HYDROLYSIS OF MANNOSE-1-PHOSPHO-6-MANNOSE LINKAGE TO PHOSPHO-6-MANNOSE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Application Serial No. 61/246,847, filed on September 29, 2009. The disclosure of the prior application is incorporated by reference in its entirety.

TECHNICAL FIELD

The invention relates to methods of hydrolyzing mannose-1-phospho-6-mannose linkages on glycoproteins, and more particularly, to using a mannosidase to hydrolyze mannose-1-phospho-6-mannose linkages to uncap the phospho-6-mannose residues on the glycoprotein.

BACKGROUND

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

U.S. Application Serial No. 12/062,469 is incorporated by reference in its entirety.

SUMMARY

The present ~~invention~~disclosure is based, at least in part, on the discovery of a mannosidase that is capable of hydrolyzing mannose-1-phospho-6-mannose linkages on glycoproteins. As such, the mannosidase can be used to obtain glycoproteins containing uncapped

terminal mannose-6-phosphate residues. *In vitro* and *in vivo* methods of obtaining such glycoproteins are described herein. Genetically engineered cells can be used in the methods to produce target molecules having uncapped terminal mannose-6-phosphate residues.

In one aspect, there is provided a method for uncapping a mannose-6-phosphate residue on an oligosaccharide, said method comprising

a) providing said oligosaccharide having a mannose-1-phospho-6-mannose linkage; and

b) contacting said oligosaccharide with a mannosidase capable of hydrolyzing said mannose-1-phospho-6-mannose residue to phospho-6-mannose, wherein said mannosidase is a member of glycosyl hydrolase family 92.

In one ~~aspect~~embodiment, this document features a method for uncapping a mannose-6-phosphate residue on an oligosaccharide. The method includes providing the oligosaccharide having a mannose-1-phospho-6-mannose linkage; and contacting the oligosaccharide with a mannosidase capable of hydrolyzing the mannose-1-phospho-6-mannose linkage to phospho-6-mannose. The contacting step can be performed using a purified mannosidase, a recombinant mannosidase, a cell lysate containing the recombinant mannosidase, or a fungal cell containing the recombinant mannosidase. The mannosidase can include a targeting sequence. The oligosaccharide can be attached to a protein (e.g., a human protein expressed in a fungal organism).

In one aspect, there is provided a method of producing a target protein having an uncapped phospho-6-mannose residue, said method comprising:

providing a fungal cell genetically engineered to comprise and express a nucleic acid encoding a mannosidase, said mannosidase being capable of hydrolyzing a mannose-1-phospho-6-mannose residue to phospho-6-mannose; and

introducing into said cell a nucleic acid encoding a target protein, wherein said cell produces said target protein in which a mannose-1-phospho-6-mannose residue on the target has been converted to a phospho-6-mannose residue by said expressed mannosidase expressed in the cell.

In another ~~aspect~~embodiment, this document features a method of producing a target protein

having terminal phospho-6-mannose residues. The method includes providing a fungal cell genetically engineered to include a nucleic acid encoding a mannosidase, the mannosidase capable of hydrolyzing a mannose-1-phospho-6-mannose linkage to phospho-6-mannose; and introducing into the cell a nucleic acid encoding a target protein, wherein the cell produces the target protein comprising the terminal phospho-6-mannose residues.

In one aspect, there is provided a method of producing a target protein having an uncapped phospho-6-mannose residue in a fungal organism, said method comprising

a) providing a fungal cell genetically engineered to comprise and express a nucleic acid encoding a mannosidase capable of hydrolyzing mannose-1-phospho-6-mannose to phospho-6-mannose, and said fungal cell further comprising a nucleic acid encoding a target protein in which a mannose-1-phospho-6-mannose residue on the target has been converted to a phospho-6-mannose residue by said expressed mannosidase expressed in the cell; and

b) isolating said target protein having said uncapped phospho-6-mannose residue.

This document also features a method of producing a target protein having terminal phospho-6-mannose residues in a fungal organism. The method includes providing a fungal cell genetically engineered to include a nucleic acid encoding a mannosidase capable of hydrolyzing a mannose-1-phospho-6-mannose linkage to phospho-6-mannose, wherein the fungal cell further includes a nucleic acid encoding a target protein; and isolating the target protein having the terminal phospho-6-mannose residues. The fungal cell further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation and/or can be genetically engineered to be deficient in OCH1 activity.

In one aspect, there is provided an isolated fungal cell genetically engineered to produce glycoproteins comprising an
uncapped phospho-6-mannose residue, said fungal cell comprising a nucleic acid encoding a mannosidase, wherein expression of said mannosidase in said fungal cell results in the production of glycoproteins comprising said terminal phospho-6-mannose residues, wherein said mannosidase is a member of glycosyl hydrolase family 92.

This document also features an isolated fungal cell genetically engineered to produce glycoproteins comprising terminal phospho-6-mannose residues. The fungal cell

includes a nucleic acid encoding a mannosidase, wherein expression of the mannosidase in the fungal cell produces glycoproteins comprising the terminal phospho-6-mannose residues. The fungal cell further can include a nucleic acid encoding a target glycoprotein protein.

In one aspect, there is provided a substantially pure culture of *Yarrowia lipolytica*, *Pichia pastoris*, *Hansenula polymorpha*, *Arxula adeninivorans*, *Pichia methanolica*, *Oogataea minuta*, or *Aspergillus niger* cells, a substantial number of which are genetically engineered to produce a glycoprotein comprising an uncapped phospho-6-mannose residue, said cells comprising a nucleic acid encoding a mannosidase capable of hydrolyzing mannose-1-phospho-6-mannose to phospho-6-mannose, wherein said mannosidase is a member of glycosyl hydrolase family 92.

In another ~~aspect~~embodiment, this document features a substantially pure culture of *Yarrowia lipolytica*, *Pichia pastoris*, *Hansenula polymorpha*, *Arxula adeninivorans*, *Pichia methanolica*, *Oogataea minuta*, or *Aspergillus niger* cells, a substantial number of which are genetically engineered to produce glycoproteins comprising a terminal phospho-6-mannose residue, the cells comprising a nucleic acid encoding a mannosidase capable of hydrolyzing a mannose-1-phospho-6-mannose linkage to phospho-6-mannose.

In any of the embodiments described herein, the fungal organism can be *Yarrowia lipolytica* or *Arxula adeninivorans*. The fungal organism can be a methylotrophic yeast such as *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, or *Hansenula polymorpha*. The fungal organism can be a filamentous fungus (e.g., a filamentous fungus selected from the group consisting of *Aspergillus caesiellus, Aspergillus candidus, Aspergillus carneus, Aspergillus clavatus, Aspergillus deflectus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus niger, Aspergillus ochraceus, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus penicilloides, Aspergillus restrictus, Aspergillus sojae, Aspergillus sydowi, Aspergillus tamari, Aspergillus terreus, Aspergillus ustus,* and *Aspergillus versicolor*).

In any of embodiments described herein, the protein can be a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein. The lysosomal protein can be a lysosomal enzyme (e.g., a lysosomal enzyme associated with a lysosomal storage disorder (LSD) such as acid alpha glucosidase or alpha galactosidase). The LSD can be Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease,

In one aspect, there is provided an isolated glycoprotein comprising an uncapped phospho-6-mannose residue, wherein the protein is produced by the method as defined above.

This document also features an isolated glycoprotein that includes terminal phospho-6-mannose residues, wherein the protein is produced by the methods described herein.

In one aspect, there is provided a composition comprising a glycoprotein, wherein at least 47% of the N-glycans on said glycoprotein comprise an uncapped phospho-6-mannose residue.

In yet another ~~aspect~~embodiment, this document features a composition that includes a glycoprotein, wherein at least 47% of the N-glycans on the glycoprotein have terminal phospho-6-mannose residues. For example, at least 50%, 75%, 80%, 85%, or 90% of the N-glycans on the glycoprotein can have terminal phospho-6-mannose residues.

In one aspect, there is provided a polypeptide comprising an amino acid sequence having at least 90% identity to residues 1 to 774 of SEQ ID NO:50 or to SEQ ID NO:50, wherein the polypeptide is capable of hydrolyzing mannose-1-phospho-6-mannose to phospho-6-mannose.

In one aspect, there is provided a nucleic acid comprising:

(a) a nucleotide sequence encoding the polypeptide as defined above; or

(b) the complement of the nucleotide sequence.

In one aspect, there is provided an expression vector comprising the nucleic acid as defined above, wherein the nucleic acid comprises (a).

This document also features an isolated nucleic acid that includes a nucleotide sequence set forth in SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, or SEQ ID NO:14, or a nucleotide sequence that is at least 90% identical to SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:20. This document also features a vector that includes a promoter operably linked to such a nucleic acid, wherein the nucleic acid encodes a mannosidase. The nucleic acid further can include a secretion signal or targeting sequence to target the mannosidase to an intracellular compartment.

In one aspect, there is provided a host cell comprising the expression vector as defined above.

In one aspect, there is provided a method of making a polypeptide, the method comprising culturing the host cell as defined above and isolating the polypeptide from the culture.

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

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

Brief Description of the Drawings7

FIG. 1 is a schematic of the pYLTmAX and pYLTmAXMnn4 constructs.

FIG. 2 is a series of electroferograms depicting sugar analysis of MTLY60Δoch1(1 wild type copy of Mnn4), MTLY60Δoch1+Hp4dMnn4 (1WT +1 extra