De-Mannosylation of Phosphorylated N-Glycans

CROSS-REFERENCE TO RELATED APPLICATIONS

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

TECHNICAL FIELD

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

BACKGROUND

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

SUMMARY

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

In one aspect, there is provided a method for demannosylating phosphorylated N-glycans on a glycoprotein, said method comprising contacting a glycoprotein comprising a terminal alpha-1,2 mannose linkage in which the underlying mannose is phosphorylated with a mannosidase, or a biologically active fragment of the mannosidase, that hydrolyzes a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated, wherein the contacting results in hydrolysis of the terminal alpha-1,2 mannose linkage, to produce a demannosylated glycoprotein.

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

mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated. The mannosidase can be from *Aspergillus satoi* or *Cellulosimicrobium cellulans*. The method further can include isolating the glycoprotein containing the demannosylated phosphorylated N-glycan. The protein can be a human protein expressed in a fungal organism. For example, the fungal organism can be *Yarrowia lipolytica* or *Arxula adeninivorans*. The fungal organism also can be a methylotrophic yeast (e.g., *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, or *Hansenula polymorpha*) or a filamentous fungus (e.g., *Aspergillus caesiellus, Aspergillus candidus, Aspergillus carneus, Aspergillus clavatus, Aspergillus deflectus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus niger, Aspergillus ochraceus, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus penicilloides, Aspergillus restrictus, Aspergillus sojae, Aspergillus sydowi, Aspergillus tamari, Aspergillus terreus, Aspergillus ustus,* or *Aspergillus versicolor*). The protein can be a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein. For example, the lysosomal protein can be a lysosomal enzyme such as a lysosomal enzyme associated with a lysosomal storage disorder (LSD). A LSD can be Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

In one aspect, there is provided a method of producing a glycoprotein comprising demannosylated phosphorylated N-glycans, the method comprising introducing into a fungal cell a nucleic acid encoding the glycoprotein and the glycoprotein comprises a terminal alpha-1,2 mannose linkage in which the underlying mannose is phosphorylated, wherein the fungal cell is genetically engineered to comprise a nucleic acid encoding a mannosidase, or a biologically active fragment of the mannosidase, that hydrolyzes a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated, to produce a demannosylated glycoprotein.

This document also features a method of producing a target protein having demannosylated phosphorylated N-glycans in a fungal organism. The method includes providing a fungal cell genetically engineered to include a nucleic acid encoding a

mannosidase capable of hydrolyzing a terminal alpha-1,2 mannose linkage or moiety when the underlying mannose is phosphorylated; and introducing into the cell a nucleic acid encoding a target protein.

This document also features an isolated fungal cell genetically engineered to produce glycoproteins that include demannosylated phosphorylated N-glycans. The fungal cell can be *Yarrowia lipolytica* or *Arxula adeninivorans*. The fungal cell also can be a methylotrophic yeast (e.g., *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, or *Hansenula polymorpha*) or a filamentous fungus (e.g., *Aspergillus caesiellus, Aspergillus candidus, Aspergillus carneus, Aspergillus clavatus, Aspergillus deflectus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus glaucus, Aspergillus nidulans, Aspergillus niger, Aspergillus ochraceus, Aspergillus oryzae, Aspergillus parasiticus, Aspergillus penicilloides, Aspergillus restrictus, Aspergillus sojae, Aspergillus sydowi, Aspergillus tamari, Aspergillus terreus, Aspergillus ustus,* or *Aspergillus versicolor*). The fungal cell further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation. The fungal cell can be genetically engineered to be deficient in OCH1 activity. The fungal cell further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation, and wherein the fungal cell is genetically engineered to be deficient in OCH1 activity.

In one aspect, there is provided an isolated fungal cell genetically engineered to express a mannosidase, or a biologically active fragment of the mannosidase, that hydrolyzes a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated.

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

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

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

In one aspect, there is provided a substantially pure culture of fungal cells, a substantial number of which are genetically engineered to express a mannosidase, or a biologically active fragment of the mannosidase, that hydrolyzes a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated.

In yet another embodiment~~aspect~~, this document features a substantially pure culture of *Yarrowia lipolytica*, *Pichia pastoris*, *Hansenula polymorpha*, *Ogataea minuta*, *Pichia methanolica*, *Arxula adeninivorans*, or *Aspergillus niger* cells, a substantial number of which are genetically engineered to produce glycoproteins that contain demannosylated phosphorylated N-glycans. The cells further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation. The cells can be genetically engineered to be deficient in OCH1 activity. The cells further can include a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation, and can be genetically engineered to be deficient in OCH1 activity.

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

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

~~9. The method of any one of claims 1-3 and 5-8, wherein the mannosidase is of the GH47 family.~~

9~~10~~. The method of any one of claims 1, 2, and 4, wherein the mannosidase comprises an amino acid sequence encoded by a nucleic acid that is at least 85% identical to SEQ ID NO:4.

10~~1~~. The method of any one of claims 1, 2, 4, and 9~~10~~, wherein the mannosidase comprises an amino acid sequence encoded by a nucleic acid that is at least 95% identical to SEQ ID NO:4.

11~~2~~. The method of any one of claims 1, 2, 4, 9~~10~~ and 10~~11~~, wherein the mannosidase comprises an amino acid sequence encoded by a nucleic acid that is at least 98% identical to SEQ ID NO:4.

12~~3~~. The method of any one of claims 1, 2, 4, and 9~~10~~-11~~12~~, wherein the mannosidase comprises an amino acid sequence encoded by SEQ ID NO:4.

~~14. The method of any one of claims 1, 2, 4, and 10-13, wherein the mannosidase is of the GH92 family.~~

13~~5~~. The method of any one of claims 1-12~~14~~, wherein the method further comprises isolating the demannosylated glycoprotein.

14~~6~~. The method of any one of claims 1-13~~15~~, wherein the glycoprotein is a human protein.

15~~7~~. The method of any of claim 1-14~~16~~, wherein the contacting occurs in a fungal organism.

16~~8~~. The method of claim 15~~17~~, wherein the fungal organism is *Yarrowia lipolytica* or *Arxula adeninivorans*.

17~~9~~. The method of claim 15~~17~~, wherein the fungal organism is a methylotrophic yeast.

1820. The method of claim 1719, wherein the methylotrophic yeast is selected from the group consisting of *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, and *Hansenula polymorpha*.

1921. The method of claim 1517, wherein the fungal organism is a filamentous fungus.

2022. The method of claim 1921, wherein the filamentous fungus is 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*, *Aspergillus versicolor*, *Neurospora*, *Trichoderma*, *Fusarium*, and *Chrysosporium*.

213. The method of any one of claims 1-2022, wherein the glycoprotein is a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein.

224. The method of claim 2123, wherein the lysosomal protein is a lysosomal enzyme.

235. The method of claim 2224, wherein the lysosomal enzyme is associated with a lysosomal storage disorder (LSD).

246. The method of claim 2325, wherein the LSD is Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren

syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

25~~7~~. An isolated fungal cell genetically engineered to express a mannosidase, or a biologically active fragment of the mannosidase, that hydrolyzes a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated.

26~~8~~. A substantially pure culture of fungal cells, a substantial number of which are genetically engineered to express a mannosidase, or a biologically active fragment of the mannosidase, that hydrolyzes a terminal alpha-1,2 mannose linkage when the underlying mannose is phosphorylated.

27~~9~~. The fungal cell of claim 25~~27~~ or the culture of claim 26~~28~~, wherein said mannosidase is from *Aspergillus satoi*.

28~~30~~. The fungal cell of claim 25~~27~~ or the culture of claim 26~~28~~, wherein said mannosidase is from *Cellulosimicrobium cellulans*.

29~~31~~. The fungal cell of claim 25~~27~~ or 27~~29~~, or the culture of claim 26~~28~~ or 27~~29~~, wherein the mannosidase comprises an amino acid sequence that is at least 85% identical to SEQ ID NO:5.

30~~32~~. The fungal cell of any one of claims 25~~27~~, 27~~29~~, and 29~~31~~, or the culture of any one of claims 26~~28~~, 27~~29~~, and 29~~31~~, wherein the mannosidase comprises and amino acid sequence that is at least 95% identical to SEQ ID NO:5.

31~~3~~. The fungal cell of any one of claims 25~~27~~, 27~~29~~, 29~~31~~, and 30~~32~~, or the culture of any one of claims 16~~18~~, 27~~29~~, 29~~31~~, and 30~~32~~, wherein the mannosidase comprises and amino acid sequence that is at least 98% identical to SEQ ID NO:5.

32~~4~~. The fungal cell of any one of claims 25~~27~~, 27~~29~~, and 29~~31~~-31~~33~~, or the culture of any one of claims 26~~28~~, 27~~29~~, and 29~~31~~-31~~33~~, wherein the mannosidase comprises SEQ ID NO:5.

~~35. The fungal cell of any one of claims 27, 29, and 31-34, or the culture of any one of claims 28, 29, and 31-34, wherein the mannosidase is of the GH47 family.~~

336. The fungal cell of claim 2527 or 2830 or the culture of 2628 or 2830, wherein the mannosidase comprises an amino acid sequence encoded by a nucleic acid that is at least 85% identical to SEQ ID NO:4.

347. The fungal cell of any one of claims 2527, 2830, and 3336, or the culture of any one of claims 2628, 2830, and 3336, wherein the mannosidase comprises an amino acid sequence encoded by a nucleic acid that is at least 95% identical to SEQ ID NO:4.

358. The fungal cell of any one of claims 2527, 2830, 3336, and 3437, or the culture of any one of claims 2628, 2830, 3336, and 3437, wherein the mannosidase comprises an amino acid sequence encoded by a nucleic acid that is at least 98% identical to SEQ ID NO:4.

369. The fungal cell of any one of claims 2527, 2830, and 3336-3538, or the culture of any one of claims 2628, 2830, and 3336-3538, wherein the mannosidase comprises an amino acid sequence encoded by SEQ ID NO:4.

~~40. The fungal cell of any one of claims 27, 30, and 36-39, or the culture of any one of claims 28, 30, and 36-39, wherein the mannosidase is of the GH92 family.~~

3741. The fungal cell of any one of claims 2527 and 2729-3640, or the culture of any one of claims 2628-3640, wherein the fungal cell is a cell, or the fungal cells are cells, of *Yarrowia lipolytica* or *Arxula adeninivorans*.

3842. The fungal cell of any one of claims 2527 and 2729-3640, or the culture of any one of claims 2628-3640, wherein the fungal cell is a cell, or the fungal cells are cells, of a methylotrophic yeast.

3943. The fungal cell or culture of claim 3842, wherein the methylotrophic yeast is selected from the group consisting of *Pichia pastoris*, *Pichia methanolica*, *Oogataea minuta*, and *Hansenula polymorpha*.

404. The fungal cell of any one of claims 2527 and 2729-3640, or the culture of any one of claims 2628-3640, wherein the fungal cell is a cell, or the fungal cells are cells, of a filamentous fungus.

415. The fungal cell or culture of claim 4044, wherein the filamentous fungus is 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*, *Aspergillus versicolor*, *Neurospora*, *Trichoderma*, *Fusarium*, and *Chrysosporium*.

426. The fungal cell of any one of claims 2527 and 2729-4145, or the culture of any one of claims 2628-4145, wherein the cell further comprises, or a substantial number of cells of the culture further comprise, a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation.

437. The fungal cell of any one of claims 2527 and 2729-4246, or the culture of any one of claims 2628-4246, wherein the cell is, or a substantial number of cells of the culture are, genetically engineered to be deficient in OCH1 activity.

448. The fungal cell of any one of claims 2527 and 2729-4347, or the culture of any one of claims 2628-4347, wherein the cell further comprises, or a substantial number of cells of the culture further comprise, a nucleic acid encoding a polypeptide capable of promoting mannosyl phosphorylation and wherein the cell is genetically engineered to be deficient in OCH1 activity.

4549. The fungal cell of any one of claims 2527 and 2729-4448, or the culture of any one of claims 2628-4448, wherein the cell further comprises, or a substantial number of cells of the culture further comprise, a nucleic acid encoding a target glycoprotein.

4650. The fungal cell or culture of claim 4549, wherein the target glycoprotein is a human protein.

4751. The fungal cell or culture of claim 4549, wherein the target glycoprotein is a pathogen protein, a lysosomal protein, a growth factor, a cytokine, a chemokine, an antibody or antigen-binding fragment thereof, or a fusion protein.

4852. The fungal cell or culture of claim 4751, wherein the lysosomal protein is a lysosomal enzyme.

4953. The fungal cell or culture of any one of claims 4549-4852, wherein the target glycoprotein is a protein associated with a LSD.

5054. The fungal cell or culture of claim 4953, wherein the LSD is Fabry's disease, mucopolysaccharidosis I, Farber disease, Gaucher disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, GM2 activator disease, Krabbe disease, metachromatic leukodystrophy, Niemann-Pick disease, Scheie disease, Hunter disease, Sanfilippo disease, Morquio disease, Maroteaux-Lamy disease, hyaluronidase deficiency, aspartylglucosaminuria, fucosidosis, mannosidosis, Schindler disease, sialidosis type 1, Pompe disease, Pycnodysostosis, ceroid lipofuscinosis, cholesterol ester storage disease, Wolman disease, Multiple sulfatase deficiency, galactosialidosis, mucolipidosis, cystinosis, sialic acid storage disorder, chylomicron retention disease with Marinesco-Sjögren syndrome, Hermansky-Pudlak syndrome, Chediak-Higashi syndrome, Danon disease, or Geleophysic dysplasia.

5155. The fungal cell or culture of claim 4246, wherein the polypeptide capable of promoting mannosyl phosphorylation is a MNN4 polypeptide.

52~~56~~. The fungal cell or culture of claim 51~~55~~, wherein the MNN4 polypeptide is a *Yarrowia lipolytica*, a *Saccharomyces cerevisiae*, an *Ogataea minuta*, a *Pichia pastoris*, or a *Candida albicans* polypeptide.

53~~57~~. The fungal cell or culture of claim 42~~46~~, wherein the polypeptide capable of promoting mannosyl phosphorylation is a *P. pastoris* PNO1 polypeptide.