H Y G A I V D Q M T L G L R F L E D
T F G S D G R P R V A W H I D P F G H S R E Q A S L F A Q M G F D
G V F F G R I D Y Q D K L V R K K R R E M E L V W R A S A S L K A
P A A D L F T G V L P N N Y G P P E G L C W D V L C A D P P V V D
D P R S P E Y N A K K L V S Y F L Q L A T A Q G R Y Y R T N H T V
M T M G S D F Q Y E N A N T W F K N L D K L I Q L V N M Q Q A N G
S R V H V L Y S T P A C Y L W E L N K A N L T W P V K E D D F F P
Y A D G P H M F W T G Y F S S R P A L K R Y E R L S Y N F L Q V C
N Q L E A Q V G P A A N V G P Y G H G D S S P L N Q A M A V L Q H
H D A V S G T S K Q H V A D D Y A R Q L A A G W G P C E V L L S N
A L A K L S G S K E T F L F C R D L N I S I C P F S Q T S E R F Q V L
V Y N P L G R K V D R M V R L P V R K G L F L I K D P G N N T V P
S T V V E L T S S G N P E L L F P A L V P A L G F S V Y S V T R V S
D Q N P Q T R S Q H S R P Q K Y S S P V L S I K N E Y L R A S F H P
D T G L L S M I E V L D R K L T L P V N Q A F F W Y N A S V G D K
R S S Q A S G A Y I F R P S Q Q W P F P V S H L A R T R L V K T A L
V Q E V H Q N F T A W C S Q V V R L Y S G Q R H L E L E W T V G P
I P V G D K W G K E I I S R F D T P L E T G G V F F T D S N G R E V
L E R R R D Y R P S W K L N Q T E P V A G N Y Y P V N S R I Y I T D
G K M Q L T V L T D R S Q G G S S M S D G S L E L M V H R R L L K
D D G R G V G E A L Q E P G S G G W V R G R H L L L L D T A R E A
A A E H R L L A E K E L L A P Q L V L A P G Q G P S Y H H D H H E
A V P R K Q F S G L R R Q L P P S V R L L T L A R W G P D T L L L R
L E H Q F A L G E D S S R N L S L P V T L D L Q D L F S T F T I T R L
Q E T T L A A N Q L R A S A S R L K W T T E I D P I S R P A V P R L
D P S S I T L Q P M E I R T F V A S V Q W E E N S

HsLmann: (SEQ ID NO: 5)

G G Y E T C P T V Q P N M L N V H L L P H T H D D V G W L K T V D
Q Y F Y G I K N D I Q H A G V Q Y I L D S V I S A L L A D P T R R F I
Y V E I A F F S R W W H Q Q T N A T Q E V V R D L V R Q G R L E F
A N G G W V M N D E A A T H Y G A I V D Q M T L G L R F L E D T F
G N V G R P R V A W H I D P F G H S R E Q A S L F A Q M G F D G F
F F G R L D Y Q D K W V R M Q K L E M E Q V W R A S T S L K P P T
A D L F T G V L P N G Y N P P R N L C W D V L C V D Q P L V E D P
R S P E Y N A K E L V D Y F L N V A T A Q G R Y Y R T N H T V M T
M G S D F Q Y E N A N M W F K N L D K L I R L V N A Q Q A K G S S
V H V L Y S T P A C Y L W E L N K A N L T W S V K H D D F F P Y A
D G P H Q F W T G Y F S S R P A L K R Y E R L S Y N F L Q V C N Q
L E A L V G L A A N V G P Y G S G D S A P L N E A M A V L Q H H D
A V S G T S R Q H V A N D Y A R Q L A A G W G P C E V L L S N A L
A R L R G F K D H F T F C Q Q L N I S I C P L S Q T A A R F Q V I V
Y N P L G R K V N W M V R L P V S E G V F V V K D P N G R T V P S
D V V I F P S S D S Q A H P P E L L F S A S L P A L G F S T Y S V A Q
V P R W K P Q A R A P Q P I P R R S W S P A L T I E N E H I R A T F
D P D T G L L M E I M N M N Q Q L L L P V R Q T F F W Y N A S I G
D N E S D Q A S G A Y I F R P N Q Q K P L P V S R W A Q I H L V K T
P L V Q E V H Q N F S A W C S Q V V R L Y P G Q R H L E L E W S V
G P I P V G D T W G K E V I S R F D T P L E T K G R F Y T D S N G R

E I L E R R R D Y R P T W K L N Q T E P V A G N Y Y P V N T R I Y I
T D G N M Q L T V L T D R S Q G G S S L R D G S L E L M V H R R L
L K D D G R G V S E P L M E N G S G A W V R G R H L V L L D T A Q
A A A A G H R L L A E Q E V L A P Q V V L A P G G G A A Y N L G A
P P R T Q F S G L R R D L P P S V H L L T L A S W G P E M V L L R L
E H Q F A V G E D S G R N L S A P V T L N L R D L F S T F T I T R L
Q E T T L V A N Q L R E A A S R L K W T T N T G P T P H Q T P Y Q
L D P A N I T L E P M E I R T F L A S V Q W K E V D

MtLMann: (SEQ ID NO: 6)
A Y T K Y N T G A G I V K G K L N V H L V P H S H D D V G W L K T
V D Q Y Y V G S N N S I Q G A C V E N V L D S I V F S L Q K D P N R
K F V F A E M A F F H R W W V E Q S P E I Q E Q V K R L V A A G Q
L E F V N G G W C M H D E A T V H Y I D M I D Q T T L G H R F I K
D Q F N T T P R A G W Q I D P F G H S A V Q G Y L L G A E L G F D
S V H F A R I D Y Q D R A K R K S D K S L E V I W R G S K T F G S S
A Q I F A N T F P V H Y S A P H G F N F E V S G D F V P L Q D D P L
L F D S N V E Q R V K D F I D A A I T Q A N V T R T N H I M W T M
G D D F Q Y Q Y A E S W F K Q M D K L I H Y V N K D G R V N A L Y
S T P S I Y T D A K N A A N Q L W P L K T D D Y F P Y A D G A N A
Y W T G F F T S R P A L K R Y V R I L S G Y Y L A A R Q L E F F A G
K R S T L D D T F G L G D A L G I A Q H H D A V T G T A K Q H T T
D D Y A K R L A I G A S K A E V V V S T S L A V L A S K K S G D Q
R S A L A S A F S Q C Q L L N I S Y C P P T E D S I P Q A K D L V V
V V Y N P L G W N R T D I V R I P V N E A N L V V K D S S G N K V
E V Q Y V D V D D D V T T D L R K L Y V K A Y L G L R P K Q A P K Y
W L L F Q V S I P P L G W S T Y F I S K A A G K G I R R K G D L S H
L N S K N G E N I D I G P G N L K M S F S S T S G Q L K R M Y N F K
T G V N I P I Q Q S Y F R Y G S S E G D N T D S Q A S G A Y I F R P
S E S S P T I V S R S V P F K V I R G P L V D E V H Q K F N S W I Y Q
V T R L Y K G K D H A E I E Y T I G P I P T D D G V G K E V I T R M
T A N M V T N K E F Y T D S N G R D F L K R V R D H R E D W P L Q
V T Q P V A G N Y Y P L N L G I Y T K D K K S E F S V L V D R A T G
G A S I K D G E V E L M L H R R L I E D D G R G V G E P L D E Q V C
I A K A D N N S T C D G L T V R G N Y Y I G I H N V G A G S R W R
R T T G Q E I Y S P I L L A F T H E K S K N W K S S H L T K G T L M
D P N Y S L P P N V A L I T L E E L D G G I V L L R L A H L Y E P N
E D A Q Y S A L A K V E L K K L F A T K T I K E L K E V S L S A N Q
E K S E M K K M T W K V E G D K G Q E P Q A V R G S P V S T S D F
V V E L G P M E I R T F L L E F

MmLMann: (SEQ ID NO: 7)
A G Y K T C P P T K P G M L N V H L L P H T H D D V G W L K T V D
Q Y Y Y G I L S D V Q H A S V Q Y I L D S V V S S L L E K P T R R F
I Y V E M A F F S R W W K Q Q T S A T Q D A V R N L V R Q G R L E
F V N G G W V M N D E A A T H Y G A I V D Q M T L G L R F L Q D T
F G S D G L P R V A W H I D P F G H S R E Q A S L F A Q M G F D G F
F L G R I D Y Q D K L N R K K K L R M E E L W R A S D S L E P P A
A D L F T G V L P N N Y N P P K Y L C W D V L C T D P P V V D N P
R S P E F N A K T L V N Y F L K L A S S Q K G F Y R T N H T V M T
M G S D F H Y E N A N M W F K N M D K L I R L V N A Q Q A N G S
L V H V L Y S T P T C Y L W E L N K A N L T W T V K E D D F F P Y

A D G P H M F W T G Y F S S R P A L K R Y E R L S Y N F L Q V C N
Q L E A L V G P E A N V G P Y G S G D S A P L Q E A M A V L Q H H
D A V S G T A R Q N V V N D Y A R Q L A A G W G P C E V L V S N A
L A R L S H Y K Q N F S F C R E L N I S I C P V S Q T S E R F Q V T L
Y N P L G R K V D Q M V R L P V Y E G N F I V K D P H D K N I S S
N V V M V P S Y Y S E T Y Q W E L L F P A S V P A L G F S T Y S V
A K M S D L N H Q A H N L L S R P R K H K S H H V L V I E N K Y M
R A T F D S G T G L L M K I E N L E Q N L S L P V S Q G F F W Y N A
S V G D E E S S Q A S G A Y I F R P N V G K P I P V S R W A Q I S L
V K T A L V Q E V H Q N F S A W C S Q V I R L Y K G Q R H L E L E
W T V G P I P V R D D W G K E V I S R F D T P M K T K G Q F F T D S
N G R E I L K R R D D Y R P T W T L N Q T E P V A G N Y Y P V N T
R I Y I T D G Q M Q L T V L T D R S Q G G S S L Q D G S L E L M V H
R R L L V D D D R G V S E P L L E T D T G D K V R G R H L V L L S S
V S D A A A R H R L L A E Q E V L A P Q V V L S L G G S S P Y H S R
A T P K T Q F S G L R Q E L P P Q V H L L T L A R W G P K M L L L R
L E H Q F A L K E D S D R N L S S P V T L N V Q N L F Q T F T I N Y
L Q E T T L A A N Q P L S R A S R L K W M T N T G P T S F P E P S K
L D P T S V T L K P M E I R T F L A S V Q W Q E H R P A

RcLMann: (SEQ ID NO: 8)
S Y V K Y N T G S G V V P G K I N V H L V A H S H D D V G W L K T
V D Q Y Y V G S N N S I Q G A C V E N V L D S V V V S L L R D P N
R K F I F V E M A F F Q R W W L E Q S E E I Q D H V R K L I D A G Q
L E F A N G G W C M H D E A T C H Y I D M I D Q T T L G H L A I K
E Q F N K T P R A G W Q I D P F G H S A V Q A Y L L G A E L G F D
S V H F A R I D Y Q D R A K R K V D K S L E V I W R G S K T F G S S
S Q I F A N T F P V H Y S P P P G F H F E V S E D F V P V Q D N P L
L F D Y N V E Q R V N D F V D A A M T Q A N V T R T N H I M W T
M G D D F Q Y Q Y A E S W F K Q M D K L I H Y V N K D G R V N A
L Y S T P S I Y T D M K N A A S E S W P L K I D D Y F P Y A D R E D
A Y W T G F F T S R P G L K R Y V R H L S G Y Y L A T R Q L E F L
V G K K S A G P S T Y R L A D A L G I A Q H H D A V T G T A K Q H
T T D D Y A K R L S I G A S E A E A T A N S A L S C L I S S K S G D
Q C T A S A S V F S Q C Q L L N I S Y C P P T E E T G S G K S L V L
V V Y N P L G W N R T D I I R I P V N D A N L A V S D S S G K T V E
S Q Y V I M D N V T S N L R N F Y L K A Y I G S S P K Q V P K Y W
L V F P V S V P P L G W S T Y F V S D A P A I G K R R N G L S V T E
S P Q N D T I E I G P G N L K M S F S S T T G Q L K R M Y N S K T G
V D L P I Q Q S Y L W Y G S S S D Y S V S L Q Q S S G A Y I F R P N
G S P P T I V A R T V P L K V N R G P V V D E V H Q Q F N S W I Y Q
V T R L Y K D K E H A E I E Y T I G P I P L D D G L G K E V I T R M
T A N M A T D K V F Y T D S N G R D F L K R I R D Y R A D W N L S
V N Q P E A G N Y Y P L N L G M F I T D Q K S E L S V L V D R A T
G G S S I R D G Q V E L M L H S E Y S M M M A E E L V N H L L K I
K E L K E M S L S A N Q Y K S G M K K M T W K V E G D N R D Q I S
P L R G G P V D S S S L V V E L G P M E I R T F L L K F

SsLMann: (SEQ ID NO: 9)
M R N I N E L E A R L I L T L G N S F R N L R Q L R W N L E N H N K
A Y L E I E G K G N S Y L L I V D H K G S G L I R L D D K P Y F E L
D R Y H T L I P I P F G N H K I S L E L S H Y M D F G E K V D I S A

G I P F Y T E I D S N A Y K L Y V Y G S Q I L D L V R S I N D N E V
K D D L I S A L S K A L H E A Y F E T I S K E Q L F I L S K L I R T T
L D V S R M V Q E I E E P L D V Y K E D E N R S K F E G A L N T L R
S E L S K L V N K Y G K R G V L V G T G H A H I D T A W L W P F D
E T R R K V L R T F S T I L T L L D K Y D F H F I Q S A A I Y Y E W
V K A D S P E L F V R I K E K V K E G K W E L A A L Y Y V E S D A N
M V S G E S L A R Q F L Y S Q R F Y L E N F G K L A N I L W L P D T
F G F S A S L P Q I A K L G G V K A F A T H K V F W N D T N K F P Y
N V F K W V G P N G D Y L P A I A F G H G K G G Y N S D F S A S S
V L E Q Y N N W A Q K D Q P M L Y S Y G Y G D G G G G P N E D M
L I R A E A V N L L P I L P K V E L S G V N S Y I Q R I V P V E E W R
G E L Y L E T H R G V L T S H S K M K L L N R S A E I A L R E A E L
W S T L A R T Y D K E V F T K L W K V V L K D Q F H D V L P G S A
I K D V Y K V A Y Q E L E E V I N K A N N V A S E A M Q K L V G G
S G D K T F V F N S L S W D R E E Y I E A D G K L V K V R V P S V G
F S L L E P V E V R D K A V I N E N N A E Y L V E N K Y F R V R I S
K S G Q V L S L F D K E A N R E V L R D K S N L L I A Y E N I P G W
A D A W D I E K G F E D R S F E I R A S S S E I V N N D G I V A S I K
F T Y K F R R S E I I Q I V R V Y A D S R R I D F I T T L R M R D R E
L L V K S W F N F D L N V E R A V S D I P F G V V E R F T W S N T S
W D K A R F E V P I Q K F V D F S E S E Y G A A L L N N G K Y G A
T L R G S S V G L S L T K T P I Y P D P S T D L E E V T F I Y S L Y P
H I G D W K R A E V I K R A Y E L N V P L R V V K G V S E V K R K
S F I R I N D S K L I L E A V K V A E D D N N S V V L R L Y E Y E N
T R G E A Y V E V P F N V T E A R S L D L L E L N E V P R D I V I E
G N R I K V K Y K N R D I L T I S V R

TcLMann: (SEQ ID NO: 10)

T V H L V A H T H D D M G W L K T V E Q Y Q Y G L N N T I Q V A D
V N G I I S S V I A G L L L N P R R K F T Y V E I G F F S R W W K E
Q G E E M R N T V R N L V A K G Q L Q F A N G G W C M H D E A T
T H F I D M I D Q T T I G H R W L W R E L K V V P R V G W Q V D P
F G H S A T Q A A M L T A R A G L V G T F F A R V D Y R D F E Y R
A S T G R R Q F W W Q P S P S L P E L Q T F A E I N L H Q T Y C P P
S K F S W D V V D Y W M S A V R N T D P L N F V E D K S S E N Y N
I P F I L E L F K E E V R R N V E Q T R G K N I M W T M G C D F N Y
F A S E L W F G K M D R L I E I V N A D G E F E V R Y S T P Y E Y A
M A K R E E H S K G I V Y D T K K G D F F P Y A S A P H E Y W T G
Y F S S R P A L K R L V R R L S S Y W T A A R Q V E F L A G V P T G
E V P M L S D A L A I A Q H H D A I T G T A K Q H V A F D Y A K R
L T E A Y K E D L S V R L R P A L S G C P F G F E N A Q H C L L S N
V S V C S A T A K A F E K E G A V V N V L V W N P N A H S V Q R T
I V H I P V P R S D V I I S G E G V L R Y S V F E S P V Q I S D Y A N
E N K E W Q P Y T L G V E L Q L V K L T I L N L F T P Q L K F A T K
K H T S R L V S L V R P S E D D L E I S N E A L V L K F G R N G L I
E S V T V R S S G Q T V A V K Q D W C Y Y I S N N G D T V S S S P
G G A Y I M R P V S N A T C N P I T D S P V E L R L V D R R M G V V
E Q R F G K D L L Q R V I L R G D L V D V E F T S F G I P I H D N F
G R E L V A R F R T S V E S G D V F Y T D S N G R E M Q R R R V D
H R S D Y P F T Q T E P V A G N F Y P V T S V F F I N D T Q T Q F N
V F P D A P M G G T S L Q S G E V L F V V H R R L L R D D F K G V
E E P L N E T A F V T S Y A D C V M A N T S N C G H H Y G P P L R

V R G T L S F S V S R S G P T A M R R V R E Q Q D E N Y Y T P L V
M F S S S S T D A A S A I A R Y N T S F G F S L P P S L Q V V T L Q
L I D Q Q K L L L R L G H R Y A V S E D P E R S L P V E V D L M T I
F K E F H W I T I L S I D E V S L T A V E I V Q R Q I Q K V T V R P M
D I R T F I F Y I K A H

ToLMann: (SEQ ID NO: 11)
Y M V Y N T S Q G I V S G K L N V H L V P H T H D D V G W L K T V
D Q Y Y V G S N N S I Q V A C V Q N V L D S L I P A L L A D K N R
K F I Y V E Q A F F Q R W W R N Q S P G M Q S T V K Q L V N S G Q
L E F I N G G W C M H D E A A T H Y I D M I D Q T T L G H K Y I K
Q Q F N V T P R I G W Q I D P F G H S A V Q A Y L L G A E V G F D S
L F F G R I D Y Q D R E K R K I E K S L E V I W R G S K S L S S S T
Q I F S G A F P Q N Y E P P S K F Y F E V N D D N S L P V Q D D V N
L F D Y N V Q E R V N D F V A A A L S Q A N I T R T N H I M W T M
G T D F K Y Q Y A H T W F R N M D K L I H Y V N Q D G R V N A L Y
S S P S I Y T D A K Y A L D E S W P L K T D D Y F P Y A D R I N A Y
W T G Y F T S R P A L K L Y V R M M S G Y Y L A A R Q L E F F K G
R I E T G P T T E I L A D A L A I A Q H H D A V S G T S K Q H V A D
D Y A K R L F I G Y K Q A E D L V S N S L A C M V E S A S A S G C
K N P Q I N F K Q C P L L N I S Y C P P T E A D L A P G K K L V V V
V Y N A L G W K R T D V V R I P V V N K N V I V E D S T G K E I E S
Q L L P I V K E S I V I R N Y Y A A A Y F G E S P T S S P K Y W L V
F T A T V P P L G F S S Y V I T S G K Q A V A A S I P Q T F Y K T D
G S Q S D A V E V G P G N L K L L Y S A N G A K F T Q Y F N K R N
Q V R S S L E Q S F S Y Y S A D D G S K D D Y K D I Q A S G A Y V F
R P N G S F P I H P E G K V P A T I L R G P L L D E V H Q N I N S W I
Y Q I T R V Y K E K E H V E V E F T V G P I P I D N G I G K E L V T
Q I Q T D I K S N K T F Y T D S N G R D F L K R V R D Y R A D W D
L Q V N Q P A A G N Y Y P I N L G L F L K D N N N E F S V L V D R S
V G G S S L V D G Q L E L M L H R R L L N D D G R G V A E A L N E
T V C A L G K C M G L T V Q G K Y Y I R I D S L G E G A K W R R S
F G Q E I Y S P L L L A F T E Q D G D K F T K F P V P T F T G M D P
S Y S L P D N V A I I T L Q E L E D H T V L L R L A H L Y E V D E D
K D L S T K A S V E L K R L F P K R K I N K I R E M S L S A N Q E R
V E M E K K R L K W K A E A P S D L R D V A R G G P V D P T K L M
V E L A P M E I R T F V I D L S Q S V P E G W K S H M S L

VvLMann: (SEQ ID NO: 12)
G Y I K Y N T G A G I V E G K L N V H L V P H S H D D V G W L K T
I D Q Y Y V G S N N S I Q G A C V E N V L D S V V E S L L R N Q N R
K F V F A E M A F F S R W W Y E Q S E E I Q E T V G K L V D G G R
L E F I N G G W C M H D E A T V H Y I D M I D Q T T L G H R L I K K
A F N K A P R A G W Q I D P F G H S A V Q A Y L L G A E L G F D S
V H F A R I D Y Q D R A K R K E D K S L E V I W R G S K T F G S T S
Q I F T N A F P V H Y S P P N G F G F E I D D F S I P V Q D N P H L F
D Y N V E Q R I N D F V A A A L V Q A N V T R T D H I M W T M G D
D F Q Y Q Y A E T W F K Q M D K L I H Y V N K D G R V N A L Y S T
P S M Y V D A K H A T N E E W P L K T H D Y F P Y A D R I N A Y W
T G Y F T S R P A L K R Y V R M L S G Y Y L A A R Q L E F L A G R
S S T G L N T F S L G D A L G I A Q H H D A V T G T A K Q H T T N D
Y A K R L A I G A S E T E A T V N S A L S C I A S K N S G Q C A A S

T S S F S Q C Q L L N I S F C P P T E E D I P E G K S L V V V A Y N P
L G W K R T E F V R I P V N D S D F V V Q D S T G N T I E A Q Y L K
V D N V T I N L R N F Y T K A Y L G R S P K N V P K F W L L F Q V S
V P P L G W N T Y F I S K A S G K E S S R S G Y I S V M D S P Q N E
S I E V G P G S L K M S F S S T T G Q L E R M F D S K T G V D L P I
Q Q S Y L W Y G S S D G G L D S Q P S G A Y I F R P N G A P P T V V
S R S V P L K V M R G P L V D E V H Q E F S P W I Y Q V T R L Y K
D K D H A E V E F T I G P I P V D D S V G K E V I T R M T A N M V T
N K V F Y T D S S G R D F L K R V R D Y R E D W S L S V N Q P E A
G N Y Y P I N L G I F T T D K K S E F S V L V D R A T G G S S I K D
G Q V E L M L H R R M I F D D S R G V G E A L D E T T C V E N T C
E G L T V R G N Y Y M S I D L L G D G A Q W R R T T G Q E I Y S P L
L L A F T H E K L E T W T A S H L T K G T V M E P N Y S L P L N V
A V I T L Q E L D D G S V L L R L A H L Y E A G E D A K Y S T L A K
V E L Q K M F R G K K I K E I R E T N L S T N Q E K S E M K T L K W
K V E G D N G D E I A P L R G G P V D N S T L V V E L G P M E I R T
F L L E

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WHAT IS CLAIMED:

1. An *in vitro* method for reducing the extent of O-mannosylation of a recombinant glycoprotein of interest comprising:

a. providing an intact glycoprotein of interest which comprises at least one O-linked glycan mannose polymer; and

b. contacting the glycoprotein of (a) with a lysosomal mannosidase (LMann) under conditions which allow for enzymatic activity of the LMann;

wherein the treated glycoprotein composition comprises a reduced level of O-mannosylation compared to the O-mannosylation of the recombinant glycoprotein that is not treated with LMann.

2. The method according to claim 1, wherein the LMann is selected from the enzyme class consisting of E.C.3.2.1.24.

3. The method of claim 1, wherein the glycoprotein of interest is expressed by a yeast or fungal host cell.

4. The method of claim 1, wherein the LMann is recombinantly expressed in *Pichia pastoris*.

5. The method of claim 1, wherein the lysosomal mannosidase is selected from GmMann (SEQ ID NO: 3), HsLMann (SEQ ID NO: 5), MtLMann (SEQ ID NO: 6) and VvLMann (SEQ ID NO:12).

6. The method of claim 1, wherein the lysosomal mannosidase is AtLMann (SEQ ID NO: 1).

7. The method of claim 1, wherein the lysosomal mannosidase is selected from DdLMann (SEQ ID NO: 2), CpLMann (SEQ ID NO: 4), MmLMann (SEQ ID NO: 7), RcLMann (SEQ ID NO: 8), SsLMann (SEQ ID NO: 9), TcLMann (SEQ ID NO: 10) and ToLMann (Seq ID NO: 11).

8. The method of claim 1, wherein the glycoprotein of interest is selected from an antibody, a hormone, a cytokine, an enzyme or a bioactive peptide.

9. The method of claim 1, wherein the O-linked glycan mannose polymer comprises at least one α1,2 mannose, α1,3 mannose and/or α1,6 mannose is linked to the initiating serine or threonine linked mannose residue.

10. The method of claim 1, wherein the recombinant glycoprotein possesses an O-linked glycan comprising a mannose residue directly linked to a serine or threonine residue of the glycoprotein by an α-bond.

11. The method of claim 1, wherein at least 10% of the original O-linked mannose is enzymatically removed.

12. The method of claim 1, wherein at least 10% of the Man-α-O-Ser/Thr glycosidic bonds are enzymatically cleaved.

13. The method of claim 1, wherein at least 74% of the Man-α-O-Ser/Thr glycosidic bonds are enzymatically cleaved.

14. The method of claim 1, wherein the LMann is added exogenously to the glycoprotein following expression and secretion of the glycoprotein, and further wherein the glycoprotein is optionally purified.

15. An *in vivo* method for reducing the extent of O-mannosylation of an intact recombinant glycoprotein comprising:

c. providing a transformed yeast production strain that is engineered to express a protein of interest which comprises at least one O-linked glycan mannose polymer;

d. transforming the yeast production strain of (a) with an expression vector comprising a lysosomal mannosidase (LMann); and

e. culturing the transformed yeast production strain of (b) under conditions which allow for the co-secretion of the protein of interest and the LMann

wherein the glycoprotein recovered from step (c) comprises a reduced level of O-mannosylation compared to the level of O-mannosylation of the glycoprotein produced by a yeast production strain that does co-express a LMann.

16. The method of claim 15, wherein the LMann is co-secreted with the glycoprotein of interest, wherein the O-linked mannose may be reduced during secretion or after secretion from the host cell.

17. The method of claim 15, wherein the host cell is wild-type or glycoengineered *Pichia pastoris*.

18. The method according to claim 15, wherein the LMann is selected from the enzyme class consisting of E.C.3.2.1.24.

19. The method of claim 15 , wherein the yeast production strain is transformed with an expression vector comprising a LMann isolated from a plant or non-plant source.

20. The method of claim 15, wherein the lysosomal mannosidase is selected from GmMann (SEQ ID NO: 3), HsLMann (SEQ ID NO: 5), MtLMann (SEQ ID NO: 6) and VvLMann (SEQ ID NO:12).

21. The method of claim 15, wherein the lysosomal mannosidases is AtLMann (SEQ ID NO: 1).

22. The method of claim 15, wherein the lysosomal mannosidase is selected from DdLMann (SEQ ID NO: 2), CpLMann (SEQ ID NO: 4), MmLMann (SEQ ID NO: 7), RcLMann (SEQ ID NO: 8), SsLMann (SEQ ID NO: 9), TcLMann (SEQ ID NO: 10) and ToLMann (Seq ID NO: 11).

23. The method of claim 15, wherein the glycoprotein is a selected from an antibody, a hormone, a cytokine, an enzyme or a bioactive peptide.

24. The method of claim 15, wherein the O-linked glycan mannose polymer comprises at least one α1,2 mannose, α1,3 mannose and/or α1,6 mannose linked to an initiating serine or threonine residue of the glycoprotein by an α-bond.

25. The method of claim 1 or 15 wherein the host cell is selected from the group consisting of *Pichia pastoris, Pichia finlandica, Pichia trehalophila, Pichia koclamae, Pichia membranaefaciens, Pichia opuntiae, Pichia thermotolerans, Pichia salictaria, Pichia guercuum, Pichia pijperi, Pichia stiptis, Pichia methanolica, Pichia sp., Saccharomyces cerevisiae, Saccharomyces sp., Hansenula polymorpha, Kluyveromyces sp., Candida albicans, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, Aspergillus oryzae, Trichoderma reesei, Chrysosporium lucknowense, Fusarium sp., Fusarium gramineum, Fusarium venenatum* and *Neurospora crassa.*

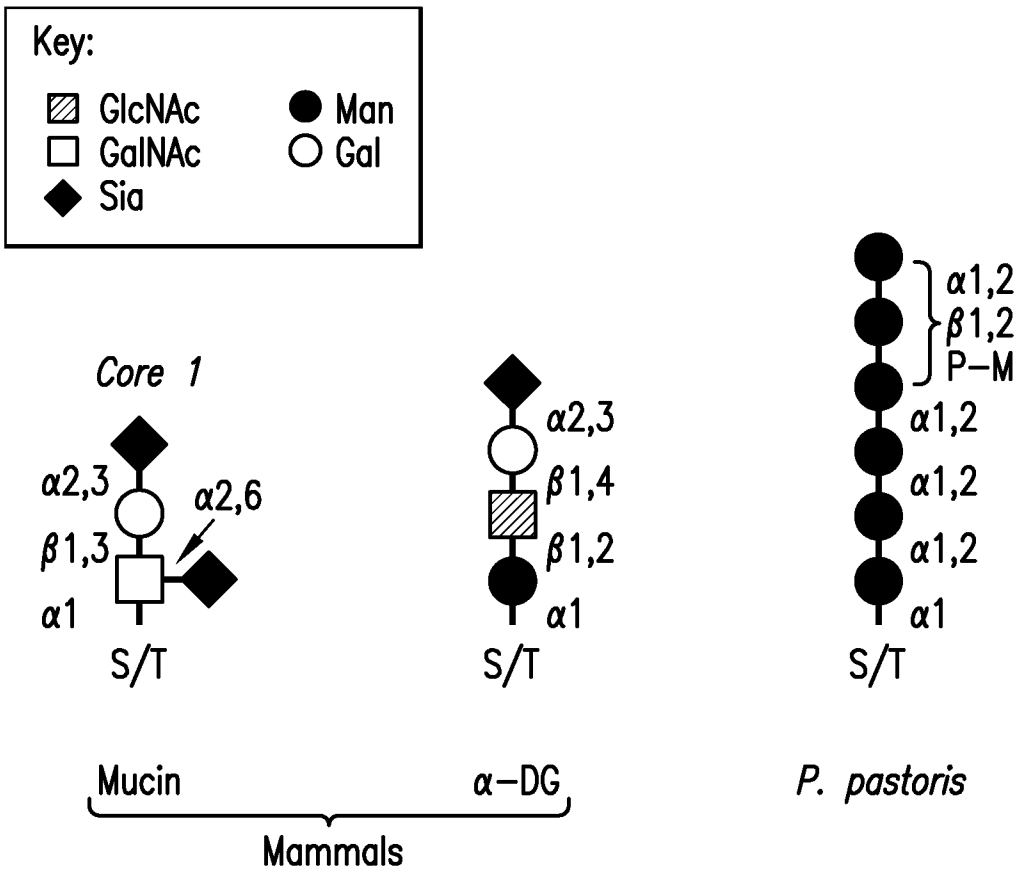
Key:
GlcNAc
GalNAc
Sia
Man
Gal
Core 1
α2,3
α2,6
β1,3
α1
S/T
α2,3
β1,4
β1,2
α1
S/T
α1,2
β1,2
P–M
α1,2
α1,2
α1,2
α1
S/T
Mucin
α–DG
Mammals
P. pastoris

FIG. 1A

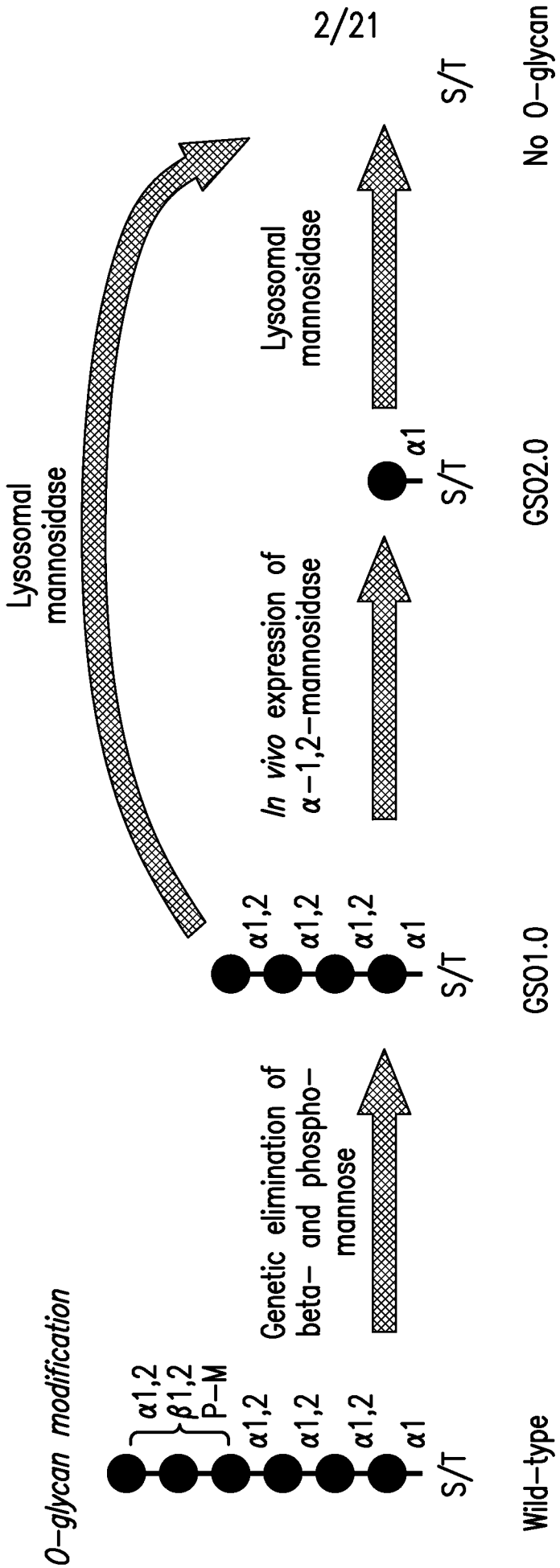
O-glycan modification
α1,2
β1,2
P-M
α1,2
α1,2
α1,2
α1
S/T
Wild-type
Genetic elimination of beta- and phospho-mannose
α1,2
α1,2
α1,2
α1
S/T
GS01.0
Lysosomal mannosidase
In vivo expression of α-1,2-mannosidase
α1
S/T
GS02.0
Lysosomal mannosidase
S/T
No O-glycan

FIG.1B

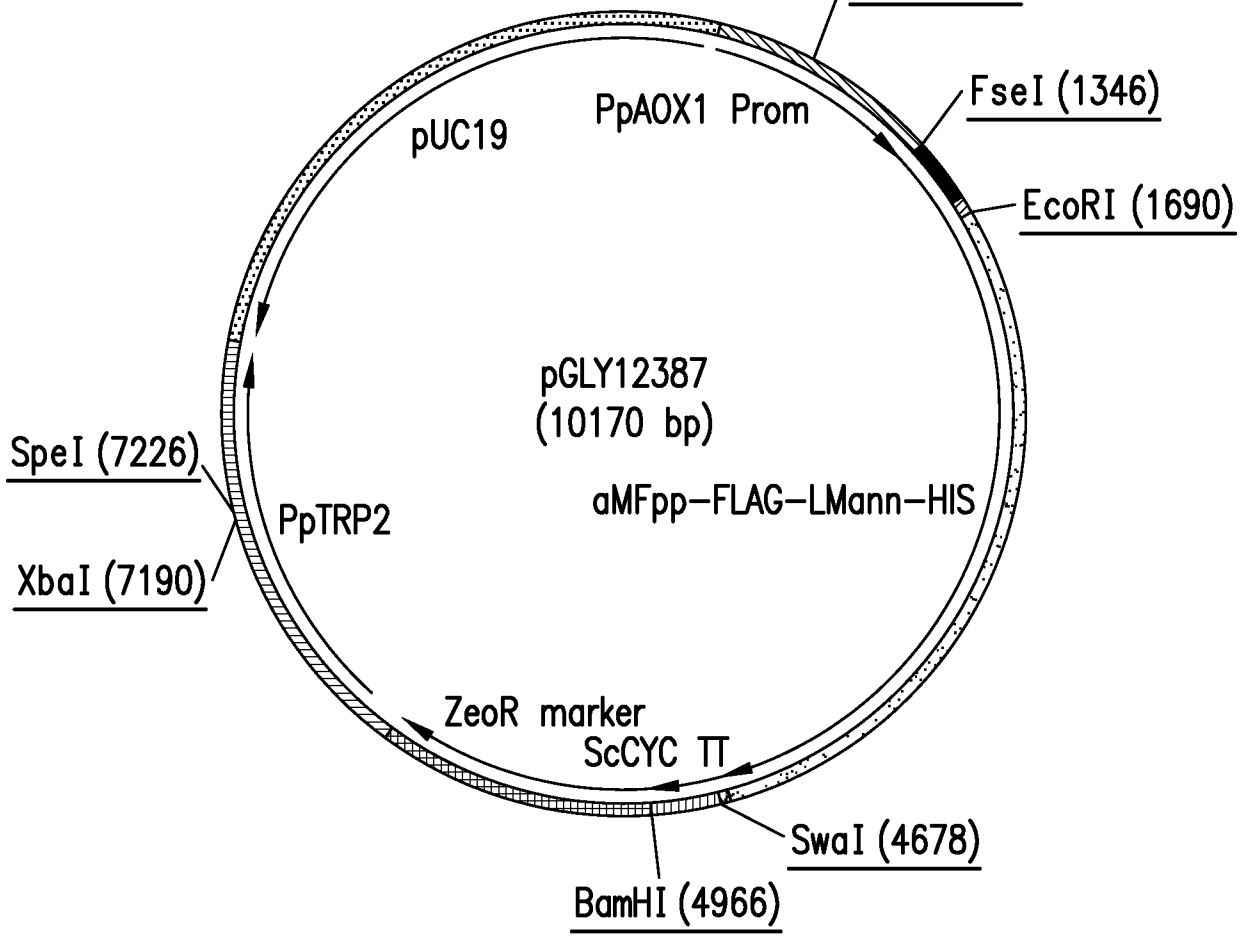
PmeI (810)
FseI (1346)
EcoRI (1690)
PpAOX1 Prom
pUC19
pGLY12387
(10170 bp)
aMFpp-FLAG-LMann-HIS
SpeI (7226)
PpTRP2
XbaI (7190)
ZeoR marker
ScCYC TT
SwaI (4678)
BamHI (4966)

FIG.2A

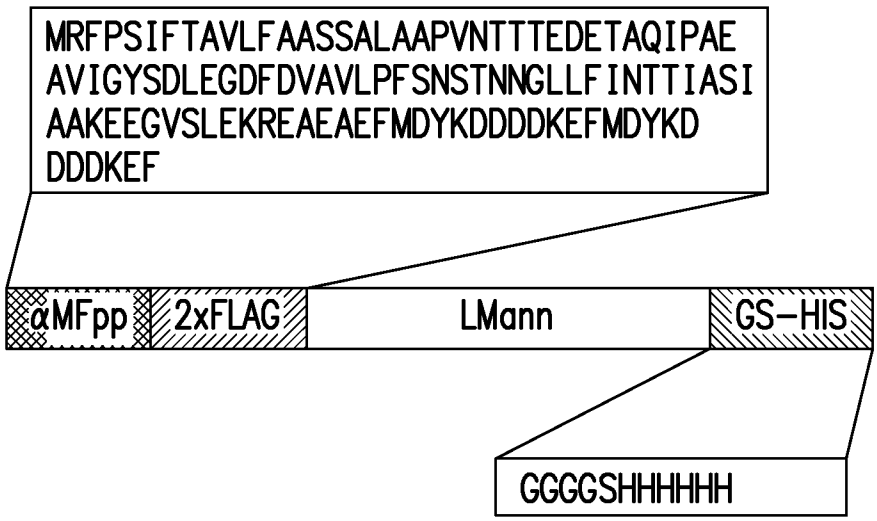
MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAE
AVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASI
AAKEEGVSLEKREAEAEFMDYKDDDDKEFMDYKD
DDDKEF
αMFpp
2xFLAG
LMann
GS-HIS
GGGGSHHHHHH

FIG.2B

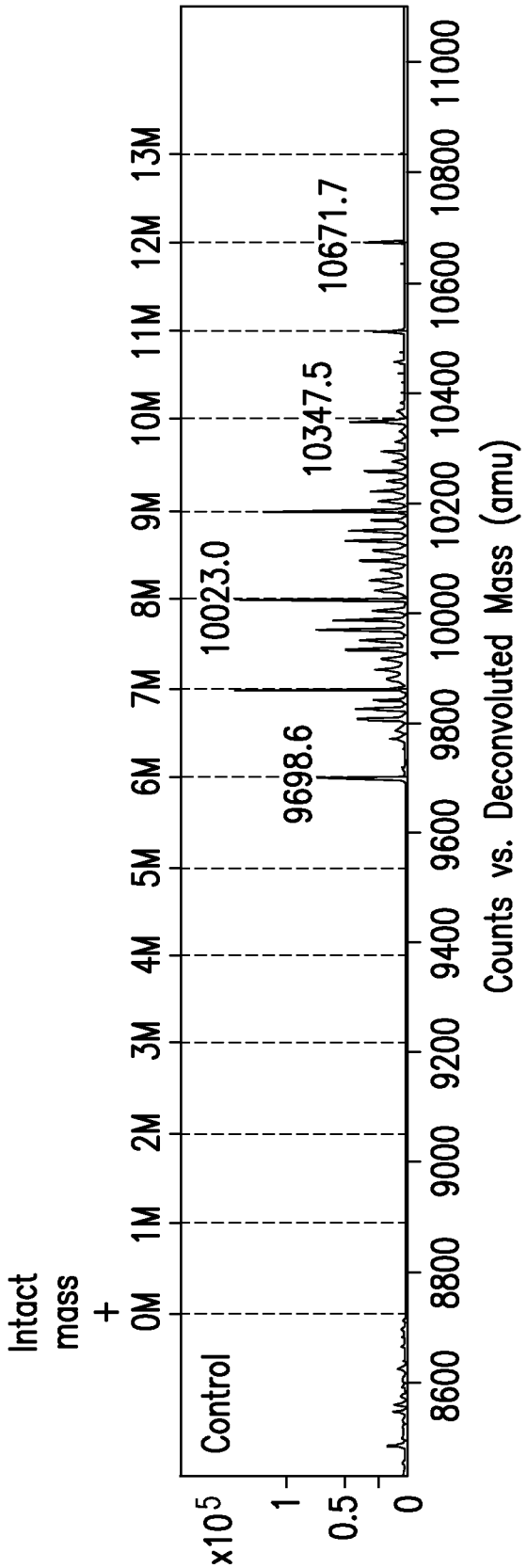
Intact mass
+
Control
x10^5
1
0.5
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M 13M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
9698.6
10023.0
10347.5
10671.7
Counts vs. Deconvoluted Mass (amu)

FIG.3A

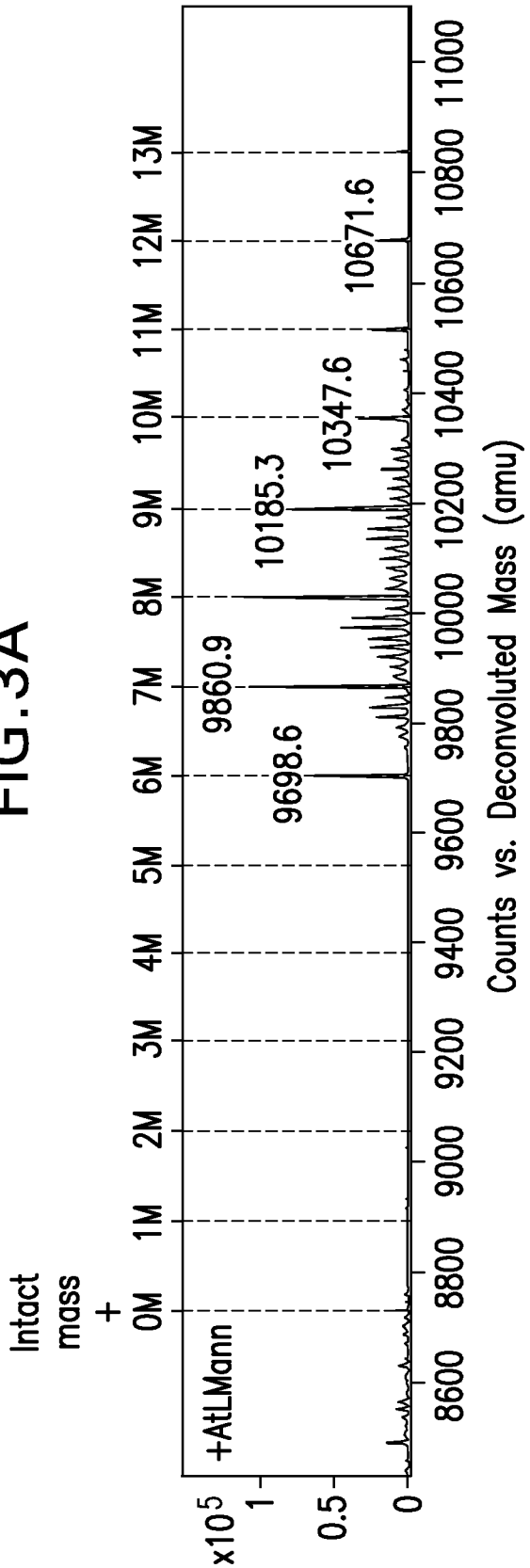
Intact mass
+
+AtLMann
x10^5
1
0.5
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M 13M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
9698.6
9860.9
10185.3
10347.6
10671.6
Counts vs. Deconvoluted Mass (amu)

FIG.3B

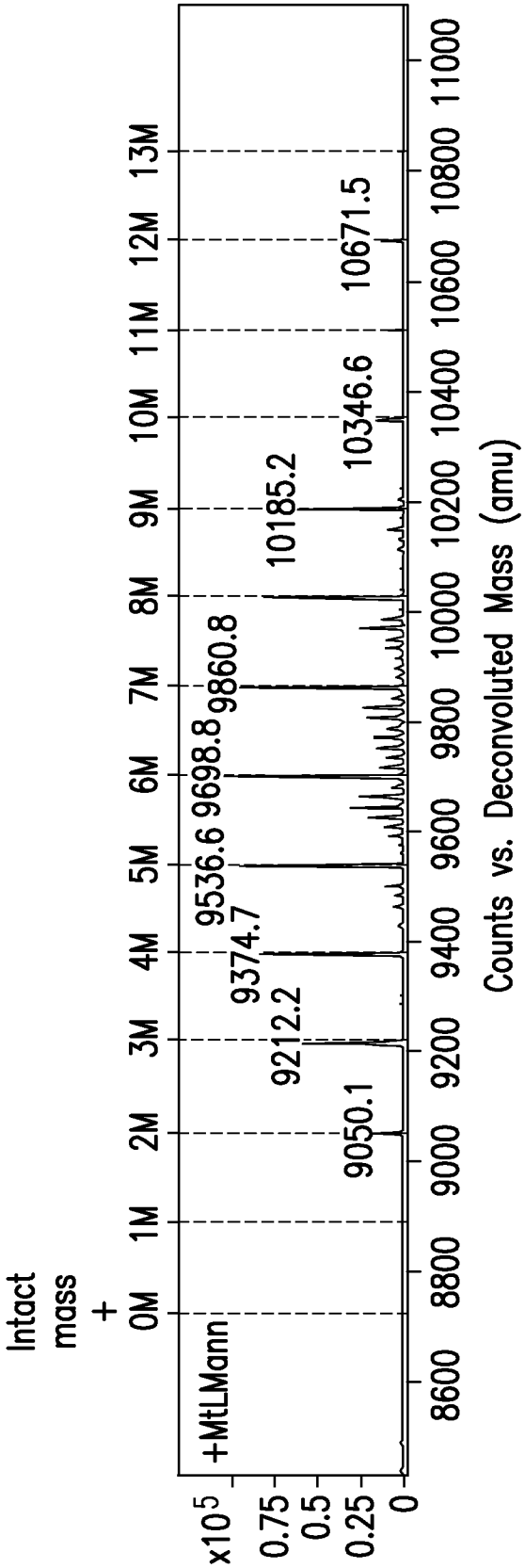
Intact mass
+MtLMann
x10^5
0.75
0.5
0.25
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M 13M
9050.1
9212.2
9374.7
9536.6
9698.8
9860.8
10185.2
10346.6
10671.5
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.3C

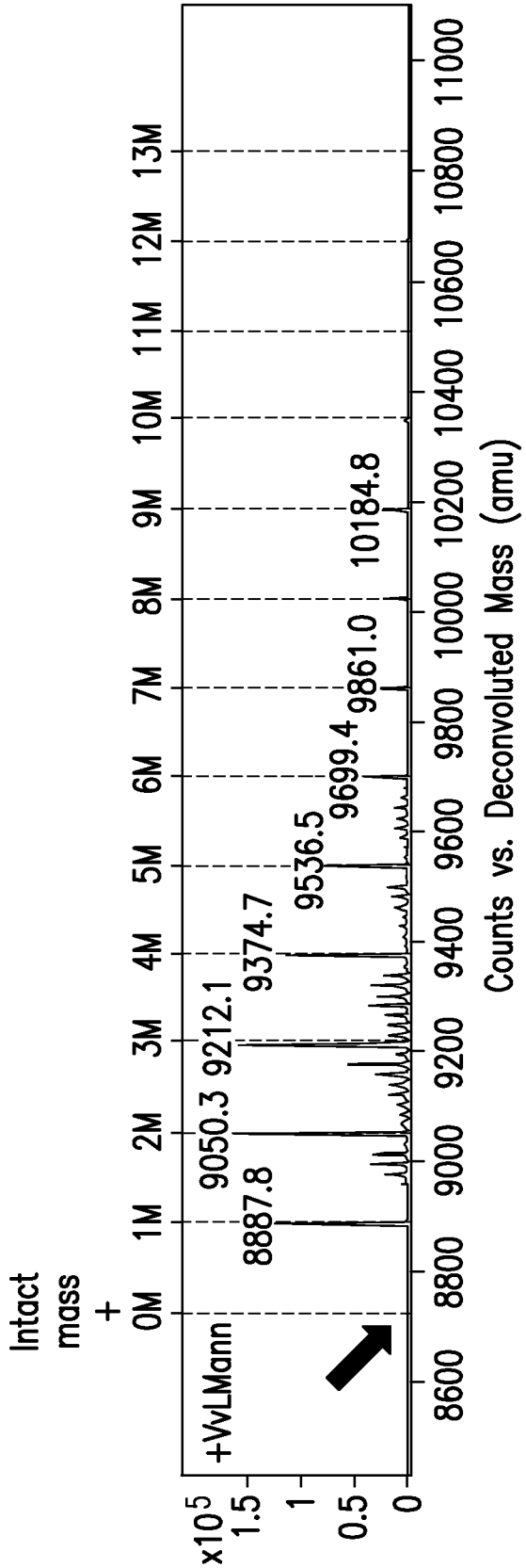
Intact mass
+VvLMann
x10^5
1.5
1
0.5
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M 13M
8887.8
9050.3
9212.1
9374.7
9536.5
9699.4
9861.0
10184.8
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.3D

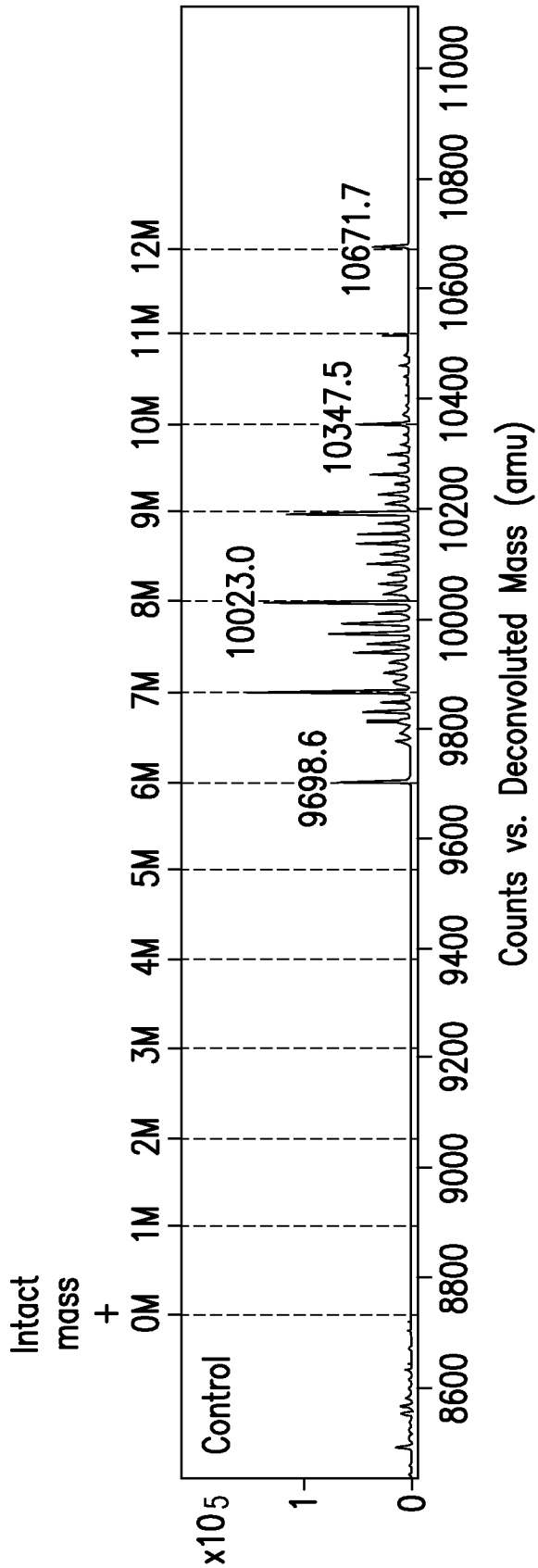
Intact mass
+
Control
x10^5
1
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
9698.6
10023.0
10347.5
10671.7
Counts vs. Deconvoluted Mass (amu)

FIG.4A

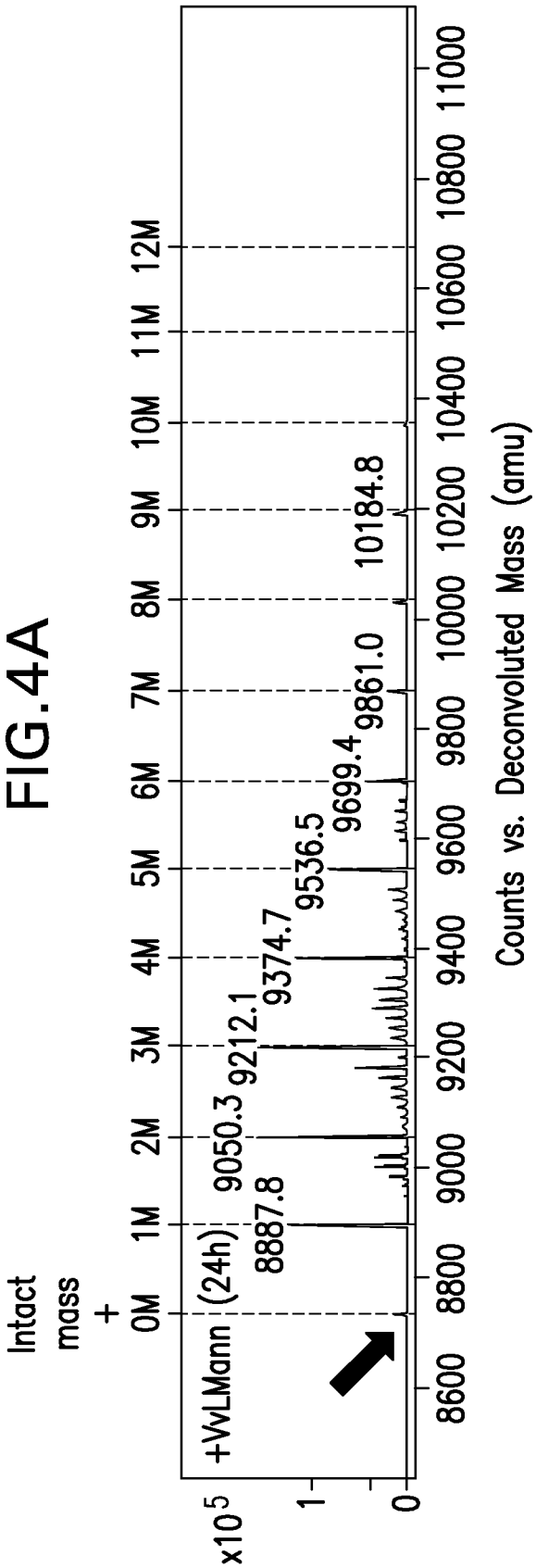
Intact mass
+
+VvLMann (24h)
x10^5
1
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
8887.8
9050.3
9212.1
9374.7
9536.5
9699.4
9861.0
10184.8
Counts vs. Deconvoluted Mass (amu)

FIG.4B

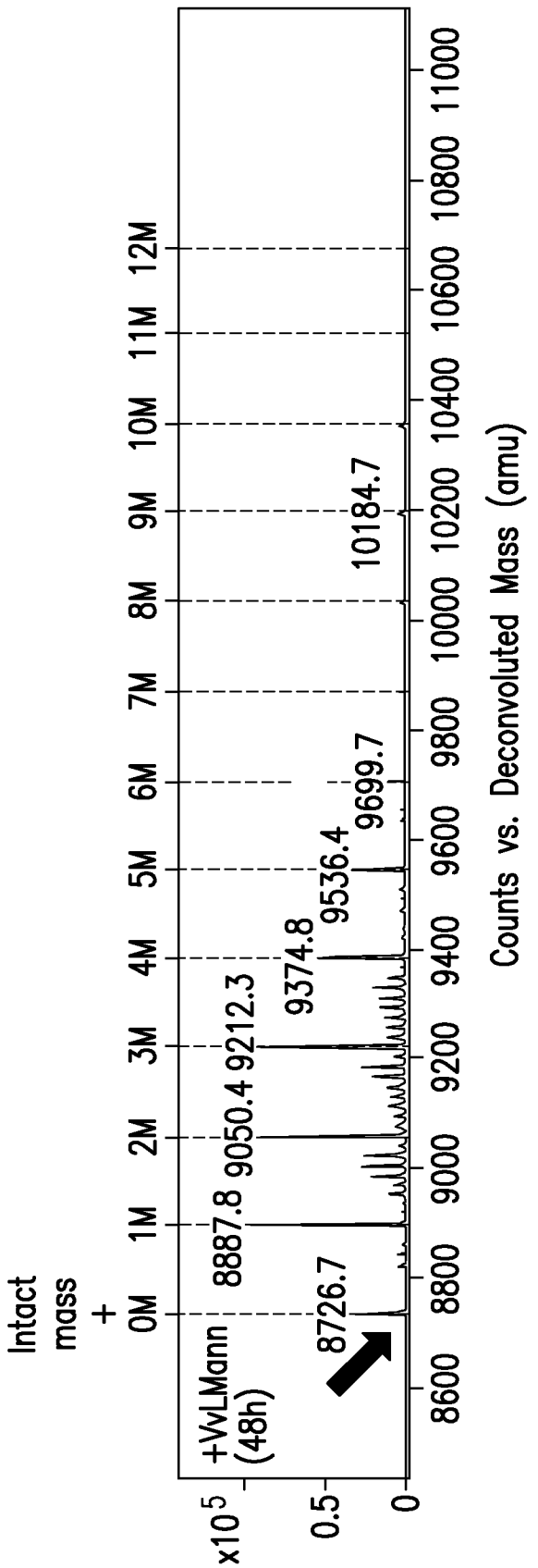
Intact mass
+
+VvLMann (48h)
8726.7
8887.8
9050.4
9212.3
9374.8
9536.4
9699.7
10184.7
x10^5
0.5
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.4C

Intact mass
+
+VvLMann (48h)
+TrMannI
8726.2
8888.0
9050.1
9212.0
9374.9
x10^5
2
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.4D

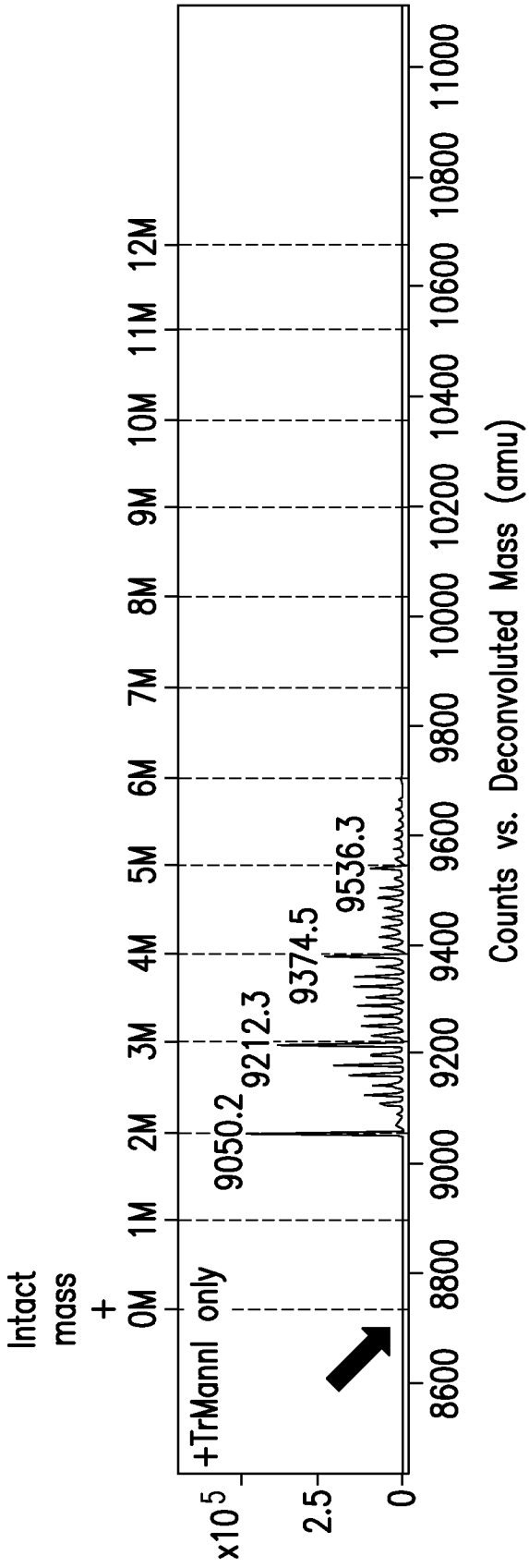
Intact mass
+TrMannI only
9050.2
9212.3
9374.5
9536.3
x10^5
2.5
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.4E

SDS-PAGE
Coomassie Stained

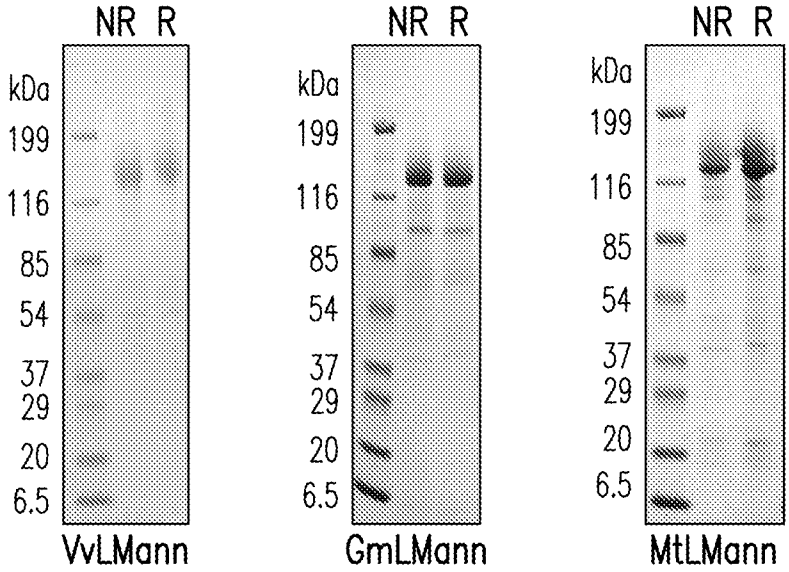

FIG.5A

Western Blot to
detect His tag

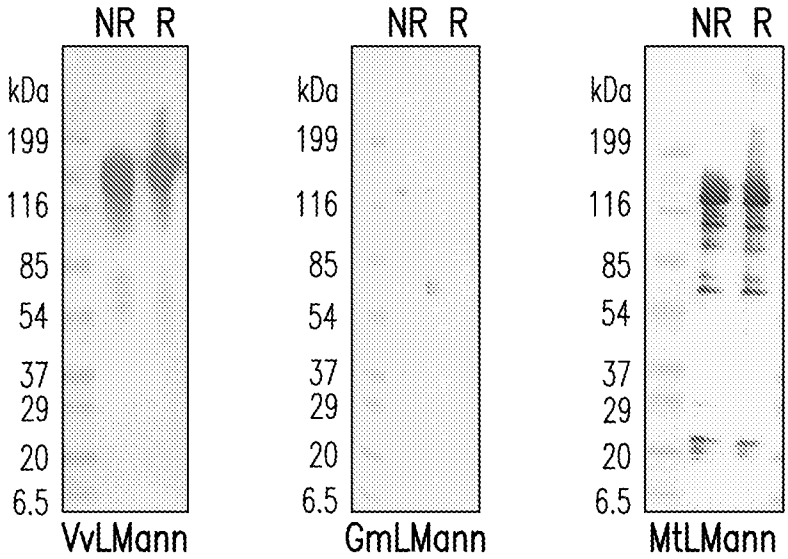

FIG.5B

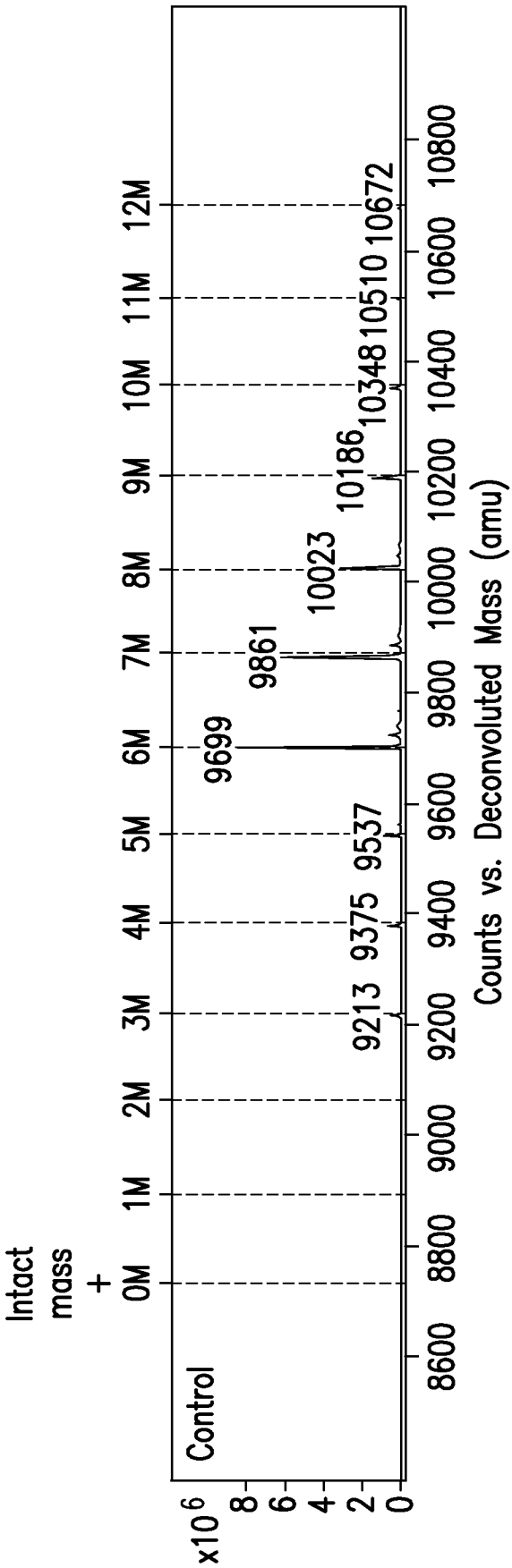
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800
Counts vs. Deconvoluted Mass (amu)
Control
x10^6
8 6 4 2 0
9213 9375 9537 9699 9861 10023 10186 10348 10510 10672

FIG.6A

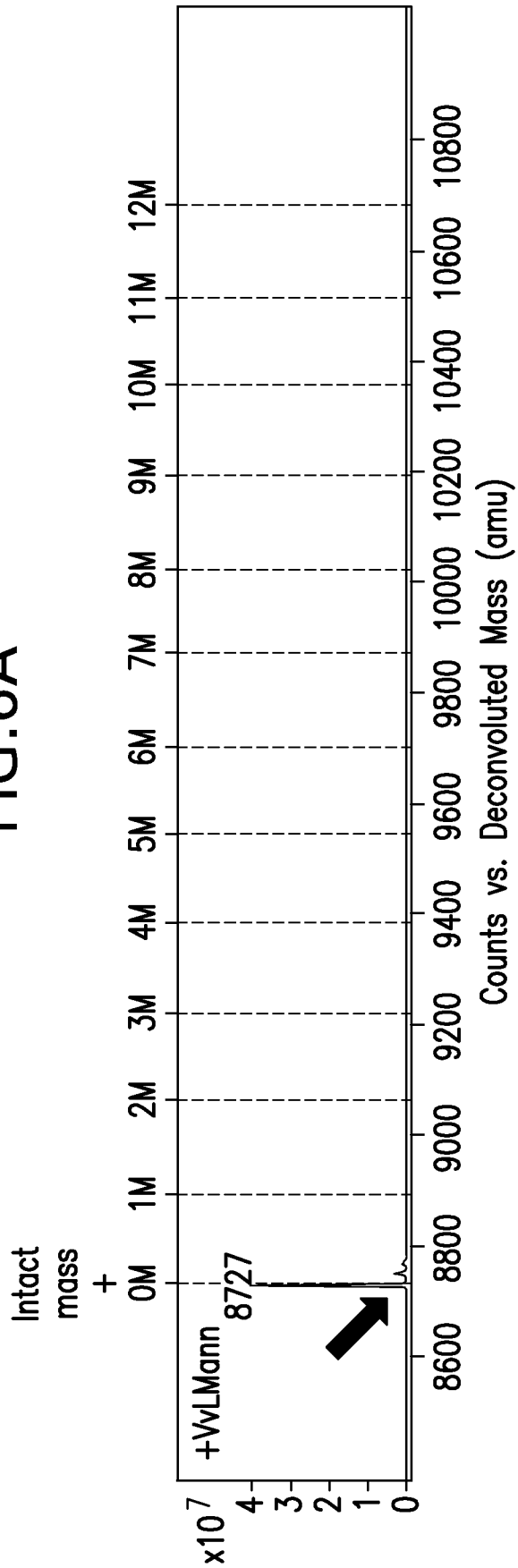
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800
Counts vs. Deconvoluted Mass (amu)
+VvLMann
8727
x10^7
4 3 2 1 0

FIG.6B

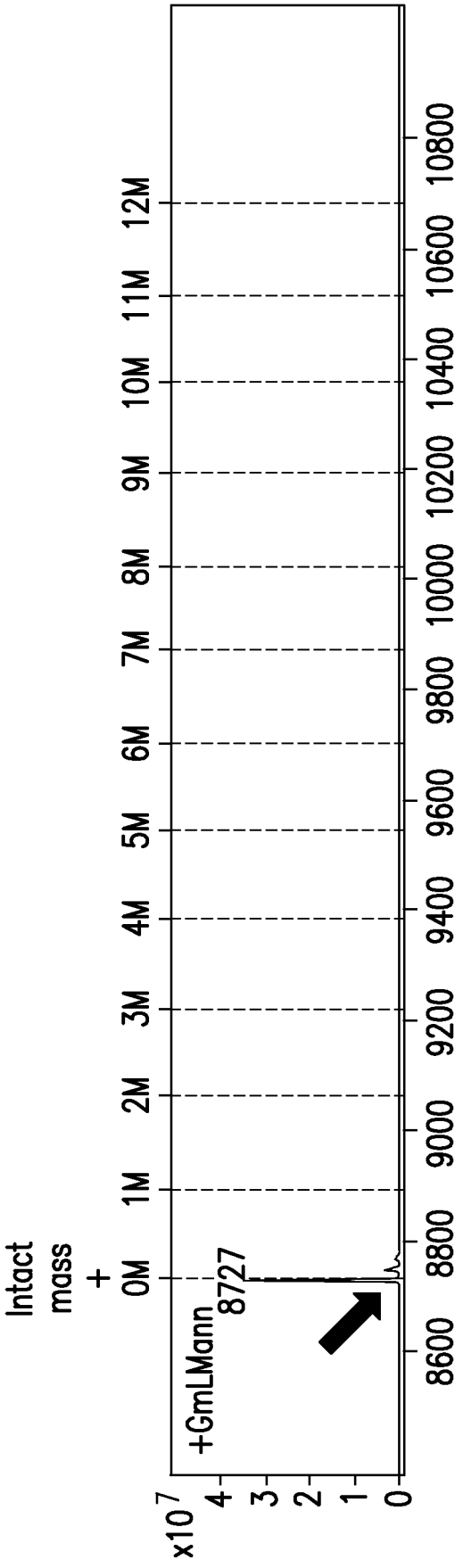
Intact mass
+GmLMann
8727
x10^7
4
3
2
1
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800
Counts vs. Deconvoluted Mass (amu)

FIG.6C

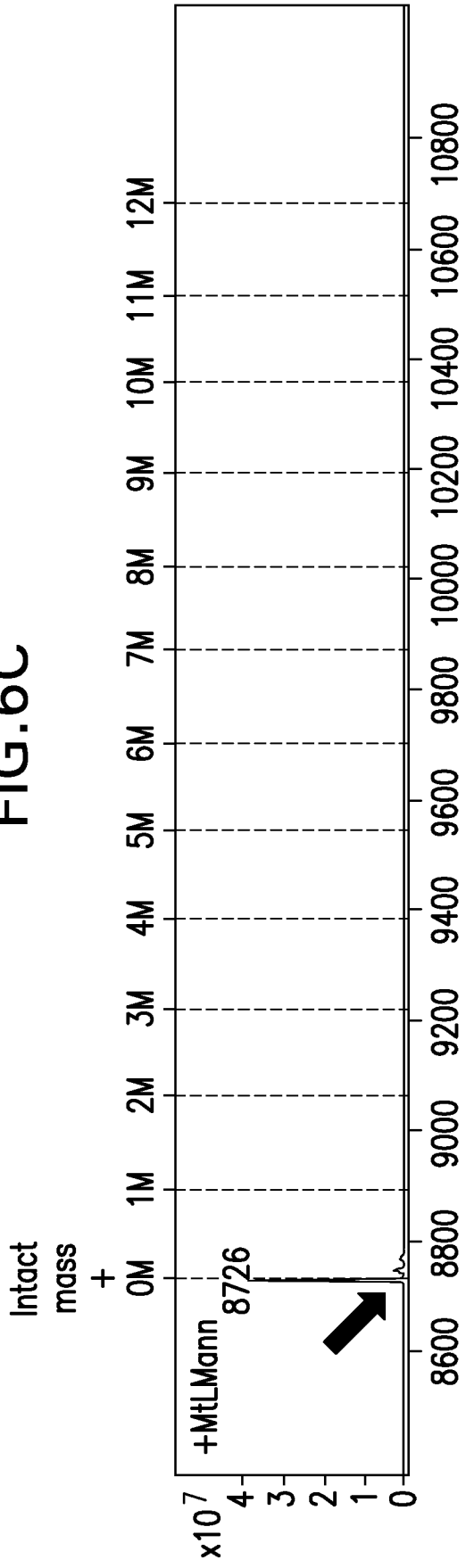
Intact mass
+MtLMann
8726
x10^7
4
3
2
1
0
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800
Counts vs. Deconvoluted Mass (amu)

FIG.6D

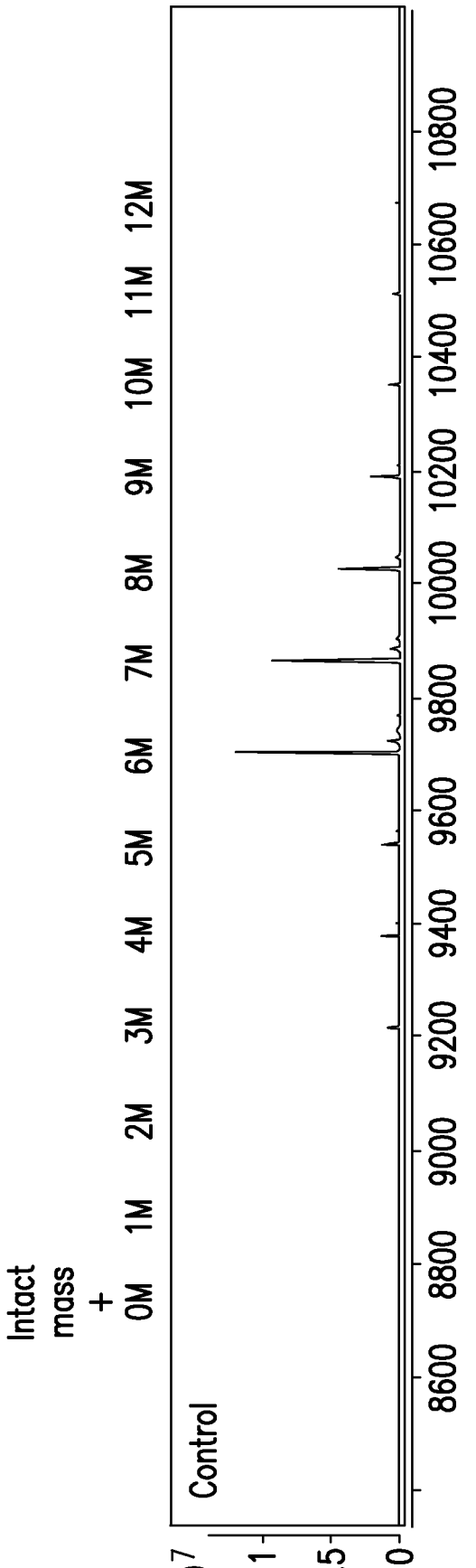
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
Control
x10^7
1
0.5
0
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800
Counts vs. Deconvoluted Mass (amu)

FIG.7A

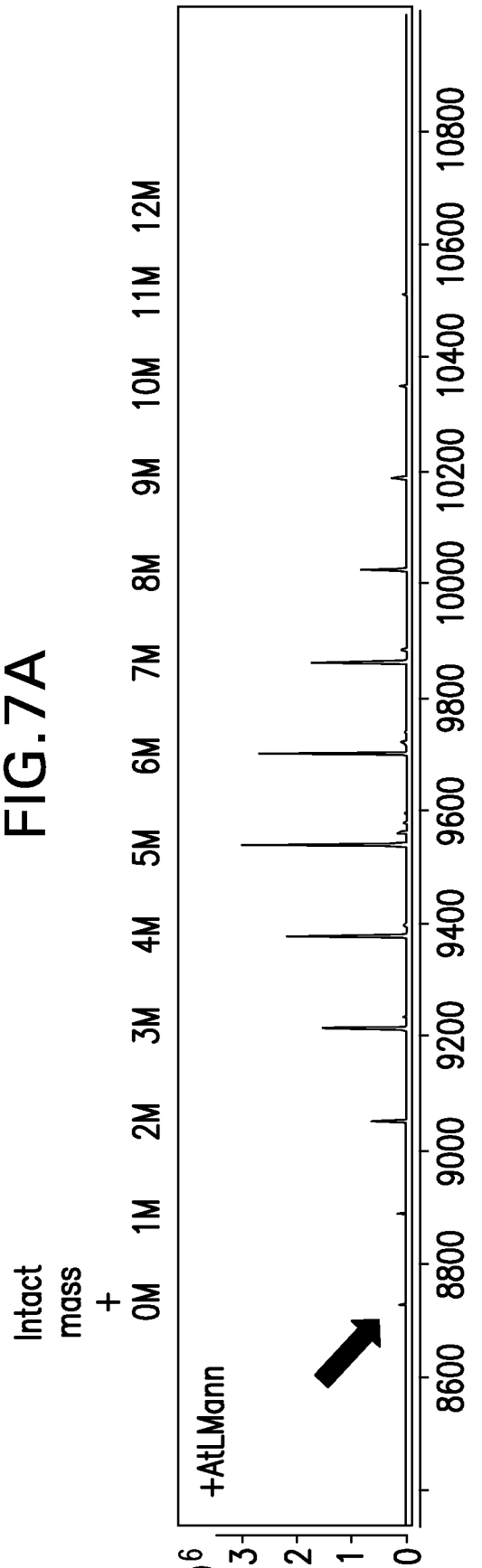
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
+AtLMann
x10^6
3
2
1
0
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800
Counts vs. Deconvoluted Mass (amu)

FIG.7B

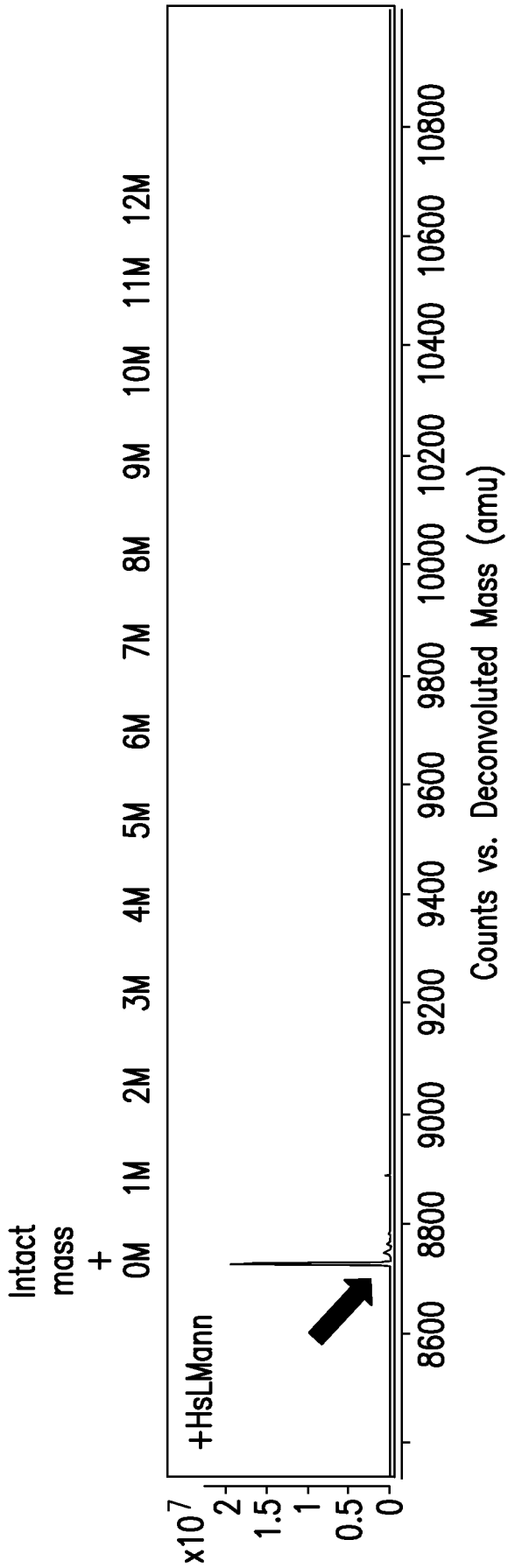
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
+HsLMann
x10^7
2
1.5
1
0.5
0
8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800
Counts vs. Deconvoluted Mass (amu)

FIG.7C

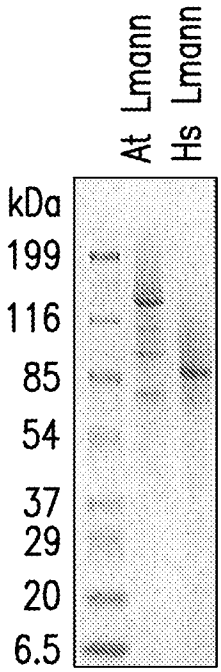
At Lmann
Hs Lmann
kDa
199
116
85
54
37
29
20
6.5

FIG.8A

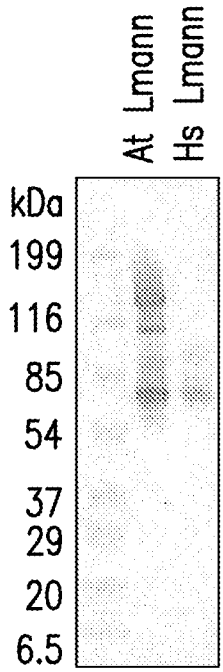
At Lmann
Hs Lmann
kDa
199
116
85
54
37
29
20
6.5

FIG.8B

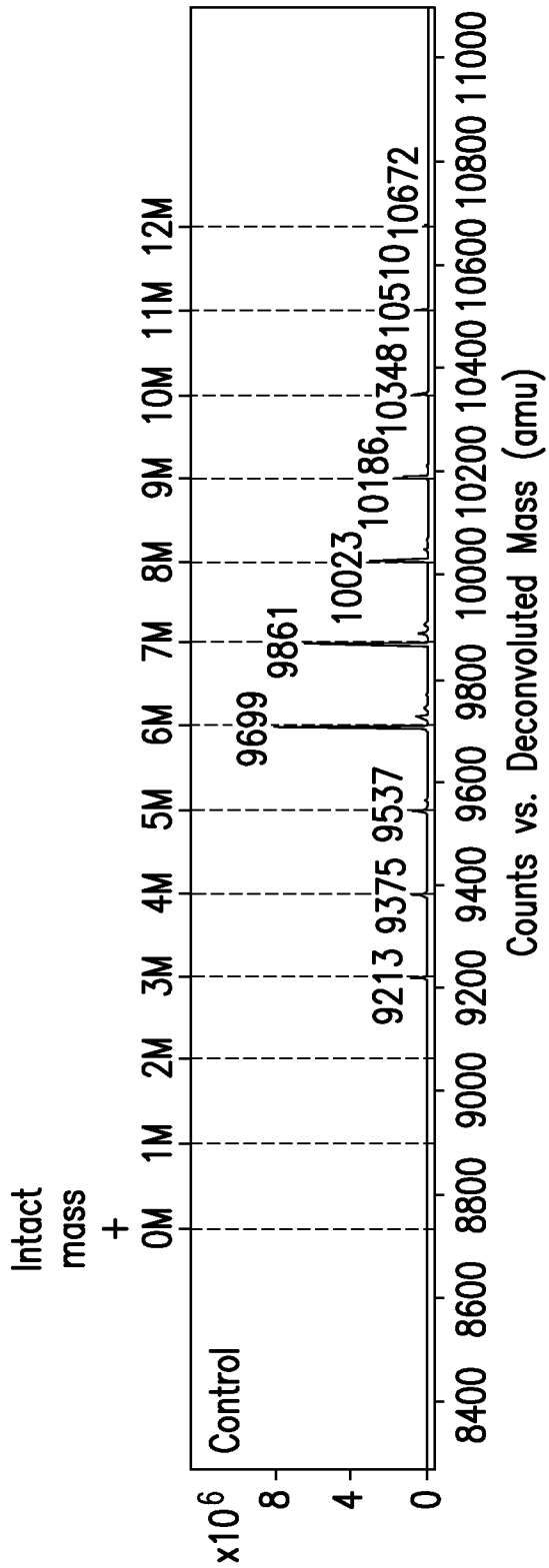
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
Control
x10^6
8
4
0
9213 9375 9537 9699 9861 10023 10186 10348 10510 10672
8400 8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.9A

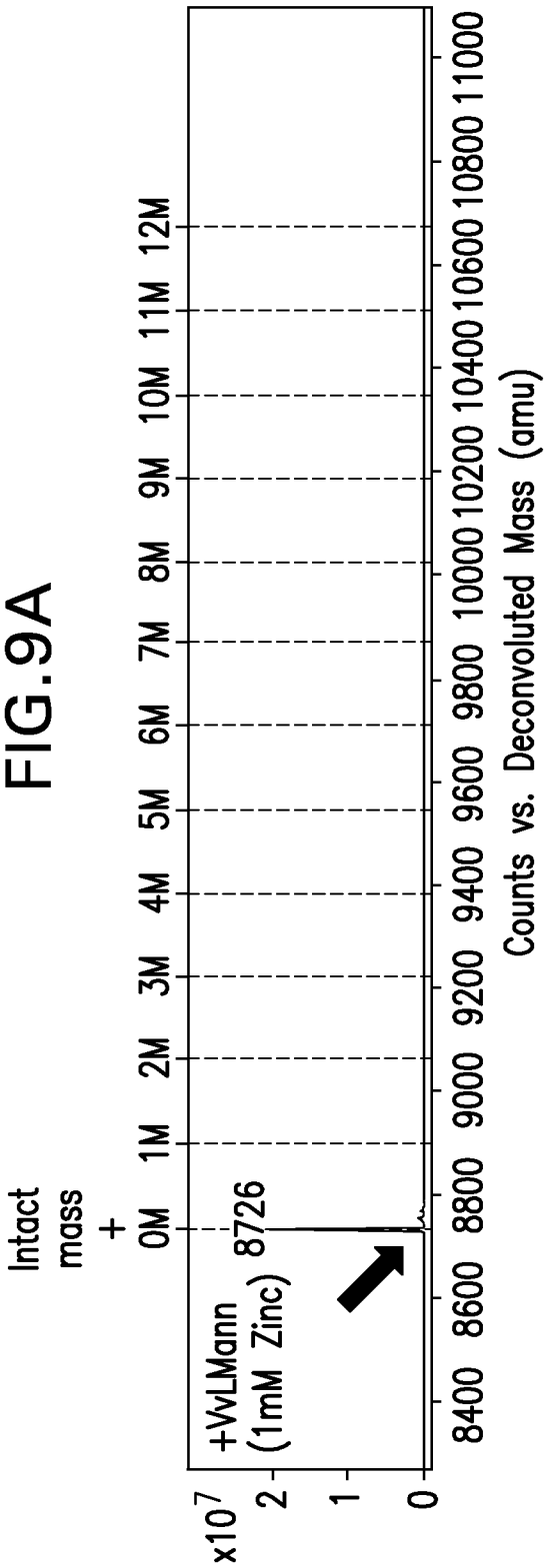
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
+VvLMann (1mM Zinc)
8726
x10^7
2
1
0
8400 8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.9B

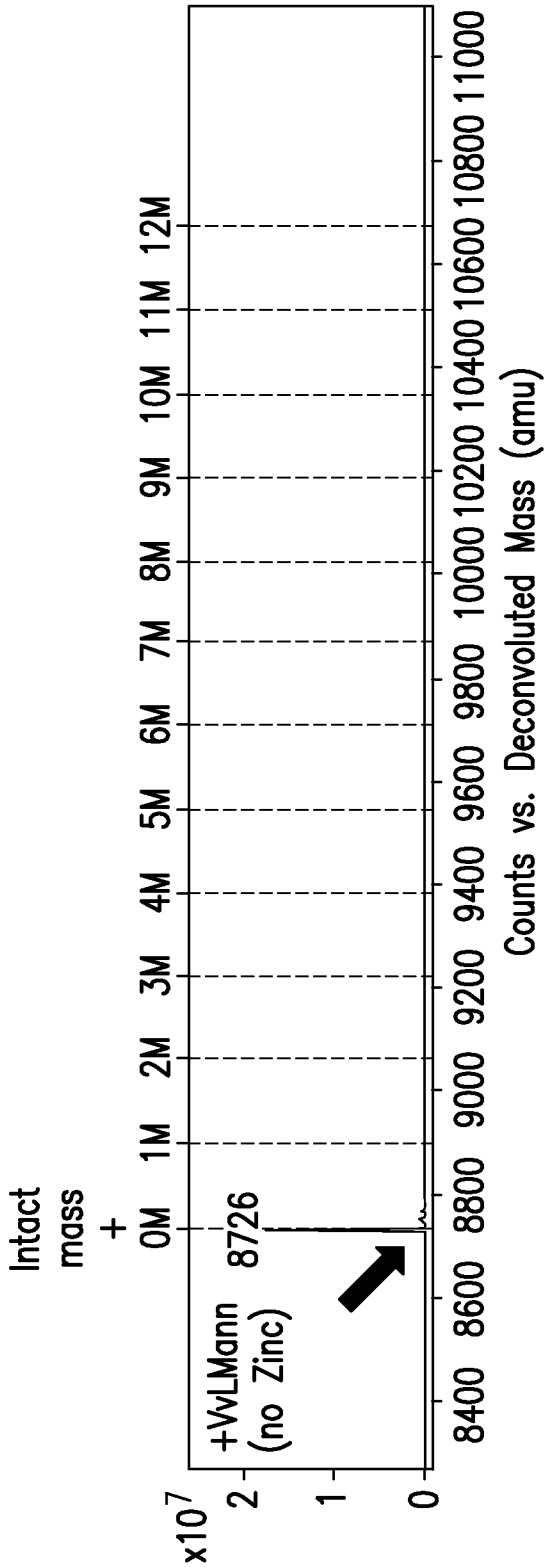
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
+VvLMann (no Zinc)
8726
x10^7
2
1
0
8400 8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.9C

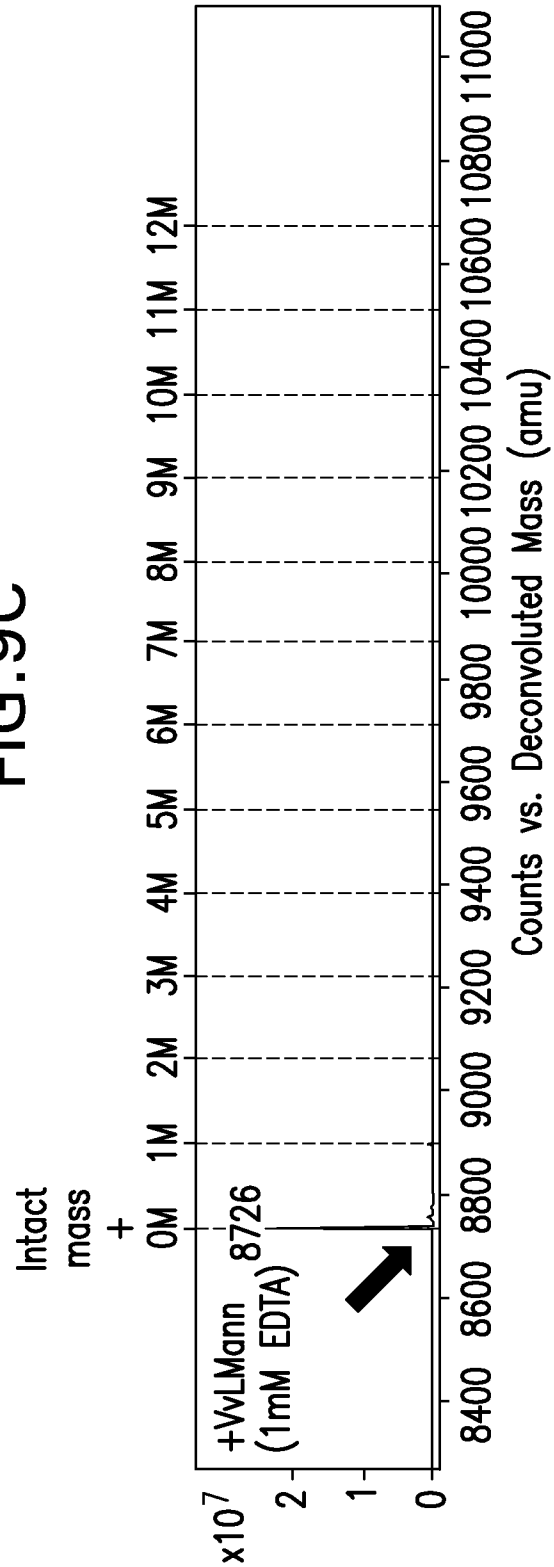
Intact mass
+
0M 1M 2M 3M 4M 5M 6M 7M 8M 9M 10M 11M 12M
+VvLMann (1mM EDTA)
8726
x10^7
2
1
0
8400 8600 8800 9000 9200 9400 9600 9800 10000 10200 10400 10600 10800 11000
Counts vs. Deconvoluted Mass (amu)

FIG.9D

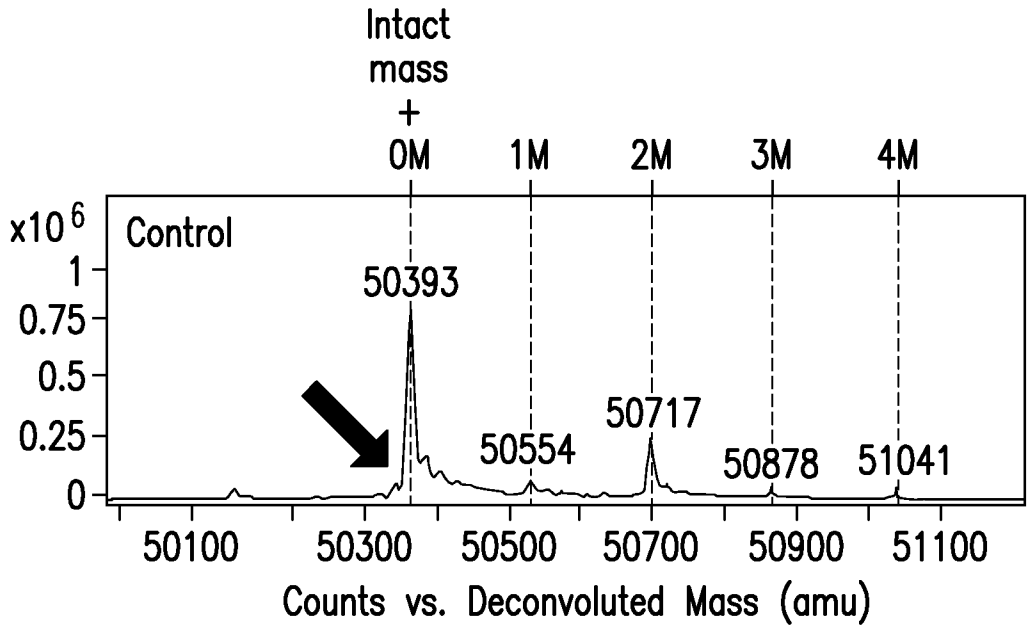

FIG. 10A

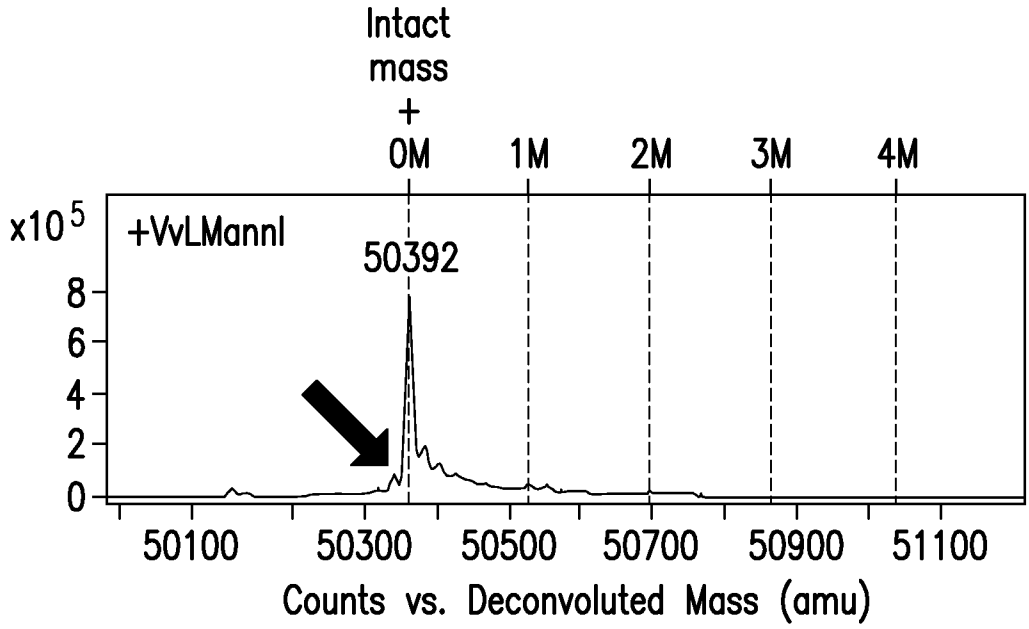

FIG. 10B

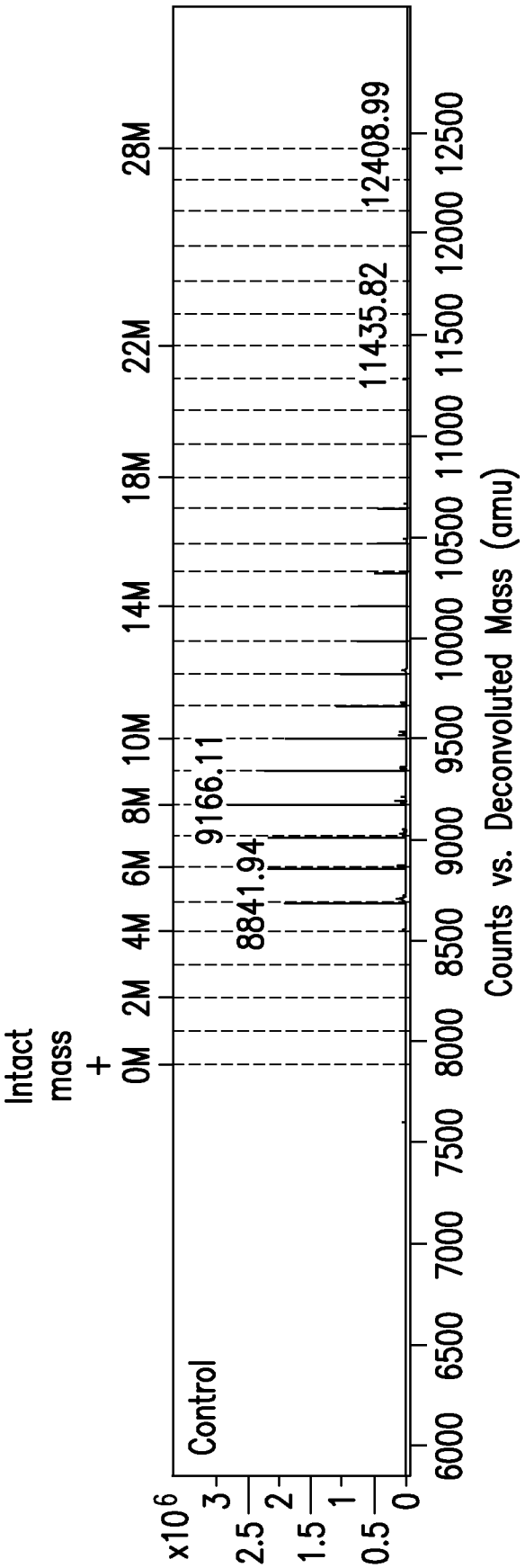

FIG.11A

FIG.11B

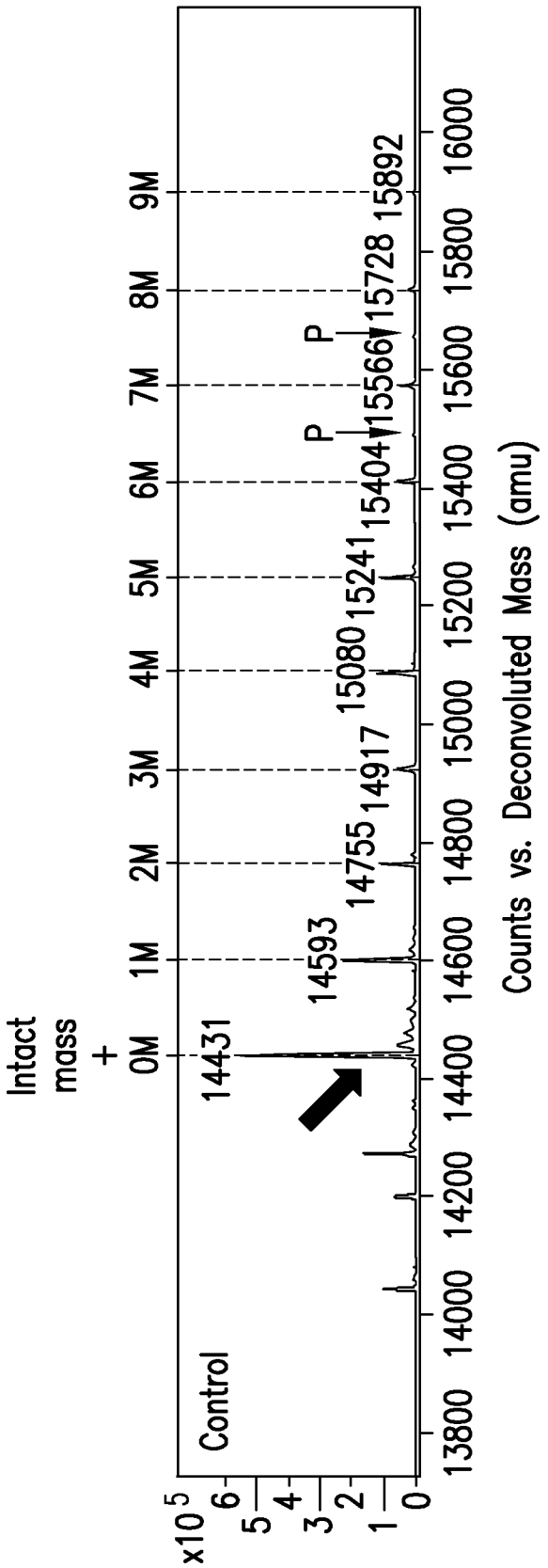

FIG.12A

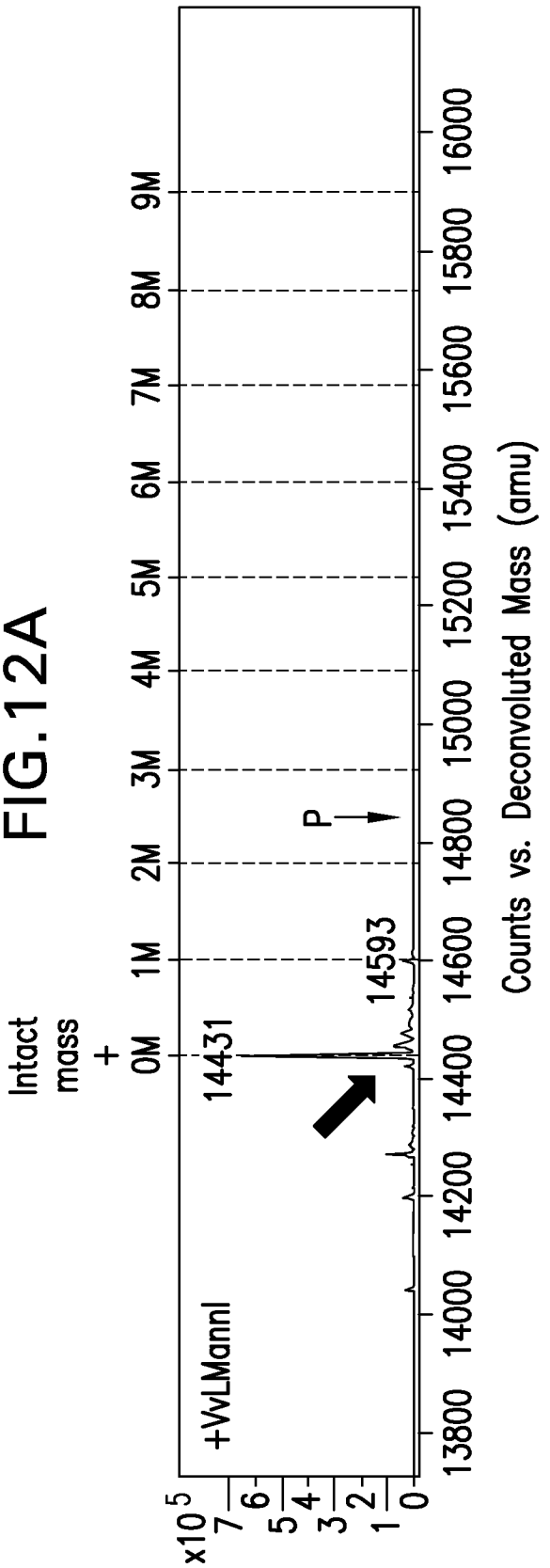

FIG.12B

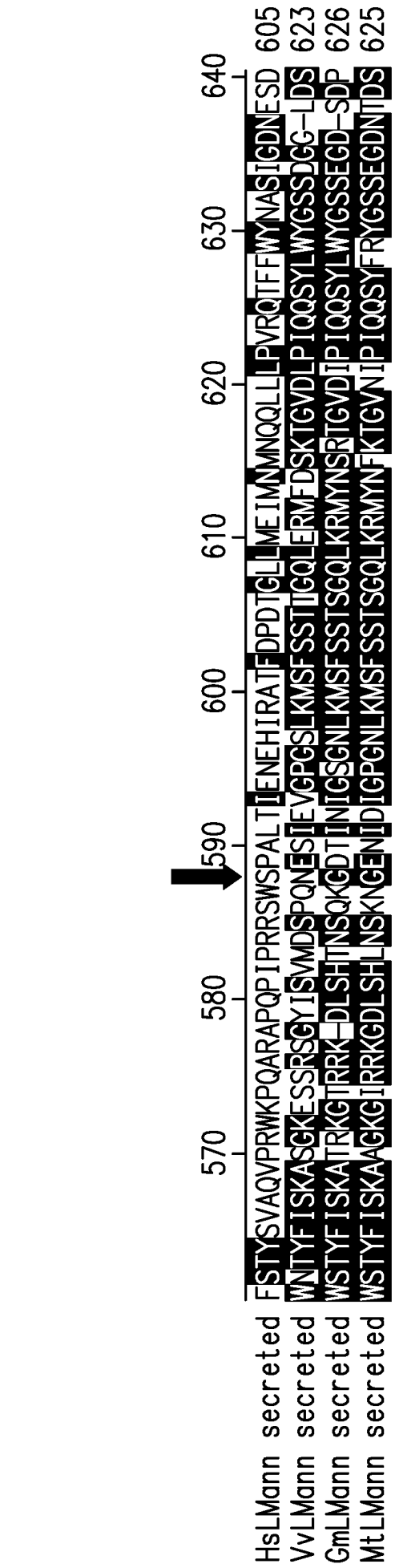
HsLMann secreted
VvLMann secreted
GmLMann secreted
MtLMann secreted
570
580
590
600
610
620
630
640
605
623
626
625

FIG. 13A

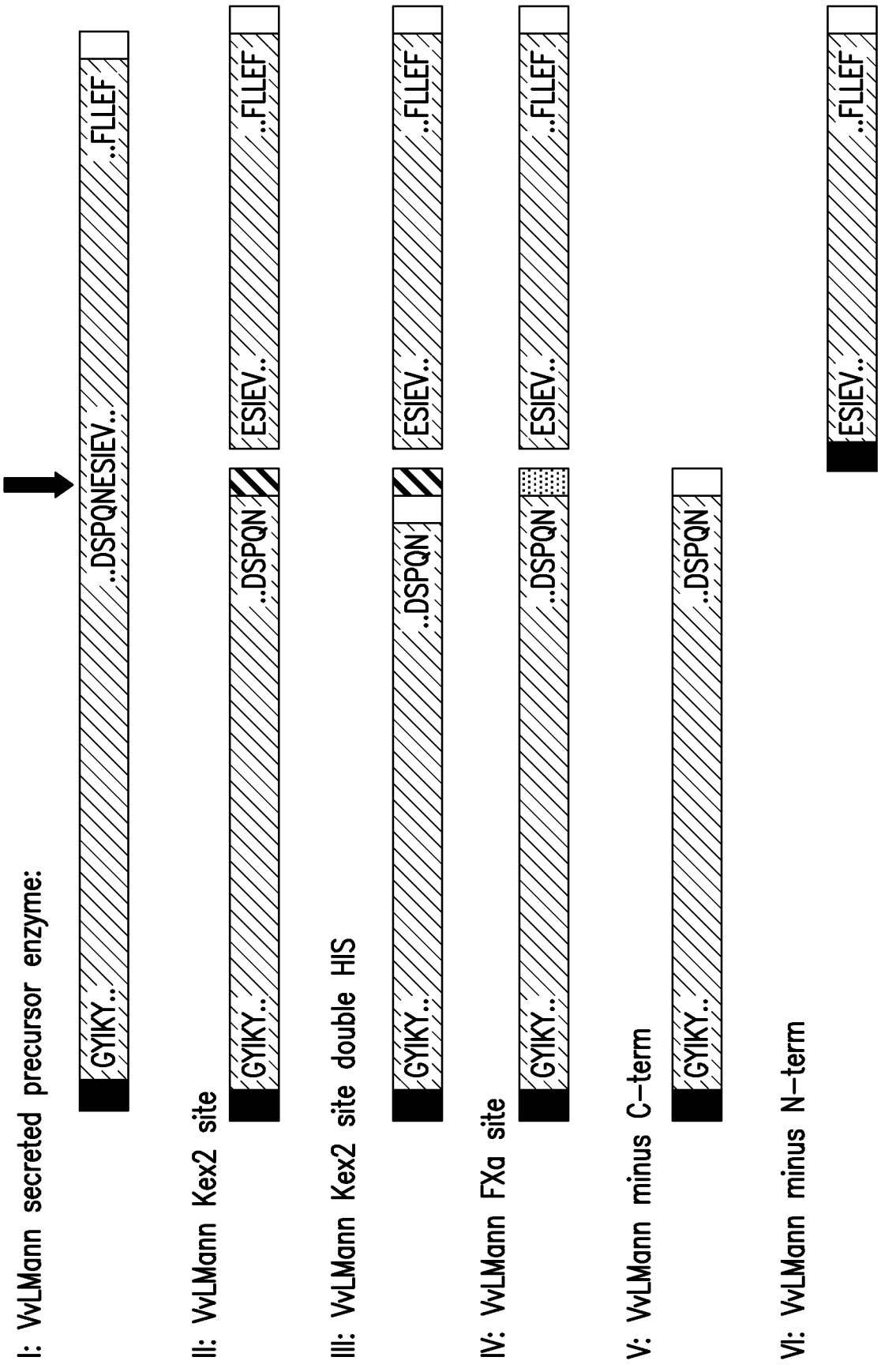
I: VvLMann secreted precursor enzyme:
GYIKY..
..DSPQNESIEV..
..FLLEF
II: VvLMann Kex2 site
GYIKY..
..DSPQN
ESIEV..
..FLLEF
III: VvLMann Kex2 site double HIS
GYIKY..
..DSPQN
ESIEV..
..FLLEF
IV: VvLMann FXa site
GYIKY..
..DSPQN
ESIEV..
..FLLEF
V: VvLMann minus C-term
GYIKY..
..DSPQN
VI: VvLMann minus N-term
ESIEV..
..FLLEF

FIG.13B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/047120

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 21/00 (2014.01)
CPC - C12P 21/005 (2014.09)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12N 15/09, 15/63, 15/79, 15/80; C12P 21/00 (2014.01)
USPC - 435/68.1, 69.1, 254.11, 254.2, 254.23

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - C12N 15/80; C12P 21/005; C12Y 302/00, 302/01, 302/01113 (2014.09)
(keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Orbit, Google Patents, PubMed, Google
Search terms used: method reduce O-mannosylation recombinant glycoprotein yeast express O-linked glycan mannose polymer transform lysosomal mannosidase vector

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 8,298,811 B2 (HAMILTON) 30 October 2012 (30.10.2012) entire document	1-4, 8, 11, 14-19, 23, 25
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Y		9, 10, 12, 13, 24
Y	US 2009/0170159 A1 (BOBROWICZ et al) 02 July 2009 (02.07.2009) entire document	9, 10, 12, 13, 24
A	US 6,890,748 B2 (GARGER et al) 10 May 2005 (10.05.2005) entire document	1-25
A	US 2012/0225453 A1 (WITHERS et al) 06 September 2012 (06.09.2012) entire document	1-25
A	WO 2012/042387 A2 (PIENS et al) 05 April 2012 (05.04.2012) entire document	1-25

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
13 November 2014	15 DEC 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)